Supplemental Materials

Please see Major Resources Table in the Supplemental Materials.

GSK-3β Knockout mice

At 10 weeks of age both male and female mice began tamoxifen chow diet (250 mg/kg Envigo TD.130855) for 5 consecutive days followed by two days of regular chow to maintain body weight, repeated for 3 weeks total. B and M-mode blinded echocardiography was performed (Vevo 2100, Fujifilm Visualsonics) prior to and following knockdown to detect any impact of the tamoxifen on global heart metrics such as ejection fraction and fractional shortening. All procedures were approved by IACUC.

Tissue preparation and protein quantification

To prepare whole tissue lysates, left ventricular tissue (human and mouse) was homogenized in lysis buffer (in mM: 50 Tris pH 7.5, 150 NaCl, 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific). Samples were then sonicated and centrifuged at 15,000xg for 10 minutes.

To prepare myofilament enrichment, left ventricular tissue (human and mouse) was skinned by homogenization in standard relax buffer (SRB) (in mM: 75 KCL, 10 Imidazole, 2 $MgCl_2$, 2 EDTA, 1 NaN_3) with 0.3% Triton X-100 supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher). Samples were centrifuged at 15,000xg for 1 minute and the supernatant was discarded. The pellets were triturated in SRB with triton, centrifuged, and the supernatant discarded. The pellets were then washed twice by triturating with SRB without triton, centrifuged, and

27 the supernatant was discarded. The pellets were solubilized in 8M urea, sonicated, and
28 centrifuged at 15,000xg for 10 minutes to clarify. Protein concentrations were quantified
29 via bicinchoninic acid protein assay (BCA) (Pierce).
30
31 *Immunoblotting*
32 Equal amounts of sample were loaded onto 4-12% Bis-Tris gradient gels (Bolt)
33 and transferred to nitrocellulose membrane. Membranes were incubated in blocking
34 buffer (Licor) for one hour, and primary antibody overnight at 4°C. Primary antibodies
35 used are as follows: GSK-3β (Cell Signaling Technology, 12456), GSK-3β pS9 (Cell
36 Signaling Technology, D28E12), GSK-3 (Y279/Y216 (EMD Millipore, 05-413), Actin
37 (Sigma-Aldrich, SAB5600071), GAPDH (Cell Signaling Technology, 2118), Tubulin (Cell
38 Signaling Technology, 2144), TnI (ipoc, MA-1040), and TnI pS23/24 (Cell Signaling,
39 4004S), Total cMyBP-C (Santa Cruz, sc-137180), abLIM-1 (Proteintech, 15129-1-AP).
40 Blots were washed with TBS-T and incubated in infared red dye conjugated secondary
41 antibodies (Licor) at room temperature for one hour in the dark prior to being imaged on
42 an Azure C600 infa-red imager at 700 and 800 nm. Signal intensity was quantified on
43 Azure analysis software.
44
45 *Skinned myocytes*
46 Myocytes were prepared by homogenizing left ventricular tissue in isolation solution (in
47 mM: 5.5Na_2ATP, 7.11 $MgCl_2$, 2 EGTA, 108.01 KCL, 891 KOH, 10 imidazole, 10 DTT)
48 with 0.3% Triton and protease and phosphatase inhibitors (Thermo Fisher Scientific)
49 and left on ice for twenty minutes. After “skinning”, the mixture was centrifuged at

50 120xg, and the pellet was re-suspended twice in isolation solution without triton to
51 remove the detergent. A single myocyte was selected and attached to a force
52 transducer and a length controller (Aurora Scientific) via pins with UV-curing glue. The
53 sarcomere length was monitored using a video camera and calculated by the High-
54 Speed Video Sarcomere Length software (Aurora Scientific) and was kept constant at
55 the experimental SL (1.9, 2.1 or 2.3) throughout the experiment. The temperature was
56 kept constant at 25°C. The myocyte was kept in relaxing solution (6.2 ATP, 6.5 $MgCl_2$,
57 10 EGTA, 100 BES, 10 phosphocreatine, 47.6 KProp) with phosphatase and protease
58 inhibitors. The force was measured by moving the myocytes to different baths
59 containing different calcium concentrations ranging from 0 to saturating conditions,
60 created by mixing relaxing and activation solutions. The Activation solution contained (in
61 mM): 5.95 Na_2ATP, 6.2 $MgCl_2$, 10 Ca^{2+}-EGTA, 100 BES, 10 phosphocreatine, 29.98
62 KProp, 10 DTT, phosphatase and protease inhibitors. Passive tension was measured in
63 cells in relax solution. The sarcomere length was increased from 1.6 to 2.6 um, with
64 force measurements being taken every 0.2 um. Each cell was then fit to an exponential
65 curve.

66 Data from skinned myocytes were statistically analyzed on a per cell basis, not
67 on a per animal basis. Approximately equal numbers of cells were analyzed from each
68 animal, although this may introduce bias from non-independence.

69

70 *Titin Gels*

71 Titin sample and gel preparation methods were adapted from established protocols[45].
72 15-20 mg pieces were obtained from each heart under liquid nitrogen. Tissues were

73 pulverized using Kontes Dounce homogenizers (Kimble Chase, Rockwood, TN) while
74 being maintained in liquid nitrogen. Tissues were solubilized at 60°C in the following
75 buffer: 4M Urea, 1M Thiourea, 1.5% SDS, 25mM Tris-HCl, 37.5mM DTT, 25% Glycerol,
76 + protease and phosphatase inhibitors (1:100) for 10 minutes. Solubilized tissue was
77 then centrifuged at 14.8k rpm for 5 minutes and then aliquoted. Protein concentrations
78 were determined using an RC/D assay (BioRad). Each sample was loaded in on a 1%
79 agarose (SeaKem™ Gold, Lonza Group, Switzerland) gel: 50mM Tris Base, 384mM
80 Glycine, 0.1% SDS, 30% Glycerol, using a large-format gel system (Hoeffer SE600X,
81 Hoefer, Inc., Holliston, MA). Gels were run at 15mA/gel for 200 minutes at 4°C and then
82 stained with Coomassie. Gels were scanned using an Azure C600 imager and
83 quantified using Image J.
84
85 *X-ray Diffraction*
86 Papillary muscles were dissected from mice and skinned at 1% Triton X in
87 relaxing solution (containing in mM: 6.3 Na_2ATP, 6.48 $MgCl_2$, 10 EGTA, 100 BES, 10
88 phosphocreatine, 49.76 KPropionate, 10 DTT, and creatine kinase 10 U/ml) overnight.
89 Next day, the muscles were washed with fresh cold relaxing solution and muscles were
90 further dissected in fiber strips, clipped on aluminum T-clips and stored in cold relaxing
91 solution for the day.
92 X-ray diffraction patterns were collected from freshly skinned muscle strips using
93 the small-angle instrument at BioCAT beamline 18ID at the Advanced Photon Source,
94 Argonne National Laboratory[32]. The X-ray beam was focused to ~0.04 × 0.10 mm at the
95 detector plane. The sample-to-detector distance was ~3.5 m, and the X-ray wavelength

96 was 0.103 nm. Isolated fiber bundles (~200 µm diameter, 2-3 mm long) were mounted
97 between a force transducer (Model 402A, Aurora Scientific) and a static hook. Force
98 was monitored using the Muscle Dynamic Control system (Model 610A, Aurora
99 Scientific). Sarcomere length was adjusted by laser diffraction using a 4-mW HeNe
100 laser. Diffraction patterns were collected at sarcomere lengths of 2.1 µm and 2.4 µm. X-
101 ray exposures were 1 s at an incident flux of ~3×10^{12} photons per second, and the
102 patterns were collected on a CCD-based X-ray detector (Mar 165; Rayonix Inc.
103 Evanston, IL). The data were analyzed using data reduction programs belonging to the
104 MuscleX software package developed at BioCAT[33]. The spacings of the 1,0 equatorial
105 X-ray reflections were measured using the "Equator" routine in the MuscleX software
106 package as described previously[34].
107
108 *Immunofluorescence*
109 Human or mouse LV was homogenized in isolation solution (see above) with
110 0.3% triton, with protease and phosphatase inhibitors (1:100). Cells were plated onto
111 chamber slides coated with Poly_L_lysine solution (Millipore Sigma, P4707) and
112 allowed to adhere for 20 minutes. Cells were fixed with ice cold methanol for 1 minute,
113 and then 4% paraformaldahyde (PFA) for 3 minutes. To skin, cells were washed twice
114 with PBS with 0.5% Triton for 20 minutes each, followed two washes in PBS with 0.1%
115 Triton for 15 minutes. The samples were then incubated in antigen-retrieval solution (0.1
116 M Glycine, pH 7.4) for 30 minutes. After washing three times with PBS, the slides were
117 incubated for 30 minutes at room temperature in blocking solution (1% BSA in PBS)
118 diluted 1:1 with PBS. Primary antibody was diluted in blocking solution at the following

119 dilutions: GSK-3β (Cell Signaling Technology, 12456, 1:150), GSK-3β pS9 (Cell
120 Signaling Technology, D28E12, 1:150), GSK-3β pY216 (Abcam, ab75745, 1:200), N-
121 cadherin (1:200) abLIM-1 (Proteintech, 15129-1-AP, 1:200), Alpha-Actinin (Sigma-
122 Aldrich, A7732, 1:500). Slides were incubated with primary antibody overnight at 4°C.
123 The following day, slides were washed three times with PBS, and incubated with
124 secondary antibody, Alexa Fluor 488 and 568, (Thermo Fisher Scientific) diluted in
125 blocking solution at room temperature for one hour. Slides were then washed with PBS
126 and mounted with hard-set Vectashield with DAPI (Vectashield) and stored in the dark
127 prior and post imaging. Slides were imaged on a Zeiss LSM 880 with a 40X oil
128 objective. To test for non-specific binding, both human and mouse myocytes were
129 incubated with (1) both rabbit and mouse IgG at a concentration which matched those
130 of primary antibodies used (followed by incubation with incubation with Alexa Fluor 488
131 and 568 secondary antibodies or (2) Alexa Fluor 488 and 568 secondary antibodies in
132 the absence of primary antibodies. This imaging was performed in parallel with imaging
133 of GSK-3β and actinin specific primary antibodies and imaging conditions (laser power
134 and gain) were kept constant across imaging of control slides.
135
136 *Recombinant Z1Z2 and abLIM-1 co-immunoprecipitation*
137 50 µl of Dynabeads Protein G (Thermo Fisher Scientific) were incubated with
138 either 10 µl GST antibody (Cell Signaling) or mouse IgG diluted into 200 µl of PBS-T for
139 10 minutes at room temperature. Antibody conjugated beads were cross-linked with 20
140 mM dimethyl pimelimidate dihydrochloride in 100 mM sodium borate (pH 9).
141 Crosslinking was inactivated by washing twice with antibody blocking buffer (200 mM

142 ethanolamine pH 8). Crosslinked beads were then blocked at 4 degrees for two hours.
143 Beads were then washed three times with IAP Buffer (50 mM MOPS (pH 7.2), 10 mM
144 sodium phosphate, 50 mM NaCl). The beads were then incubated with recombinant
145 Z1Z2 and abLIM-1 diluted in PBS-T for 15 minutes at room temperature. Treatment
146 groups were as follows: IgG= 0.24 µg of Z1Z2 (a kind gift from Siegfried Labeit) and 0.5
147 µg of GST-tagged abLIM-1 (Novus Biologicals, H00003983-P01), BL= 0.24 µg of Z1Z2
148 and 0.5 µg of GST-tagged abLIM-1, GSK = 0.24 µg of Z1Z2 and 0.5 µg of GST-tagged
149 abLIM-1 that had been pre-treated with 0.5 µg of recombinant GSK-3β for 10 minutes).
150 The beads were then gently washed 6 times with PBS-T. After the final wash, the beads
151 were eluted in 0.15% Formic acid. Samples were treated with 5 mM DTT, followed by
152 10 mM Iodoacetamide, and then digested with 0.02 ug of trypsin overnight. Samples
153 were run through C18 spin columns (Thermo Fisher) and then dried in a speed vacuum.
154 Samples were then reconstituted in HPLC water with 0.15% formic acid and run on an
155 Ultimate 3000 nHPLC coupled to an Orbitrap XL Mass Spectrometer (Thermo Scientific)
156 using a 250mm EASY-Spray LC column. Data were analyzed on PEAKs software, and
157 spectra were searched against a custom Z1Z2 and abLIM-1 database.
158
159 *GSK-3β co-immunoprecipitation (IP)*
160 Myofilament enriched samples from human non-failing LV (n=5) were used for
161 these experiments. To bind the antibody to the beads, 10µg of antibody: (GSK-3β (Cell
162 Signaling Technology, 12456), GSK-3β pS9 (Cell Signaling Technology, D28E12),
163 GSK-3 (Y279/Y216 (EMD Millipore, 05-413) diluted in PBS-T were combined with 50 µl
164 of Dynabeads Protein G (Thermo Fisher Scientific) and incubated with rotation, at room

165 temperature, for 10 minutes. The beads were then separated from supernatant with a
166 magnetic stand, and the supernatant was discarded. Beads were gently washed in
167 PBS-T. To allow binding of protein to the antibody-conjugated beads, 400 µg of protein
168 was added to the beads and incubated with rotation for 10 minutes at room
169 temperature. The supernatant was kept to run as the flow-through fraction. The beads
170 were then gently washed 6 times with PBS-T. After the final wash, the beads were
171 suspended in loading buffer and boiled, and the eluted supernatant was loaded on a 4-
172 12% Bis-Tris gel (Bolt) with the corresponding flow-through fractions. The gels were
173 then fixed and silver-stained (BioRad). The reaction was stopped with 5% acetic acid
174 when the background became saturated.
175
176 *Adenovirus and pcDNA*
177 WT mouse GSK-3β (NM_019827) was purchased from Origene (Cat: MR206669) and
178 216 F and E oligonucleotides were purchased from IDT. Y216F and Y216E were
179 subcloned into the WT GSK-3β plasmid using standard methods. The WT GSK-3β
180 contained a c-terminal Flag tag, and the two mutants contained by c-terminal Flag and
181 Myc tags. Adenoviruses were generated using the Agilent Adeasy XL system (cat #
182 240009) following manufacturer protocols. Viruses were CsCl purified prior to dialysis and
183 use.
184
185 *NRVM Isolation*
186 Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 1-day old
187 rat pups. Hearts were digested with trypsin and collagenase, using the Worthington

188 Neonatal Cardiomyocyte Isolation System (Worthington). Cells were plated and allowed
189 to incubate at 37ºC for 24 hours prior to continuing experiments.
190
191 *NRVM Viral overexpression*
192 RNVMs were infected with either wild type GSK-3β, GSK-3β Y216F, GSK-3β
193 Y216E, or an empty control vector. Samples incubated for 24 hours before harvest. A
194 portion of the cell lysate was used for immunoblotting (as described above) to verify and
195 quantify the overexpression. The primary antibodies were as follows: GSK-3β (Cell
196 Signaling Technology, 12456), GSK-3β pS9 (Cell Signaling Technology, D28E12),
197 GSK-3 (Y279/Y216 (EMD Millipore, 05-413)) and tag antibodies: Myc tag (Cell Signaling
198 Technology, 2278) and Flag tag: (Cell Signaling Technology, 8146).
199 Prior to performing co-immunoprecipitation experiments, cell lysis was skinned in
200 0.3% triton for 15 minutes. The samples were centrifuged and washed twice with PBS,
201 and then solubilized in 8M urea. The samples were then sonicated and centrifuged. Co-
202 immunoprecipitation was performed as described above, with the myc-tag antibody
203 used to pull down proteins, as this tag was found on all three virus constructs. A subset
204 of the cell lysis was incubated with beads that lacked antibody, to control for non-
205 specific binding. The eluted samples and flow-throughs were run on a 4-12% Bis-Tris
206 gel and silver-stained.
207
208 *RNA extraction and cDNA synthesis*
209 RNA was extracted from LV from GSK-3β CON and KO mice using an RNeasy
210 Fibrous Tissue Kit (Qiagen), following the manufacturer protocol. 5 ug RNA was

211 synthesized to cDNA using SuperScript IV Reverse Transcriptase (Thermofisher)
212 following the manufacturers protocol.
213
214 *Real-time PCR*
215 SYBR Green PCR Master Mix (Applied Biosystems), 2 ul of cDNA, and 0.25 uM
216 of primers were used for each real-time qPCR reaction. Data was analyzed using the Δ
217 ΔΔCT method in which ABLIM-1 RNA expression was compared to that of beta actin.
218 ABLIM-1 primer Sequence:
219 F: CAC ATC AGG CTA TGA GGA CAA G
220 R: CGA TCC CGG ACA TCT TGA TAA C
221
222 Beta Actin Primer Sequence:
223 F: GAG GTA TCC TGA CCC TGA AGT A
224 R: CAC ACG CAG CTC ATT GTA GA
225
226 *siRNA*
227 abLIM-1 siRNA (Sigma-Aldrich, SASI,Rn02_00205714) or scrambled control
228 RNA (Sigma Aldrich) was transfected into iPSC cells with Lipofectamine RNAiMAX
229 Reagent (Thermofisher) following manufacturer's instructions. Briefly, Lipofectramine
230 was diluted into Opti-MEM, and then combined with diluted siRNA (10 uM). After a 10-
231 minute incubation at room temperature, the siRNA-lipid complex was added to the cells.
232 After two hours, the media was replaced with standard maintenance media. Cells were
233 harvested or used for experiments after 72 hours.
234
235 *Engineered Heart Tissues (EHTs)*

236 To prepare decellularized porcine scaffolds, fresh pig hearts (Latella Farm, West Haven,
237 CT) were preserved in ice-cold DPBS with 5% pen-strep. Left ventricular free wall
238 portion of the hearts was trimmed and frozen on dry ice before cryostat sectioning
239 (Leica CM3050 S) into 150-*µm* slices. 2.5 mm × 6 mm scaffolds were laser-cut from the
240 slices, affixed to laser-cut PTFE holders, lysed (10mM Tris and 0.1% 0.5M EDTA in DI
241 water), decellularized (0.5% wt/vol sodium dodecyl sulfate in DPBS), and incubated
242 overnight before seeding (10% FBS and 2% pen-strep in DMEM).

243 For seeding, one million cells of iPSC-CMs (derived from a healthy control line
244 GM23338, Coriell Institute) and human adult cardiac fibroblasts (PromoCell 306-05A)
245 were seeded per EHT at a 9:1 ratio. Both cell types were dissociated with TrypLE and
246 resuspended in seeding media (10% FBS, 1% pen-strep, 1% NEAA, 1% L-glutamine,
247 and 1% sodium pyruvate in DMEM). Tissues were maintained in DMEM supplemented
248 with B27 with insulin and media were changed every other day.

249 A custom mechanical testing setup composed of a force transducer and linear
250 actuators was used to characterize the EHT biomechanical properties. EHTs were
251 maintained in Tyrode's solution at physiological conditions during testing (1 Hz pacing,
252 35°C, and continuous perfusion). MATLAB scripts were used to record and process the
253 mechanical data.

254

255

Tables

Table 1

	Normality		Var
	NF	HF	
Age	0.72	0.25	0.22
EF (%)	0.035	0.31	0.7

S Table 1

	Normality		Var
	CON	KO	
Weight	0.11	0.18	0.15
HR	0.92	0.06	0.85
LVAW:d	0.22	0.96	0.08
LVAW;s	0.67	0.90	0.12
LVID;d	0.37	0.75	0.96
LVID;s	0.30	0.08	0.82
LVPW;d	0.001	0.026	0.60
LVPW;s	0.054	0.049	0.52
LV Mass AW	0.64	0.79	0.28
LV vol;d	0.66	1.00	0.96
LV Vol;s	0.57	0.41	0.58
EF	0.24	0.34	0.74
FS	0.17	0.15	0.59

T-Tests

	Normality		Var
Figure	CON	KO	
1A	0.33	0.09	0.036
1B	0.27	0.52	0.91
1F	0.89	0.78	0.84
1G	0.75	0.28	0.24
2D	0.33	0.19	0.27
2E	0.79	0.38	0.43
5C	0.90	0.33	0.82
S3A	0.32	0.76	0.72
S3B	0.51	0.86	0.79
S3C	0.77	0.49	0.41
S3D	0.88	0.99	0.84
s6A	0.18	0.43	0.81
2B			
SL	CON	KO	
1.8	0.39	0.79	0.53
2.0	0.013	0.79	0.51
2.2	0.37	0.089	0.51
2.4	0.68	0.62	0.90
2.6	0.23	0.45	0.77
	Scrambled	abLIM1	
5G	0.13	0.45	0.37
5H	7.00E-04	0.38	0.45
5I	0.98	0.24	0.10
	NF	HF	
6A	0.46	0.95	0.48
6B	0.10	0.19	0.089
S8B	7.10E-03	7.00E-04	0.05
S8C	0.53	0.86	0.57
S8D	0.51	0.25	0.46
S8E	0.97	0.12	0.07
S7A	0.053	0.91	0.19
S7B	0.14	0.12	0.40
	Hi GSK-3β	Lo GSK-3β	
S9B	0.79	0.56	0.28
S9C	0.89	0.26	0.90

One-Way ANOVAs					
		Normality			Var
Figure	CON	KO	AP		
3D	0.47	0.38	0.15		0.67
	CON	WT	Y216F	Y216E	
4D	NA	0.22	0.48	0.58	0.095
4F	0.82	0.34	0.71	0.76	0.15
	CON	KO	Delta		
s4E	0.15	0.26	0.89		0.54
s4F	0.96	0.99	0.41		0.26
s4G	0.47	0.64	0.57		0.59

256

Two-Way ANOVAs				
Figure	CON 1.9	CON 2.3	KO 1.9	KO 2.3
2A	0.44	0.38	0.044	0.55
	NF Short	NF Long	HF Short	HF Long
6E	0.088	0.079	0.97	0.44
6F	0.87	0.018	0.79	0.50
	CON BL	CON GSK	KO BL	KO GSK
S2	0.16	0.90	0.24	0.95
	Myo CON	Myo KO	Sol CON	Sol KO
s6C	0.44	0.11	0.66	0.31

257

258 **Table S1**: P-values for normality and equal variance (var) tests for all statistical tests
259 presented in the study.

260

261

Parameter	CON	KO	p-value
Weight (g)	20.76 ± 2.10	19.40 ± 3.29	0.20
HR (BMP)	376.67 ± 72.30	355.88 ± 31.24	0.48
LVAW;d (mm)	0.73 ± 0.10	0.84 ± 0.18	0.087
LVAW;s (mm)	1.0 ± 0.14	1.18 ± 0.23	0.036
LVID;d (mm)	3.87 ± 0.50	3.87 ± 0.49	0.99
LVID;s (mm)	2.84 ± 0.55	2.78 ± 0.52	0.77
LVPW;d (mm)	0.90 ± 0.39	0.87 ± 0.33	0.99*
LVPW;s (mm)	1.17 ± 0.42	1.19 ± 0.51	0.99*
LV Mass AW (mg)	113.84 ± 27.88	123.82 ± 38.93	0.48
LV Vol;d (µl)	66.04 ± 18.8	66.02 ± 18.49	0.99
LV Vol;s (µl)	32.54 ± 13.78	30.58 ± 11.63	0.71
EF (%)	52.42 ± 11.30	54.52 ± 12.52	0.67
FS (%)	26.84 ± 7.20	28.37 ± 8,50	0.64

262

263 **Table S2**: LV metrics measured by echocardiography. *n*=12 mice/group*.* P-values
264 calculated by unpaired t-test, * indicates p-values calculated by Mann-Whitney tests

265

	CON			KO		
	1.9 μm	*2.3 μm*	*p-value*	*1.9 μm*	*2.3 μm*	*p-value*
F_{max} (mN/mm^2)	17.36 ± 1.90	22.05 ± 1.97	0.00064	18.90 ± 1.67	21.38 ± 1.57	0.069
EC_{50} (μM)	1.66 ± 0.14	1.18 ± 0.13	0.000497	1.46 ± 0.14	1.43 ± 0.12	0.96

266

267 **Table S3**. LDA Measurements - F_{max} and EC_{50} average and SEM at 1.9 μm and 2.3 μm
268 SL in CON and KO mice. P-values were calculated from two-way ANOVA with multiple
269 comparisons and represent pairwise comparisons within genotypes.

270

Sarcomere Length (µm)	CON	KO
1.8	4.23 ± 0.77	3.53 ± 0.64
2.0	7.20 ± 0.91	4.89 ± 0.75
2.2	10.18 ± 1.22	7.33 ± 1.01
2.4	15.43 ± 1.28	10.31 ± 1.24
2.6	20.01 ± .62	13.87 ± 1.76

271

Sarcomere Length (µm)	CON BL	CON GSK-3β
1.8	0.666 ± 0.448	0.763 ± 0.452
2.0	1.679 ± 0.012	1.721 ± 0.011
2.2	8.763 ± 0.862	11.07 ± 1.205
2.4	12.60 ± 0.982	15.53 ± 1.248
2.6	18.12 ± 0.62	13.87 ± 1.76

Sarcomere Length (µm)	KO BL	KO GSK-3β
1.8	0.868 ± 0.688	1.378 ± 0.658
2.0	3.538 ± 0.772	5.182 ± 0.645
2.2	7.845 ± 1.101	9.668 ± 1.358
2.4	11.76 ± 1.322	14.64 ± 1.743
2.6	13.97 ± 1.427	18.00 ± 1.907

272

273 **Table S4**. Passive Tension Measurements (mN/mm^2). Average Force and SEM for
274 each sarcomere length (µm) in Con and KO mice, and Con and KO mice before (BL)
275 and after exogenous recombinant GSK-3β treatment.

276

277 **Table S5**: Mass spectrometry analysis of Con and GSK-3β KO myofilament-enriched
278 samples.

279

280 Presented in attached Excel Spreadsheet

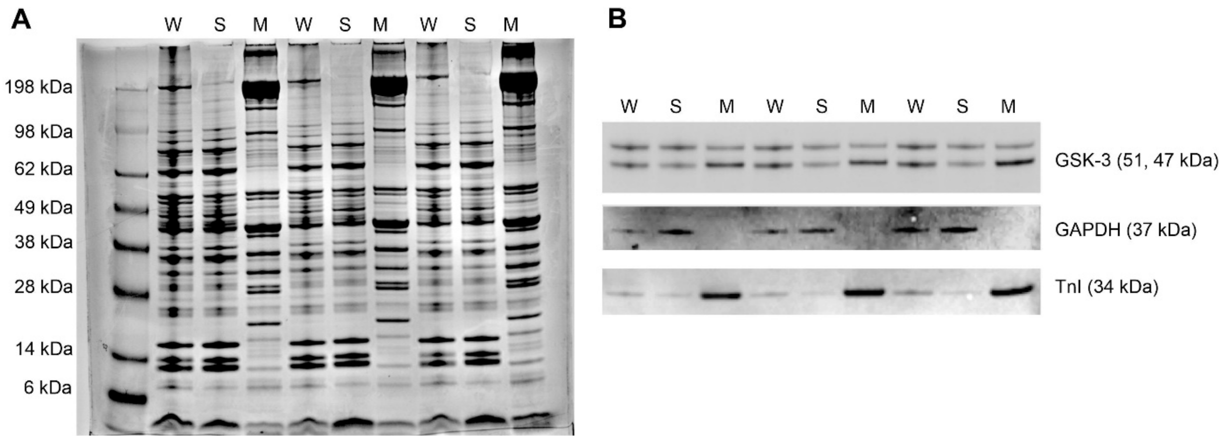
281

	NF		HF	
	1.9 µm	*2.3 µm*	*1.9 µm*	*2.3 µm*
F_{max} (mN/mm^2)	17.01± 1.63	24.52 ± 2.34	13.61 ± 1.43	20.90 ± 2.78
EC_{50} (µM)	1.28 ± 0.05	0.94 ± 0.80	0.85 ± 0.06	0.81 ± 0.07

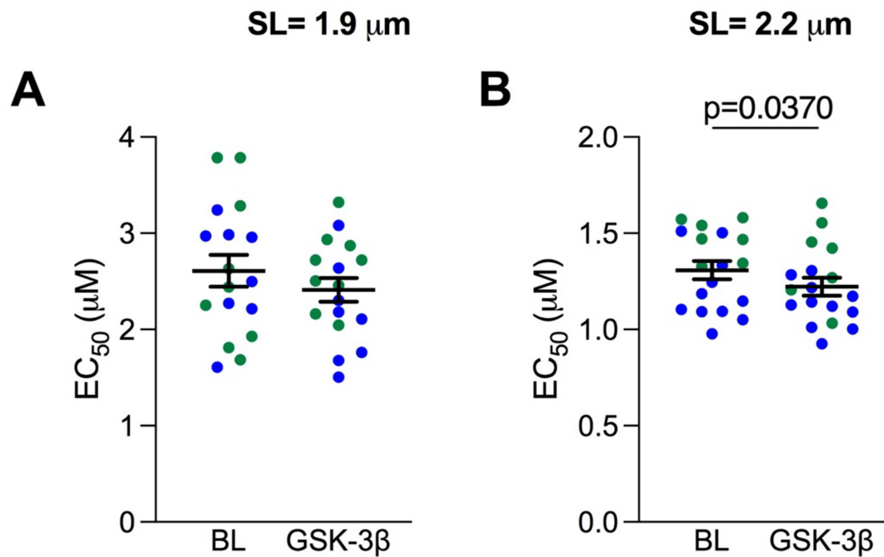
284
285

286 **Table S6.** LDA Measurements- F_{max} and EC_{50} averages and error at 1.9 µm and 2.3 µm
287 SL in NF and HF samples.

288

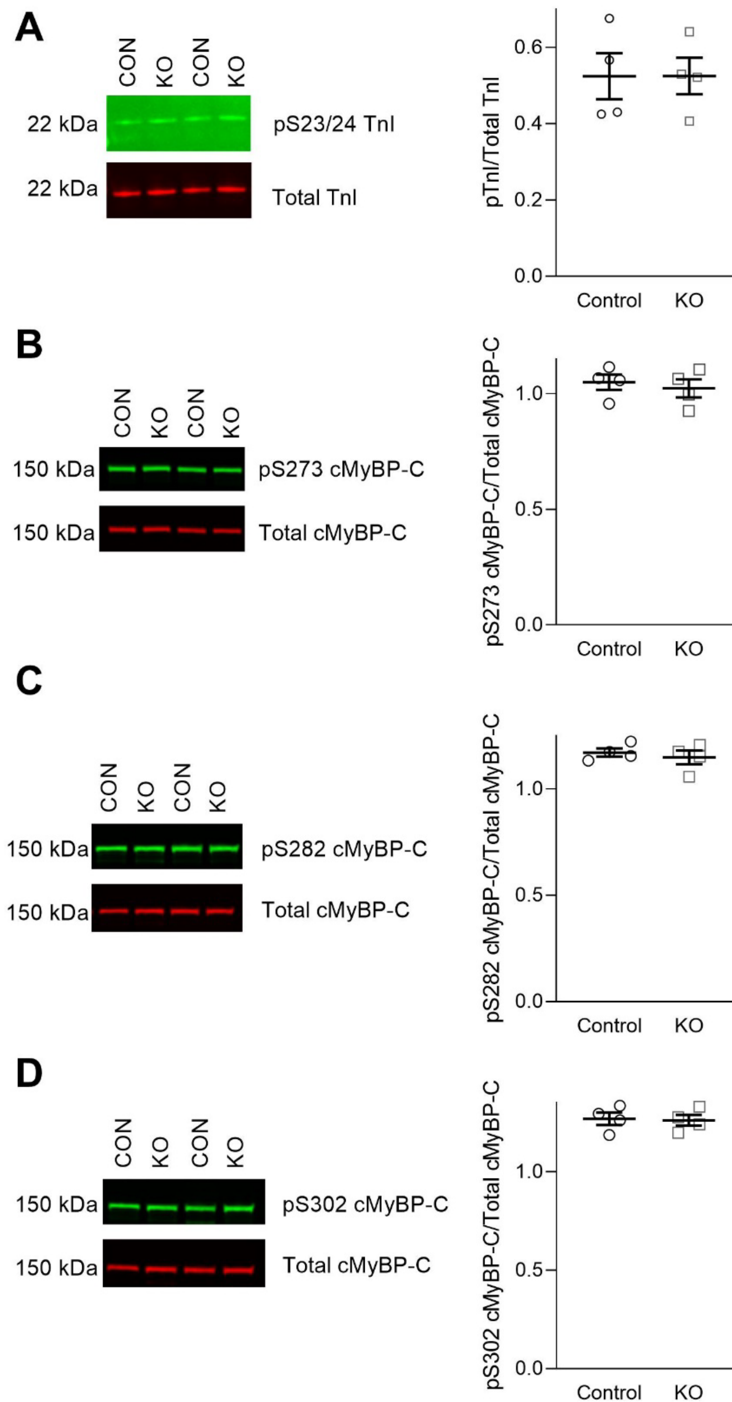


290
291 **Figure S1. Myofilament preparation of left ventricle myocardium enriches for**
292 **myofilament proteins while excluding cytosolic contents**. **(A)** Coomassie stained
293 SDS-Page gel depicting left ventricle prepared as whole tissue lysis (W), soluble
294 fraction (S), myofilament enrichment (M) **(B)** Western blot of W, S, and M samples
295 blotted for GSK-3, GAPDH, and Troponin I (TnI).
296

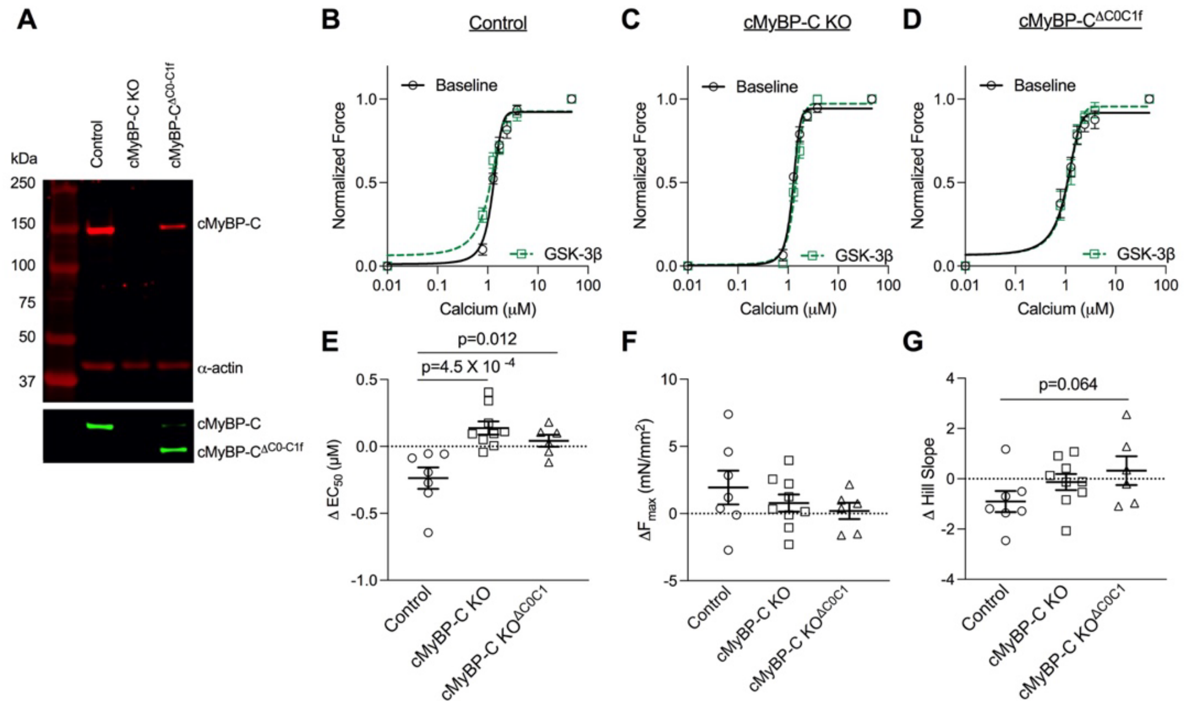


298 **Figure S2**. Summary data of EC_{50} of CON (Blue) and KO (Green) mice at baseline (BL)
299 and after GSK-3β treatment (GSK-3β) depicted as mean± SEM at a (**A**) short SL of 1.9
300 µm in Con (*n*= 8 myocytes from 3 mice) and KO (*n*= 8 myocytes from 3 mice). P-values
301 were calculated by paired t-test. Mean ± SEM is as follows: BL: 2.609 ± 0.1651, GSK-
302 3β: 2.412 ± 0.1236. (**B**) The same experiments were performed on a separate cohort of
303 animals at a long SL of 2.2 µm in CON (*n* = 11 myocytes from 4 mice) and KO (*n* = 7
304 myocytes from 3 mice). P-values were calculated by paired t-test. Mean ± SEM is as
305 follows: BL: 1.308 ± 0.047, GSK-3β: 1.222 ± 0.047.

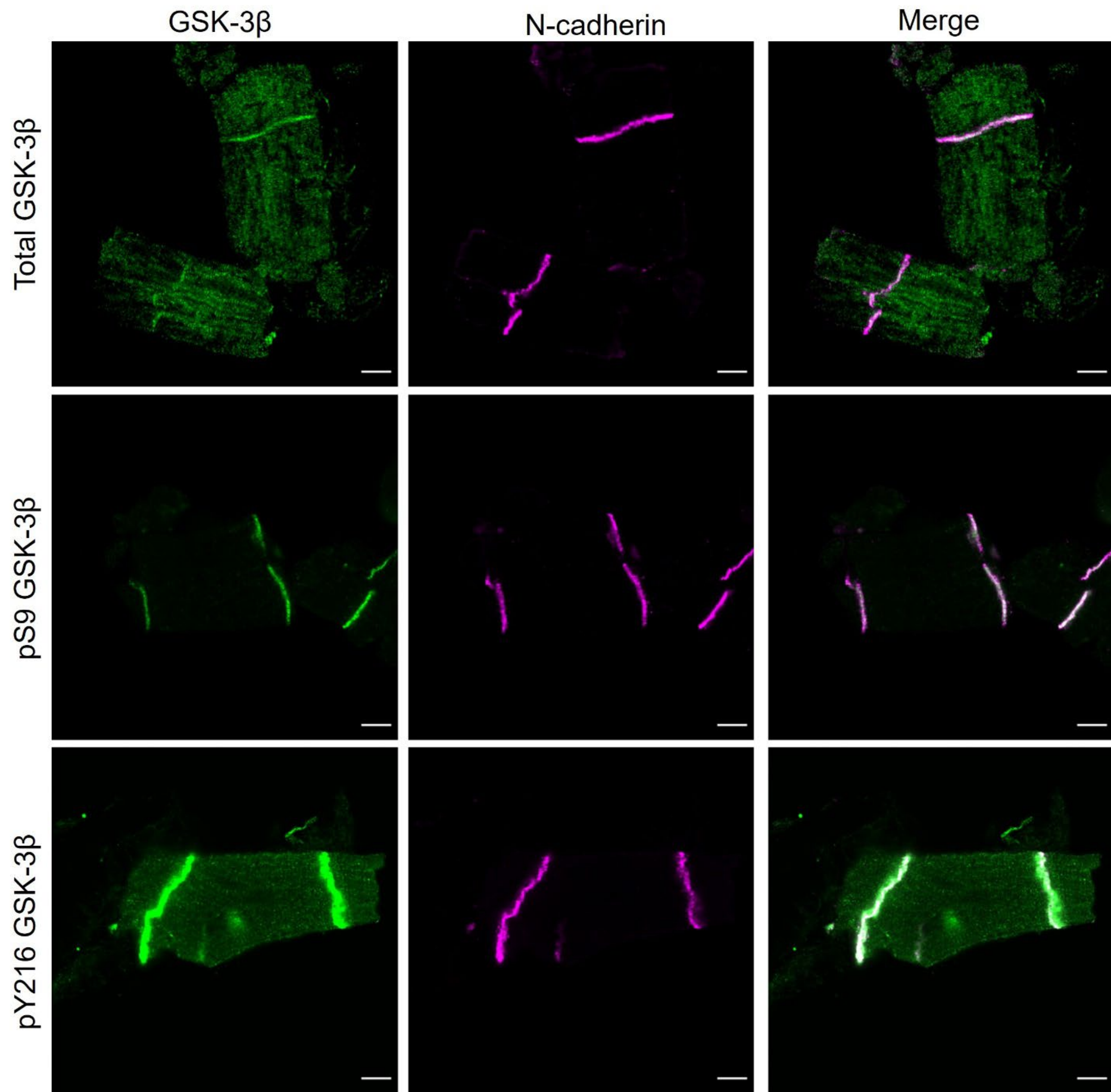
306



308 **Figure S3. Troponin I and cMyBP-C phosphorylation is unchanged in GSK-3β KO**
309 **mice (A)** Western blot depicting total Troponin I (TnI) and pS23/24 TnI in CON and
310 GSK-3β KO mice. Phosphorylation sites on cMyBP-C in control and GSK-3β KO mice
311 **(B)** pS273, **(C)** pS282, and **(D)** pS302 normalized to total cMyBP-C (*n*=4/group). Groups
312 were compared with Mann-Whitney tests.
313

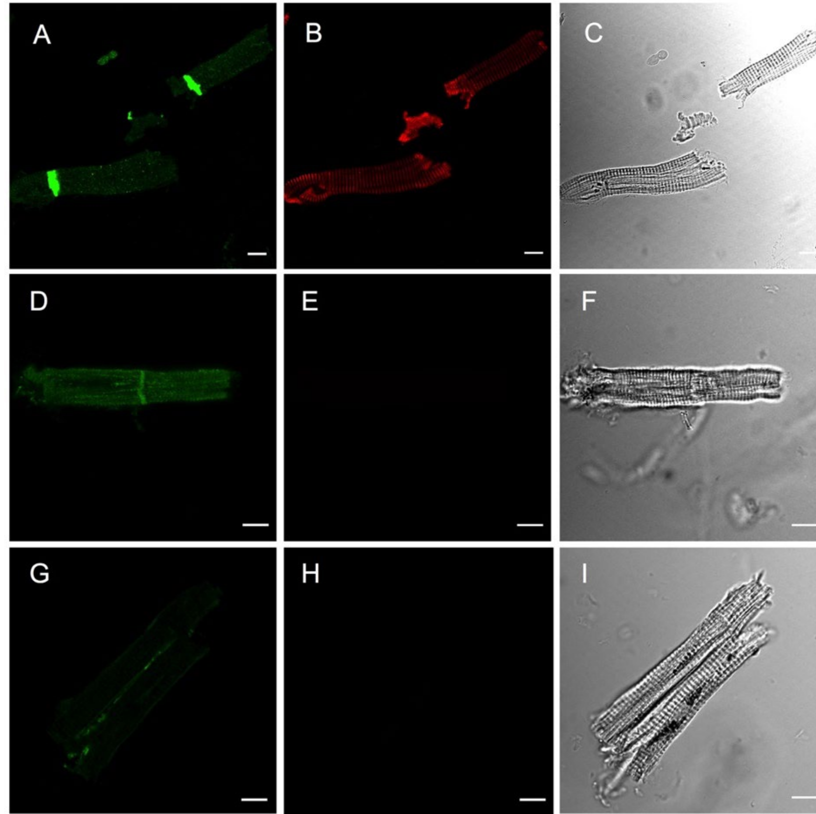


315 **Figure S4. GSK-3β is unable to modulate calcium sensitivity in cMyBP-C KO or**
316 **cMyBP-C$^{\Delta C0\text{-}C1f}$ mice**. **(A)** Representative western blot analyses demonstrating the
317 level of cMyBP-C in cMyBP-C KO and cMyBP-C$^{\Delta C0\text{-}C1f}$ mouse hearts, compared to
318 control mouse hearts. The upper panel was performed with both amino terminal-specific
319 cMyBP-C[31] and actin antibodies. The bottom panel was treated with carboxy terminal-
320 specific cMyBP-C antibodies[31]. **(B)** Mean force as a function of calcium concentration
321 and fitted curves for skinned myocytes at baseline and with exogenous GSK-3β
322 treatment from LV from Control (*n*= 3 mice, 2-3 cells/mouse) **(C)** MyBP-C KO (n= 3
323 mice, 3 cells/ mouse) and (**D**) MyBP-C$^{\Delta C0C1f}$ (*n*= 3 mice, 2 cells/mouse) mice. The SL
324 was set to 2.1 µm for all experiments. Summary data of **(E)** ΔEC_{50} Mean ± SEM:
325 Control: -0.237 ± 0.080, cMyBP-C KO: 0.137 ± 0.064, MyBP-C$^{\Delta C0C1f}$: 0.042 ± 0.045 **(F)**
326 ΔF_{max} Mean ± SEM: Control: 1.939 ± 1.26, cMyBP-C KO: 0.78 ± 0.77, MyBP-C$^{\Delta C0C1f}$:
327 0.198 ± 0.609 and **(G)** ∆Hill slope Mean ± SEM: Control: -0.901 ± 0.419, cMyBP-C KO:
328 -0.128 ± 0.405, MyBP-C$^{\Delta C0C1f}$: 0.321 ± 0.057 . Statistics were calculated by one-way
329 ANOVA.
330
331



333 **Figure S5. GSK-3β localizes to the intercalated disc.** Human NF myocytes depicting
334 either Total, pS9, or pY216 GSK-3β (green) counterstained against n-cadherin (pink).
335 Scale bar = 10 um.

336



338 **Figure S6. Antibody controls in human LV.** Human LV stained with primary
339 antibodies (**A**) total GSK-3β and (**B**) and actinin, with (**C**) corresponding brightfield
340 image. IgG controls of (**D**) rabbit IgG and (**E**) mouse IgG and (**F**) corresponding
341 brightfield image. Secondary controls of (**G**) alexa-fluor 488 anti-rabbit and (**H**) alexa-
342 fluor 568 anti-mouse with (**I**) corresponding brightfield image. Scale bars = 10 microns.

343

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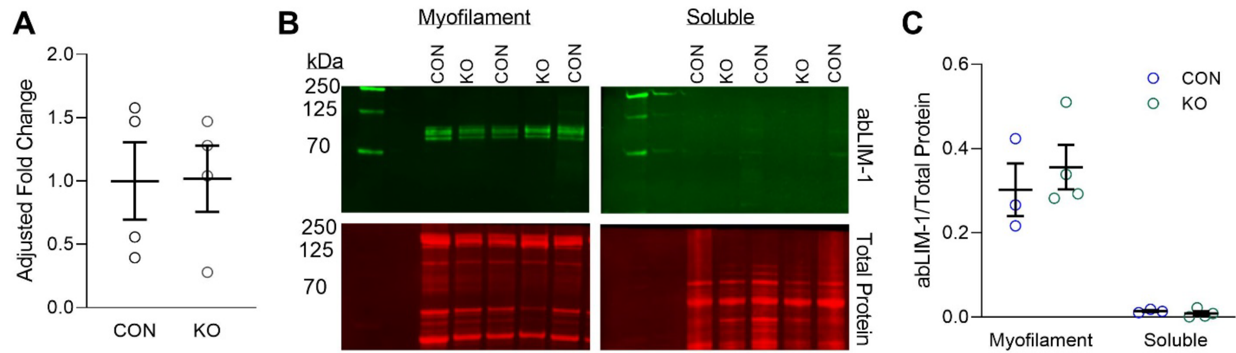
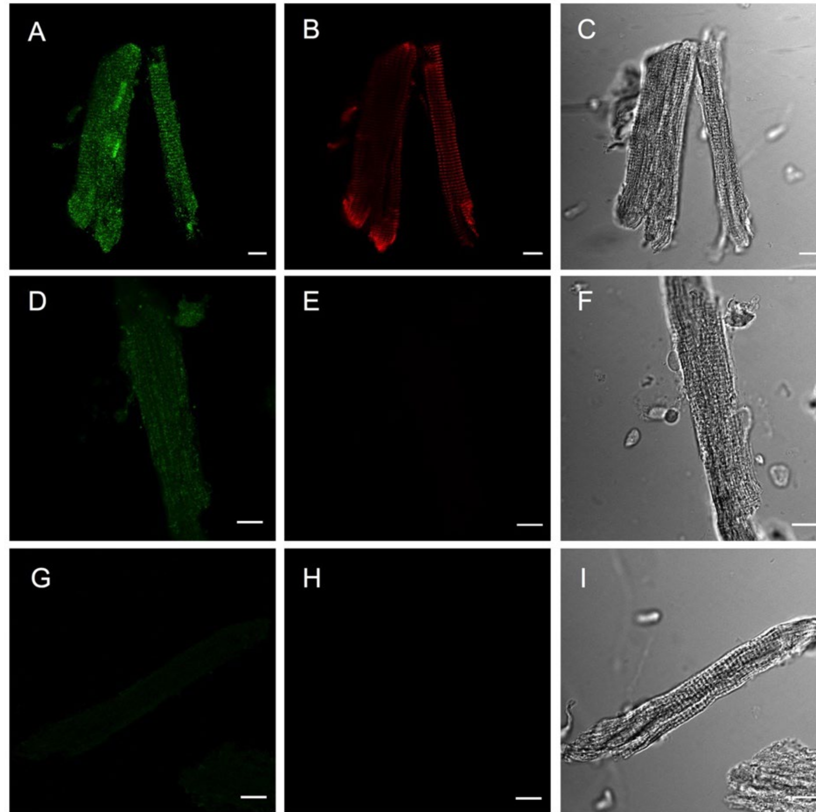


346 **Figure S7. abLIM-1 characterization in GSK-3β KO mice (A)** RNA transcript levels of
347 abLIM-1 in GSK-3β CON and KO mice (*n*=4). Statistics were calculated by Mann-
348 Whitney test. **(B)** abLIM-1 and total protein in myofilament and soluble fractions **(C)**
349 myofilament and soluble abLIM-1 normalized to total protein in GSK-3β CON (*n*=3) and
350 KO (*n*=4) mice. Statistics were calculated by two-way ANOVA.
351



353 **Figure S8**. **Antibody controls in mouse LV**. Mouse LV stained with primary antibodies
354 (**A**) total GSK-3β and (**B**) and actinin, with (**C**) corresponding brightfield image. IgG
355 controls of (**D**) rabbit IgG and (**E**) mouse IgG and (**F**) corresponding brightfield image.
356 Secondary controls of (**G**) alexa-fluor 488 anti-rabbit and (**H**) alexa-fluor 568 anti-mouse
357 with (**I**) corresponding brightfield image. Scale bars = 10 microns.

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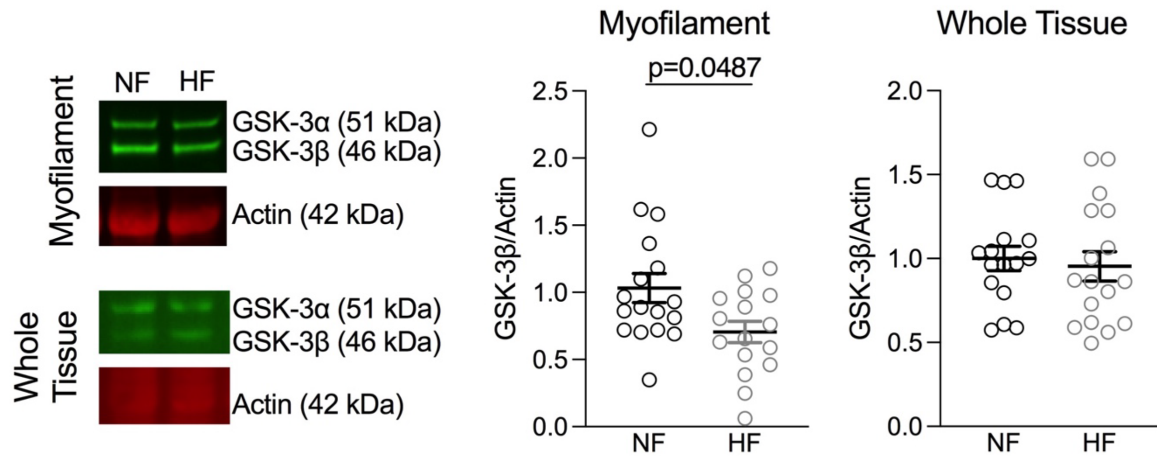
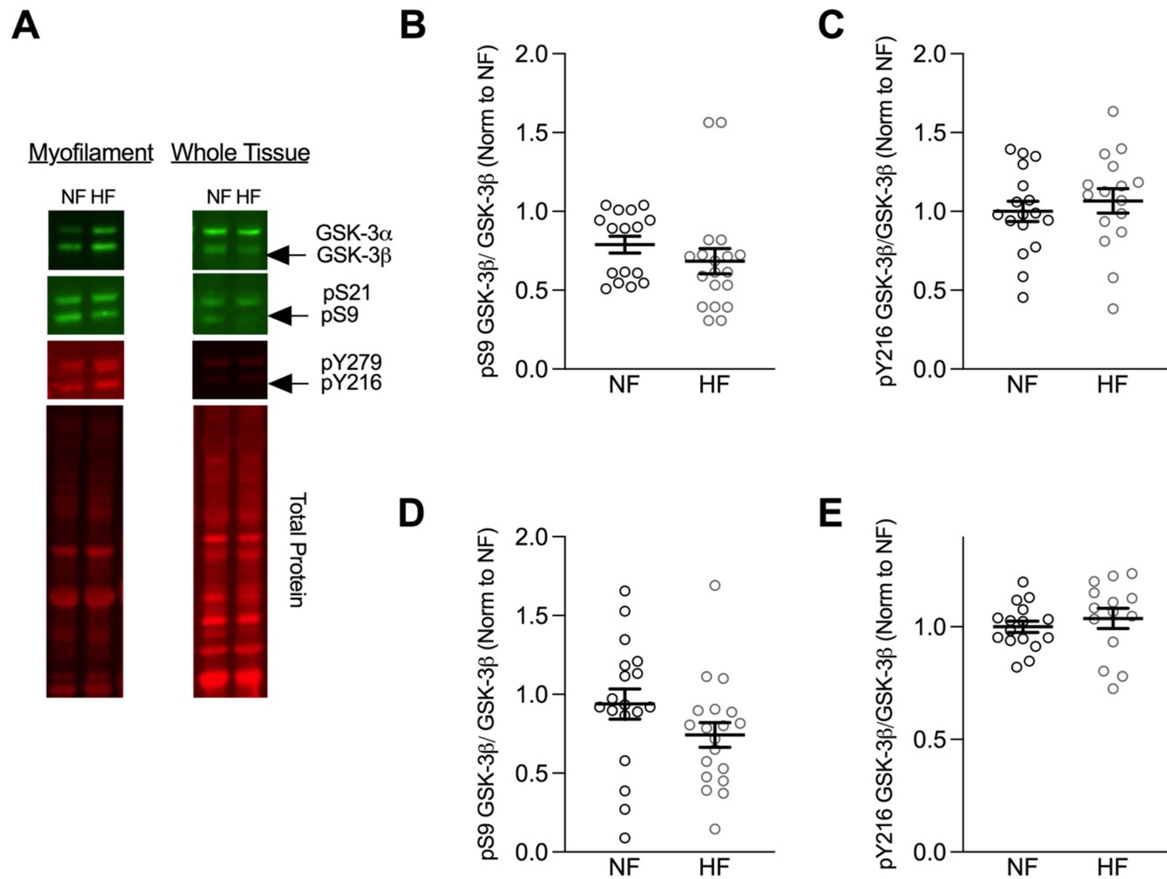
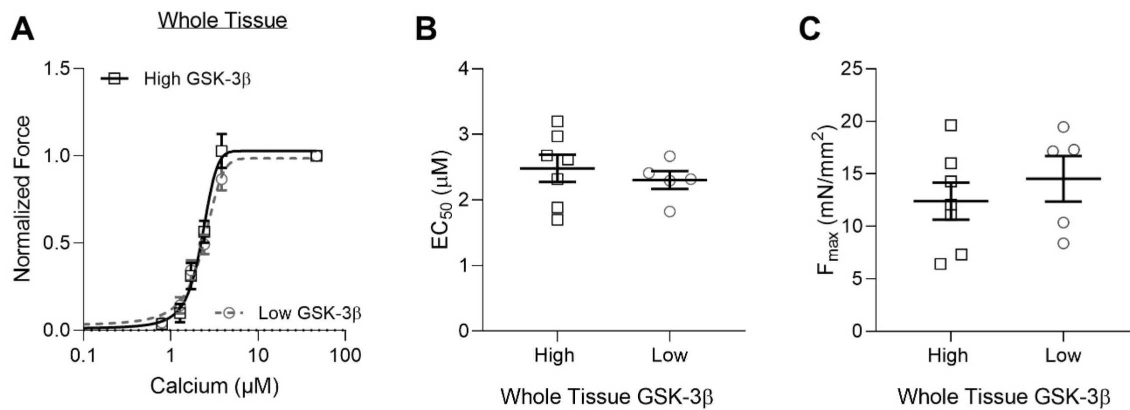


361
362 **Figure S9. Myofilament GSK-3β is reduced when normalized to actin.** Myofilament
363 (p=0.049 by Mann Whitney test*)* and Whole tissue preparations of NF and HF LV,
364 normalized to the myofilament protein Actin. *n* values are as follows: NF myofilament =
365 17, KO myofilament = 16, NF whole tissue = 16, HF whole tissue = 17.
366



368 **Figure S10. pS9 and pY216 are unchanged in heart failure.** (**A**) Representative
369 western blot from myofilament enriched and whole tissue preparations from NF and HF
370 patients. **(B**) pS9 GSK-3β normalized to total GSK-3β in myofilament enriched tissue
371 from NF (*n*=16) and HF (*n*=19) patients. P-value was calculated from a non-parametric
372 t-test (**C**) pY216 GSK-3β normalized to total GSK-3β in myofilament enriched tissue in
373 NF (*n*= 17) and HF (*n*= 16) patients. (**D**) pS9 GSK-3β normalized to total GSK-3β in
374 whole tissue prep from NF (*n*= 18) and HF (*n*= 19) patients. (**E**) pY216 GSK-3β
375 normalized to total GSK-3β in whole tissue lysis in NF (*n*=16) and HF (*n*=14) patients.
376
377



379 **Figure S11. Whole tissue GSK-3β does not correlate with calcium sensitivity in**
380 **human myocytes. (A)** Force-calcium curves of human LV myocytes sorted by whole
381 tissue GSK-3β, low (*n*= 3 samples, 7 cells) and low (*n*= 3 samples, 5 cells). **(B)** EC_{50}
382 and **(C)** F_{max} for whole tissue sorted samples. Statistics were calculated by Mann-
383 Whitney test.
384