Supplemental Material

Supplemental Figures

Figure S1. Experimental design and pilot study of chronic AAV9-TTL treatment in mice *in vivo*. A) Plasmid map of TTL-IRES-dsRed under the control of the human cardiac troponin T (cTnT; *TNNT2*) promoter (PENN.AAV.cTNT.TTL.IRES.dsRed). **B)** Protocol: wild-type young adult male mice (6 weeks of age) received either an adeno-associated virus serotype 9 (AAV9) encoding TTL-dsRed at a dose of 5.625E11 vg/g (N=6) or a sham injection (N=6). After 6 weeks, ventricular myocardium was harvested and crude proteins were extracted **C)** Representative Western blot and **D)** quantification of TTL, α-tubulin (α-tub) and detyrosinated tubulin (dTyr-tub), normalized to GAPDH or α-tubulin. Data are expressed as mean ± SEM. Statistical significance was assessed with the unpaired Student's t-test. **E)** Schematic linear representation of the vector expressing the HA-tagged human WT *TTL* cDNA (AAV9-TTL; upper part) or nothing (AAV9-Empty; lower part) under the control of the *TNNT2* promoter. **F)** Experimental protocol of AAV9-mediated gene transfer in mice, including the number of *Mybpc3*-targeted KI and WT mice used and the timeline of experiments. Abbreviations: N, number of mice; ns, non-significant, WB, Western blot.
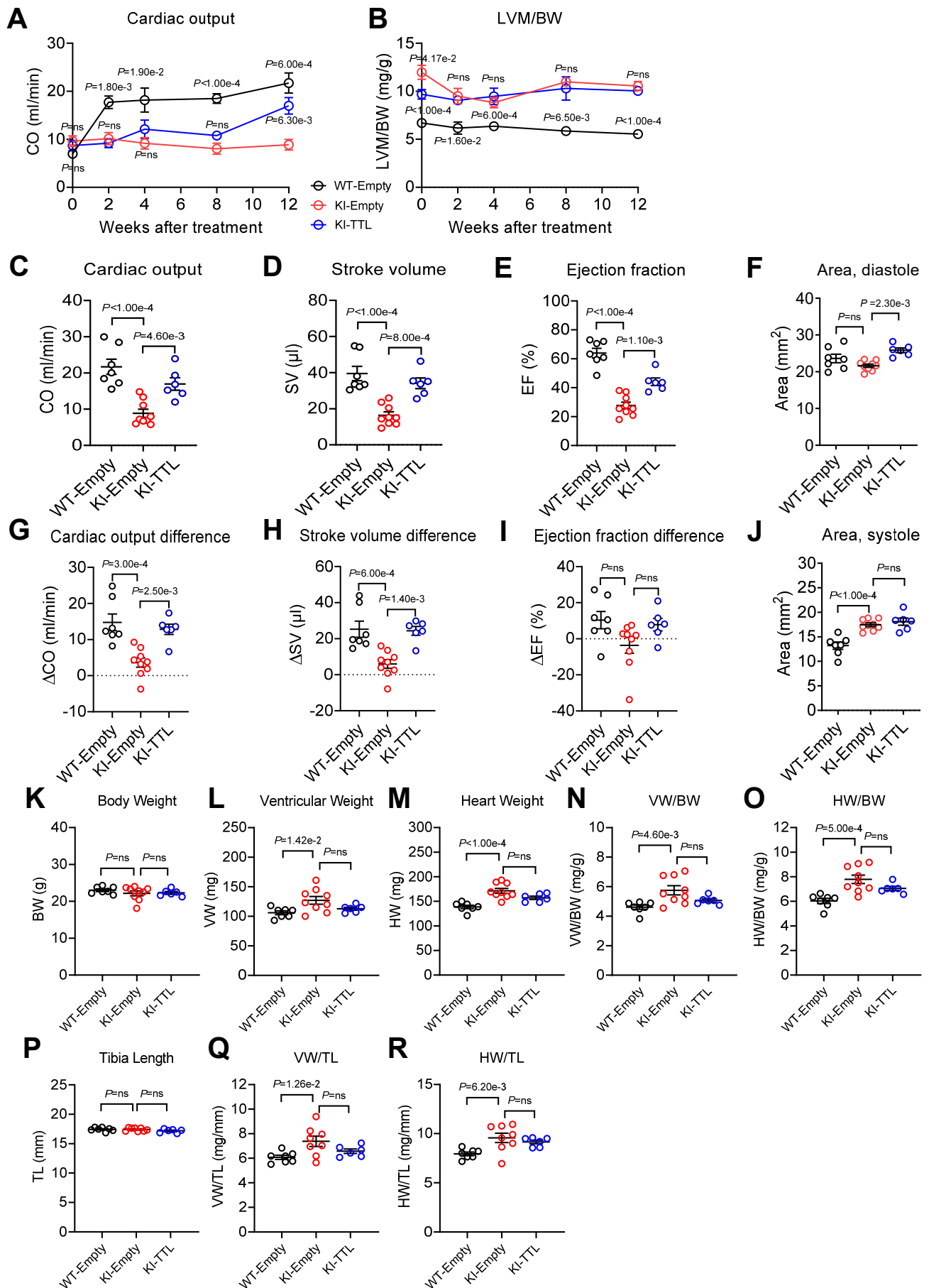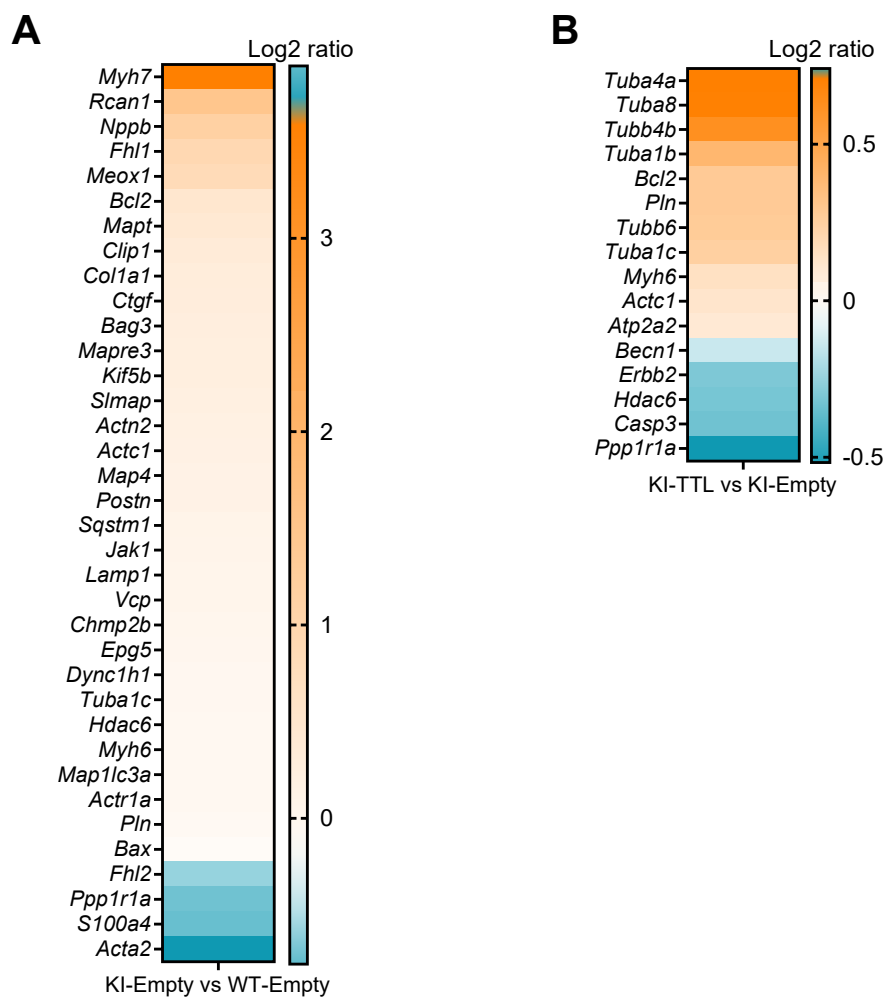
Figure S2. Generation and characterization of SVBP-KO and TTL-KO hiPSC lines. A) Schematic of knock-out (KO) approach for SVBP (left) and TTL (right) panel. **B)** Sequencing of KO DNA clones. PCR fragments for amplicons internal (Int) and external (Ext) of the large deletion are shown for SVBP-KO and WT DNA (upper) and for TTL-KO and WT DNA (lower). Alignment of the sequencing result for external fragments of the KO clones are shown on the right, confirming large, homozygous deletions (9,352 bp for SVBP-KO and 20,604 bp for TTL-KO. **C)** Nanostring karyotype panel analysis for WT and KO cell lines. **D)** Measurement of pluripotency by flow-cytometry SSEA-3 staining of hiPSC cultures. RT-qPCR results for **E)** *SVBP* and **F)** *TTL* mRNA levels in 30-day-old WT and KO hiPSC-derived cardiomyocytes (N=3). Data are expressed as mean ± SEM, with *P* values obtained with Brown-Forsythe & Welch ANOVA test and Dunnett's T3 multiple comparisons test. Abbreviations: N, number of samples; ns, non-significant (*P*>0.05); *P*, adjusted P-value.
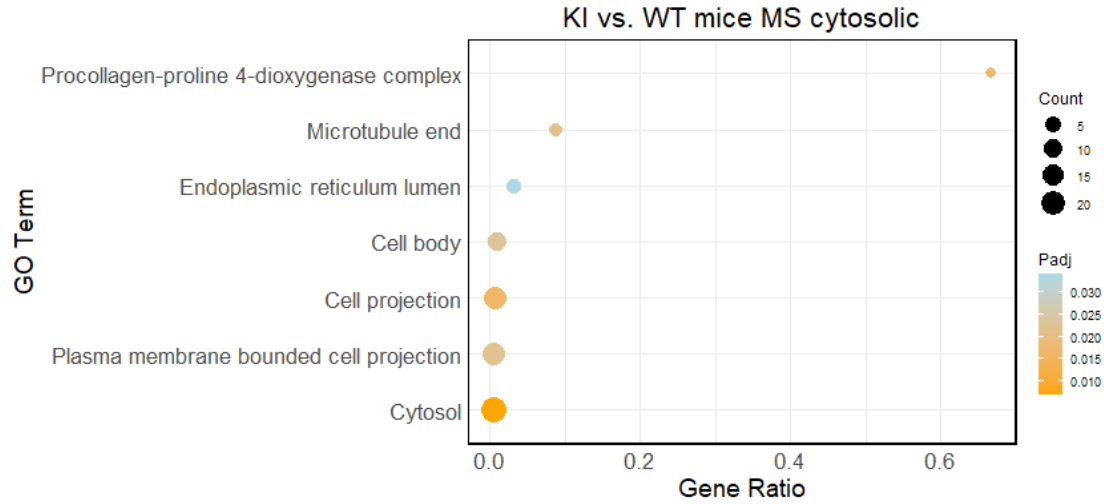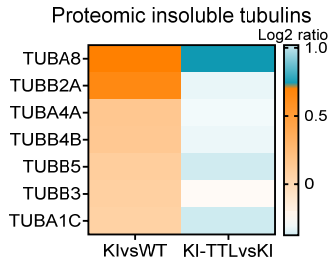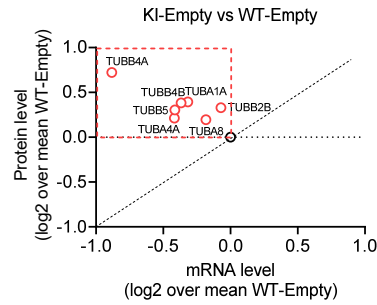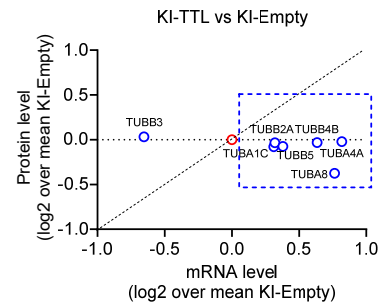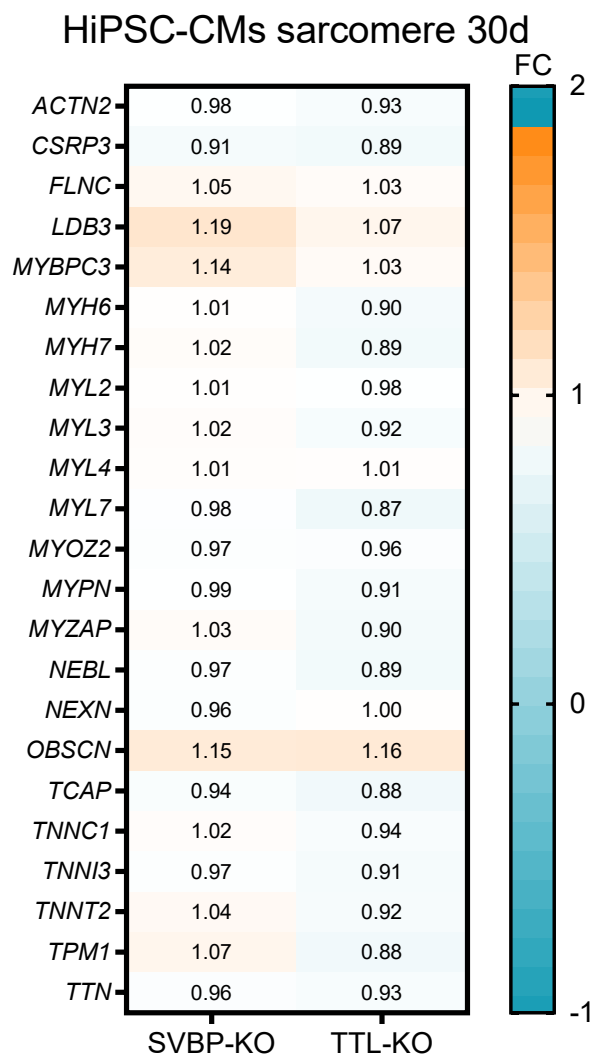
Figure S3. Evaluation of the cardiac phenotype by echocardiography overtime and tissue weights in female mice. Three-week-old wild-type (WT) and *Mybpc3*-targeted knock-in (KI) mice were included and received either AAV9-Empty (no insert) or AAV9-TTL (HA-tagged human tubulin tyrosine ligase). **(A-J)** Selected echocardiographic parameters overtime for **A)** Cardiac output (CO) and **B)** Left ventricular mass to body weight (LVM/BW) ratio overtime. After 12 weeks, parameters of **C)** CO, **D)** Stroke volume (SV), **E)** Ejection fraction (EF), **F)** LV area in diastole, **J)** LV area in systole. Difference between parameters obtained at 12 weeks and day 0 (Δ) for **G)** CO, **H)** SV and **I)** EF. **(K-R)** Cardiac tissue weights in relation to body weight (BW) or tibia length (TL): **K)** BW, **L)** Ventricular weight (VW), **M)** Heart weight (HW), **N)** VW/BW, **O)** HW/BW, **P)** TL, **Q)** VW/TL and **R)** HW/TL. Data are expressed as mean ± SEM. N = 7 (WT-Empty), 9 (KI-Empty) and 6 (KI-TTL), except in panels P-R where 1 WT-Empty TL was broken. Statistical significance was assessed vs KI-Empty by fitting a mixed-effect model with the Geisser-Greenhouse correction (panels A and B) or by one-way ANOVA (panels C-R), each followed by Dunnett's multiple comparisons test. Abbreviations: N, number of mice; ns, non-significant (*P*>0.05); *P*, adjusted P-value.
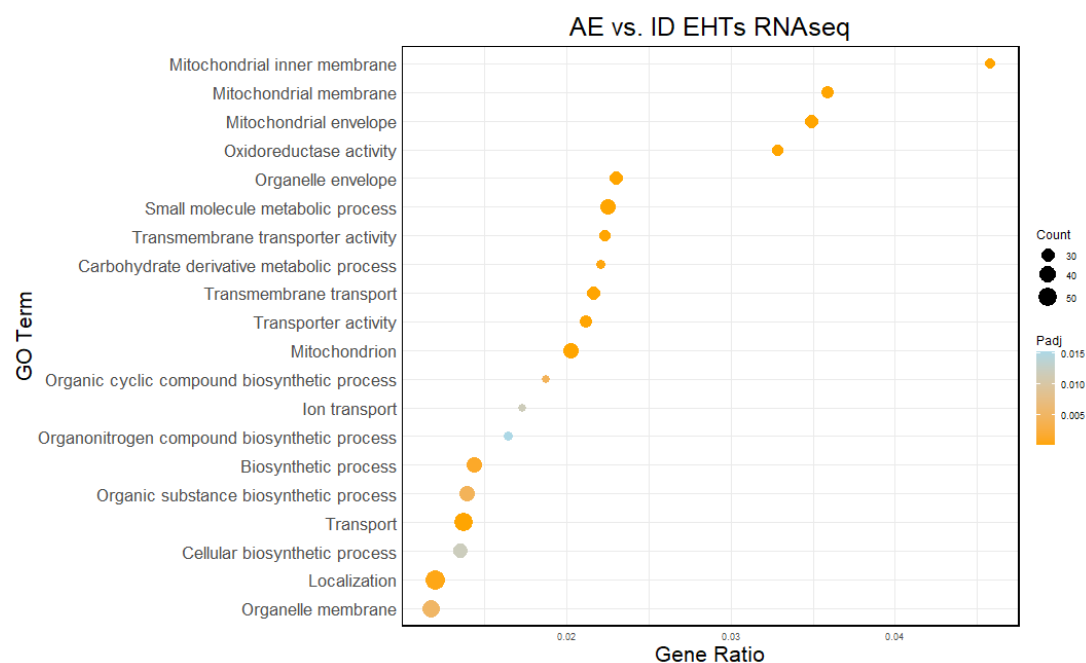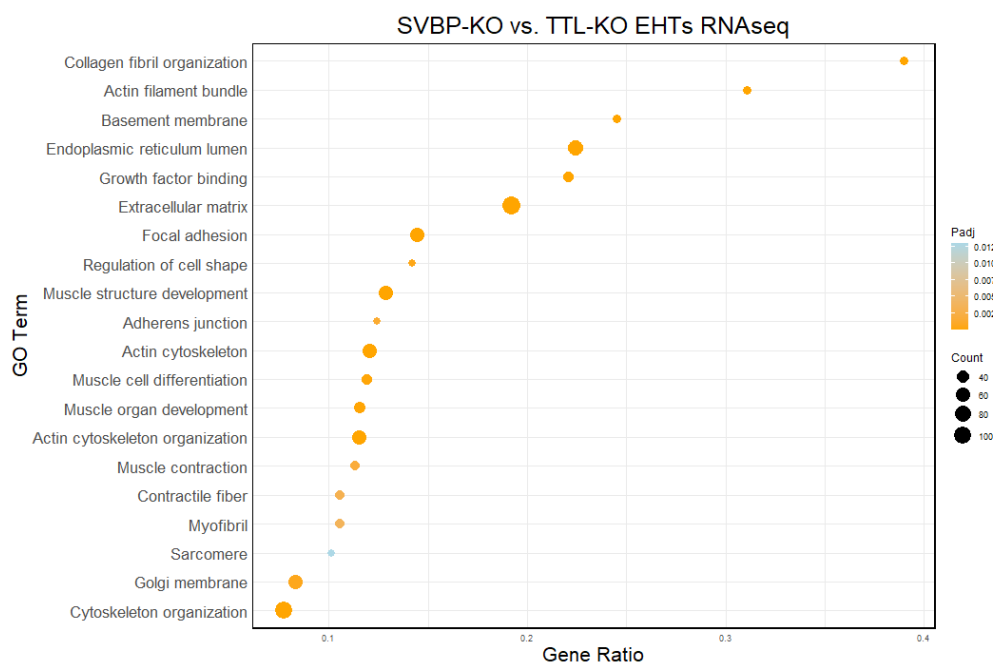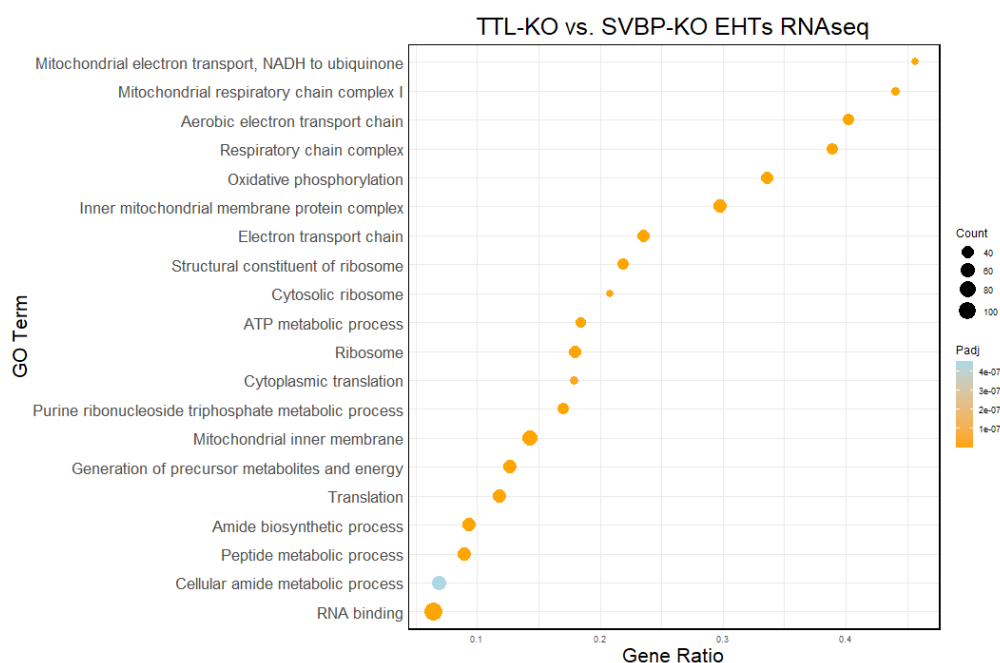
Figure S4. mRNA quantification in mice with the nanostring technology. Quantification of levels of mRNAs encoding protein dysregulated in heart failure or involved in microtubules and autophagy using the nanostring nCounter Elements technology and customized mouse-specific panels in **A)** KI-Empty vs WT-Empty and **B)** KI-TTL vs KI-Empty (p<0.05; N=7 (WT-Empty), 9 (KI-Empty) and 6 (KI-TTL)). Data are expressed as log2 ratio, which represents the difference in log2 mean value in tested samples vs reference mean, which was set to 0. Statistical significance was assessed with the unpaired Student's t-test for each gene of interest and only significantly dysregulated genes are shown. Abbreviations: N, number of female mouse myocardial samples.
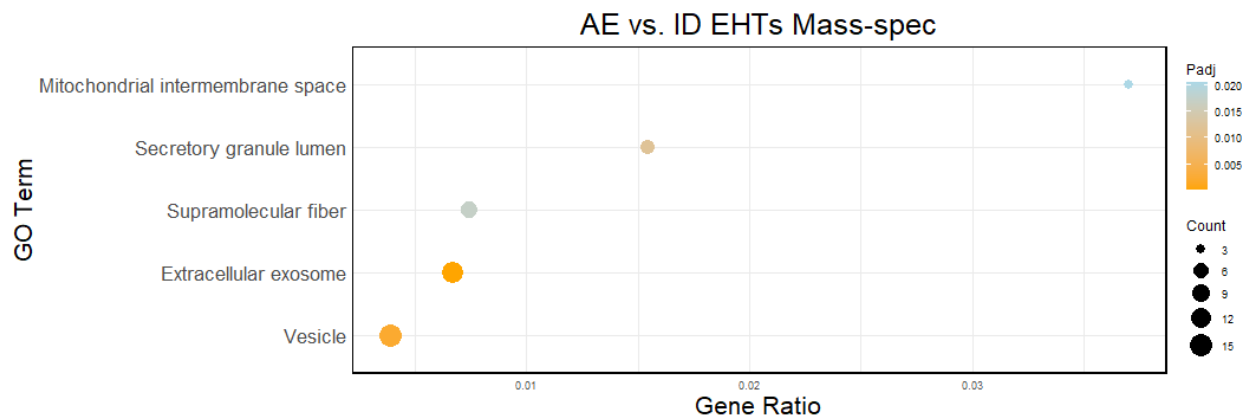
A

B

C

D

Figure S5. Selected proteome and transcriptome analysis in mice. A) Dot plot of GO pathway enrichment based on MS analysis of cytosolic LV protein fractions with significantly overrepresented pathways (*Padj*<0.05) in KI-Empty vs WT-Empty (log2 ratio >0.58). The dot size is proportional to the number of gene or protein counts, the x-axis shows the gene or protein ratio, and the heatmap colour shows the extent of adjusted P-value (*Padj*) from the lowest (orange) to the highest (blue). **B)** Heatmap of log2 ratio of tubulin isoforms in KI-Empty vs. WT-Empty and in KI-TTL vs. KI-Empty in the insoluble protein fraction. *Padj* were <0.05 only in KI-Empty vs. WT-Empty **C)** Correlation of dysregulated proteins with their mRNA levels in KI-Empty vs WT-Empty. **D)** Correlation of proteins and mRNA levels in KI-TTL vs. WT-Empty. Data from RNA-seq were obtained from 3 female mice per group, whereas data obtained from mass spectrometry analysis were obtained from N = 11 (WT-Empty), N=9 (KI-Empty) and N=10 (KI-TTL). Abbreviations: LV, left ventricular; N, number of mice; *Padj*, adjusted p-value.

HiPSC-CMs sarcomere 30d

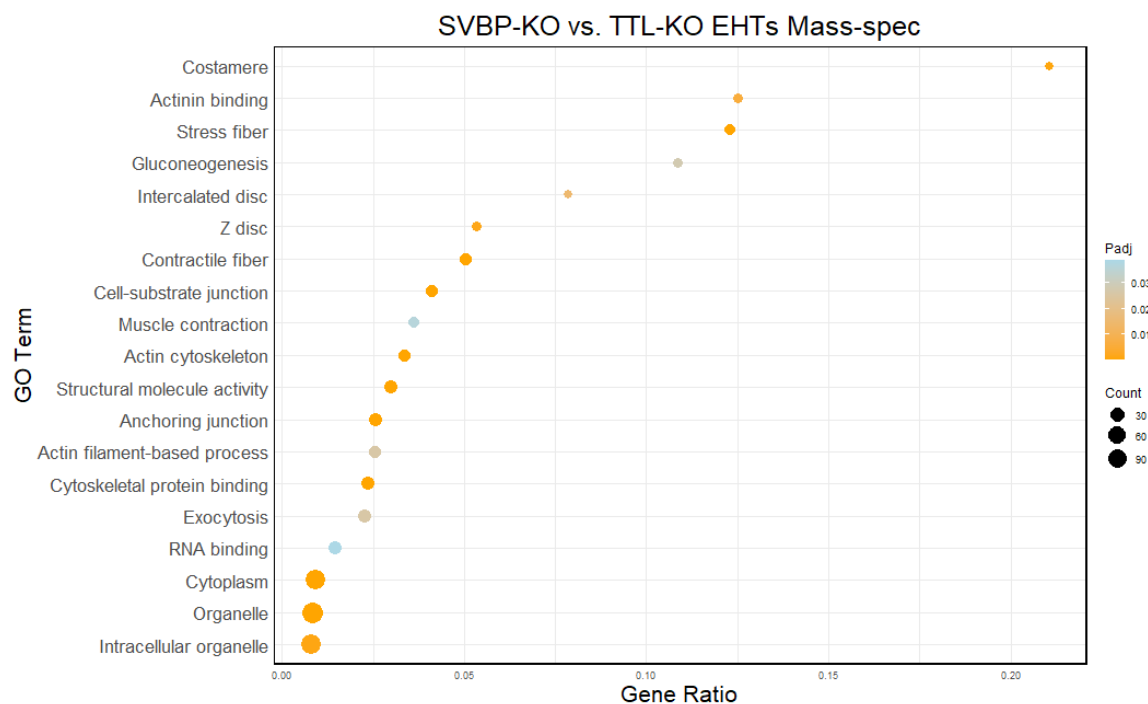|	SVBP-KO	TTL-KO
ACTN2	0.98	0.93
CSRP3	0.91	0.89
FLNC	1.05	1.03
LDB3	1.19	1.07
MYBPC3	1.14	1.03
MYH6	1.01	0.90
MYH7	1.02	0.89
MYL2	1.01	0.98
MYL3	1.02	0.92
MYL4	1.01	1.01
MYL7	0.98	0.87
MYOZ2	0.97	0.96
MYPN	0.99	0.91
MYZAP	1.03	0.90
NEBL	0.97	0.89
NEXN	0.96	1.00
OBSCN	1.15	1.16
TCAP	0.94	0.88
TNNC1	1.02	0.94
TNNI3	0.97	0.91
TNNT2	1.04	0.92
TPM1	1.07	0.88
TTN	0.96	0.93

Figure S6. Quantification of levels of mRNAs encoding protein of the cardiomyocyte sarcomere in 30-day-old hiPSC-derived cardiomyocytes. Wild-type (WT), SVBP-KO and TTL-KO human iPSC lines were differentiated in cardiomyocytes and maintained for 30 days in complete medium. Extraction of RNA was performed (n=3 per group), pooled and analyzed with the nanostring nCounter Elements technology and a customized human-specific sarcomeric panel. Data are expressed as fold-change over the mean of WT (not shown). Overall, no major difference in the gene expression level was observed for all analysed sarcomeric genes between SVBP-KO and TTL-KO when compared to WT. These data support the high and not different percentage of TNNT2+ cells as determined by FACS analysis between the groups.
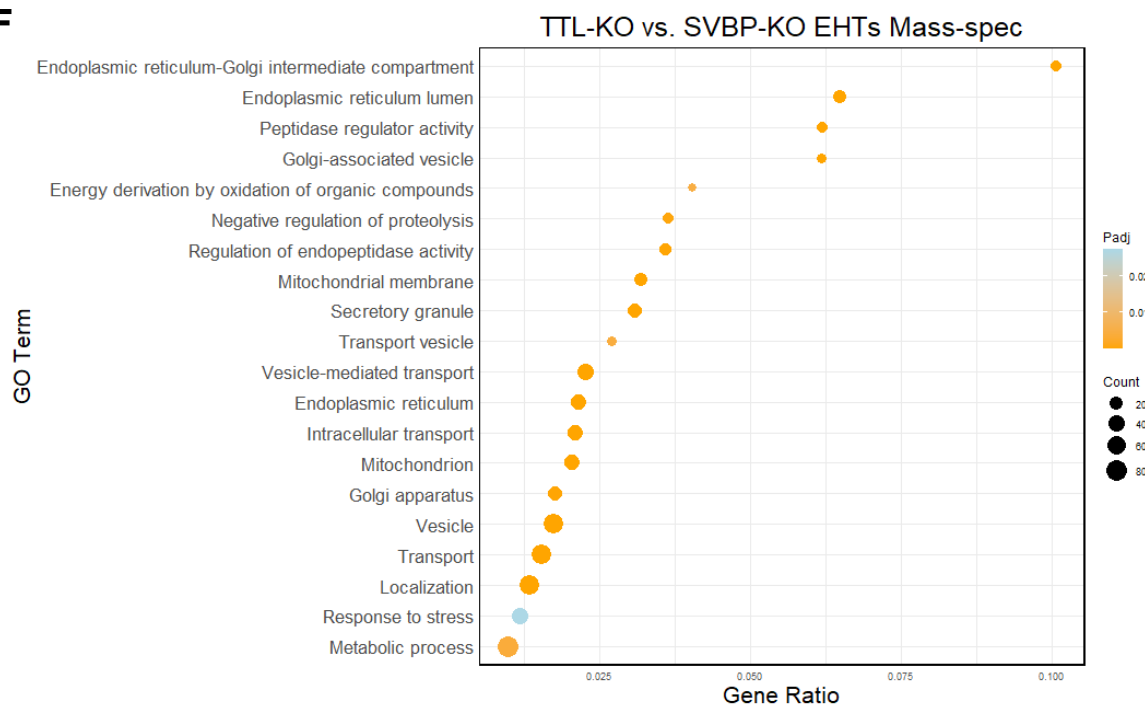
A

B

C

Figure S7…. Continued on next page with the legend

Figure S7. GO pathway enrichment analysis in RNA-seq and mass spectrometry in hiPSC-derived engineered heart tissues (EHTs). Dot plot of GO pathway enrichment map based on RNA-seq with significantly overrepresented pathways (*Padj*<0.05) in **A)** WT-EHTs cast on stiff vs. standard posts (log2 ratio>0.58, N=3 per group), **B)** SVBP-KO vs. TTL-KO cast on stiff posts (log2 ratio>0.58, N=2 vs. 3, respectively) and **C)** TTL-KO vs. SVBP-KO cast on stiff posts (log2 ratio>0.58, N=3 vs. 2, respectively). One SVBP-KO sample was excluded from the analysis. Dot plot of ALL pathway enrichment map based on MS analysis with significantly overrepresented pathways (*Padj*<0.05) in **D)** WT-EHTs cast on stiff vs. standard posts (log2 ratio>0.58, N=3 per group), **E)** SVBP-KO vs. TTL-KO cast on stiff posts (log2 ratio>0.58, N=3 per group) and **F)** TTL-KO vs. SVBP-KO cast on stiff posts (log2 ratio>0.58, N=3 per group). The dot size is proportional to the number of gene or protein counts, the x-axis shows the gene or protein ratio, and the heatmap colour shows the extent of adjusted P-value (*Padj*) from the lowest (orange) to the highest (blue).

Supplemental Tables

Table S1. Customized NanoString's nCounter® Elements TagSet panels for mouse or human RNA analysis

Heart failure panel

Acronym	Full name
Abcf1	ATP binding cassette subfamily F member 1
Actb	Actin, beta
Acta1	Actin, alpha, skeletal muscle
Actc1	Actin, alpha, cardiac muscle 1
Actn2	Actinin alpha 2
Atp2a2	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2
Bax	BCL2 associated X, apoptosis regulator
Bcl2	BCL2, apoptosis regulator
Casp3	Caspase 3
Casq2	Calsequestrin-2
Cdh5	Cadherin 5, type 2, VE-cadherin
Cltc	Clathrin heavy chain
Col1a1	Collagen type I alpha 1
Col3a1	Collagen type III alpha 1
Ctgf	Connective tissue growth factor
Fhl1	Four-and-a-half-LIM-domains 1
Fhl2	Four-and-a-half-LIM-domains 2
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Meox1	Mesenchyme homeobox 1
Myh6	Myosin heavy chain 6
Myh7	Myosin heavy chain 7
Nfkb1	nuclear factor kappa B subunit 1
Nppa	Natriuretic peptide A
Nppb	Natriuretic peptide B
Pgk1	Phosphoglycerate kinase 1
Pln	Phospholamban
Postn	Periostin
Ppp1r1a	Protein phosphatase 1, regulatory (inhibitor) subunit 1A (PP-I-1)
Rcan1	Regulator of calcineurin 1
Ryr2	Ryanodine receptor 2
S100a4	S100 calcium binding protein A4 (=FSP1)
Srf	Serum response factor
Vwf	von Willebrand factor

Autophagy panel

Acronym	Full name
Bag3	BCL2 associated athanogene 3
Becn1	Beclin-1
Chmp2b	Charged multivesicular body protein 2B
Epg5	Ectopic P-granules autophagy protein 5 homolog
Erbb2	Erb-b2 receptor tyrosine kinase 2
Foxo1	Forkhead box O1
Fyco1	FYVE and coiled-coil domain containing 1
Hdac6	Histone deacetylase 6

Table S1…. Continued on next page

Jak1	Janus kinase 1
Lamp1	Lysosomal-associated membrane protein-1
Lamp2	Lysosomal-associated membrane protein-2
Map1lc3a	Microtubule associated protein 1 light chain 3 alpha
Map1lc3b	Microtubule associated protein 1 light chain 3 beta
Mtor	Mechanistic target of rapamycin
Nbr1	Neighbor of BRCA1 gene 1
Nfkb1	Neuregulin 1
Rab7	RAB7A; RAS oncogene family
Sh3tc2	SH3 domain and tetratricopeptide repeats 2
Sqstm1	sequestosome 1 ; p62
Stat1	Signal transducer and activator of transcription 1
Stat3	Signal transducer and activator of transcription 3
Tfeb	Transcription factor EB
Trp53	Tumor protein p53
Vcp	Valosin containing protein (p97)

Microtubule panel

Acronym	Full name
Actr1a	ARP1 actin related protein 1 homolog A
Clip1	CAP-Gly domain containing linker protein 1 (CLIP-170)
Clip2	CAP-GLY domain containing linker protein 2 (CLIP-115)
Dync1h1	Dynein cytoplasmic 1 heavy chain 1
Kif2a	Kinesin family member 2A (KHC, kinesin family 13)
Kif5b	Kinesin family member 5B (KHC, kinesin family 1)
Map4	Microtubule associated protein 4
Mapre1	Microtubule associated protein RP/EB family member 1 (EB1)
Mapre3	Microtubule associated protein RP/EB family member 3 (EB3)
Mapt	Microtubule associated protein tau
Slmap	Sarcolemma associated protein
Svbp	Small vasohibin-binding protein
Ttl	Tubulin tyrosine ligase
Tuba1a	Tubulin alpha 1a
Tuba1b	Tubulin alpha 1b
Tuba1c	Tubulin alpha 1c
Tuba4a	Tubulin alpha 4a
Tuba8	Tubulin alpha 8
Tubb4b	Tubulin beta 4B class Ivb (TUBB2C)
Tubb5	Tubulin, beta 5 class I
Tubb6	Tubulin beta 6 class V
Vash1	Vasohibin 1

Table S2. Echocardiographic parameters overtime in *Mybpc3*-targeted knock-in (KI) and wild-type (WT) mice treated with AAV9-TTL and/or AAV9-Emtpy.

Baseline / 3-week-old				One-way ANOVA and Tukey's multiple comparisons test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	12	12			
F/M	7/5	12/2	7/5			
BW (g)	8.9±0.2	7.6±0.3	8.3±0.3	3.20E-03	ns	ns
IVSd (mm)	0.39±0.03	0.46±0.03	0.45±0.03	ns	ns	ns
LVPWd (mm)	0.38±0.02	0.46±0.03	0.50±0.02	5.45E-02	4.80E-03	ns
LVEDD (mm)	3.21±0.06	3.50±0.10	3.61±0.06	1.89E-02	9.00E-04	ns
LVESD (mm)	2.41±0.07	3.00±0.07	3.13±0.06	<1.00E-4	<1.00E-4	ns
FAC (%)	44.0±2.7	25.1±2.1	25.1±1.6	<1.00E-4	<1.00E-4	ns
AET (msec)	nd	32.6±1.1	31.4±1.5	nd	nd	ns
MV Decel Time (msec)	nd	35.5±5.3	35.5±4.8	nd	nd	ns
MV E/A	nd	0.95±0.06	1.21±0.08	nd	nd	4.24E-02
E'/A'	nd	0.73±0.17	0.69±0.05	nd	nd	ns
E/E'	nd	37.9±3.9	38.9±8.4	nd	nd	ns
IVRT (msec)	nd	27.0±2.9	26.6±1.1	nd	nd	ns
VTI (msec)	22.7±0.9	17.0±0.7	19.0±0.8	3.00E-04	4.07E-02	ns
LVM (mg)	57.4±3.2	90.0±7.1	92.4±5.4	5.00E-04	2.00E-04	ns
LVM/BW (mg/g)	6.5±0.3	11.8±0.65	11.2±0.6	<1.00E-4	<1.00E-4	ns
EF (%)	55.8±4.0	28.7±3.3	31.8±3.1	<1.00E-4	<1.00E-4	ns
SV (µl)	15.9±1.4	9.3±1.4	10.2±1.4	5.80E-03	1.94E-02	ns
CO (ml/min)	7.8±0.8	4.6±0.7	4.8±0.7	1.17E-02	1.82E-02	ns

1 week treatment / 4-week-old				One-way ANOVA and Tukey's multiple comparisons test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	12	12			
F/M	7/5	12/2	7/5			
BW (g)	14.1±0.4	12.0±0.4	13.1±0.5	5.50E-03	ns	ns
IVSd (mm)	0.49±0.04	0.54±0.03	0.51±0.02	ns	ns	ns
LVPWd (mm)	0.50±0.03	0.59±0.03	0.55±0.03	ns	ns	ns
LVEDD (mm)	3.67±0.06	3.76±0.07	3.95±0.08	ns	2.10E-02	ns
LVESD (mm)	2.80±0.08	3.26±0.08	3.48±0.08	1.20E-03	<1.00E-4	ns
FAC (%)	42.5±1.6	24.5±1.8	22.8±2.2	<1.00E-4	<1.00E-4	ns
AET (Msec)	nd	25.4±1.3	28.2±2.0	nd	nd	ns
MV Decel Time (msec)	nd	20.6±1.0	23.8±1.1	nd	nd	ns
MV E/A	nd	1.23±0.08	1.28±0.09	nd	nd	ns
E'/A'	nd	0.68±0.15	0.69±0.05	nd	nd	ns
E/E'	nd	42.4±8.9	40.4±4.5	nd	nd	ns
IVRT	nd	21.0±1.4	23.8±1.1	nd	nd	ns
VTI (msec)	23.1±0.7	20.8±1.9	22.4±1.3	ns	ns	ns
LVM (mg)	100.6±6.0	112.6±6.3	116.9±7.1	ns	ns	ns
LVM/BW (mg/g)	7.2±0.5	9.5±0.6	9.0±0.6	1.94E-02	ns	ns
EF (%)	51.9±1.9	32.9±2.0	37.0±3.1	<1.00E-4	8.00E-04	ns
SV (µl)	27.2±1.6	13.6±1.2	19.0±2.8	<1.00E-4	1.72E-02	ns
CO (ml/min)	13.9±0.8	7.5±0.6	9.8±1.4	3.00E-04	2.52E-02	ns

2 weeks treatment / 5-week-old				One-way ANOVA and Tukey's multiple comparisons test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	12	12			
F/M	7/5	12/2	7/5			
BW (g)	18.4±0.6	16.3±0.4	17.7±0.3	5.90E-03	ns	ns
IVSd (mm)	0.46±0.03	0.57±0.03	0.60±0.03	4.90E-02	7.20E-03	ns
LVPWd (mm)	0.45±0.02	0.60±0.03	0.61±0.03	3.50E-03	1.16E-03	ns
LVEDD (mm)	3.61±0.08	4.14±0.06	4.40±0.04	<1.00E-4	<1.00E-4	2.09E-02
LVESD (mm)	2.52±0.11	3.56±0.07	3.66±0.11	<1.00E-4	<1.00E-4	ns
FAC (%)	51.7±2.2	26.0±1.8	28.9±2.0	<1.00E-4	<1.00E-4	ns
AET (msec)	35.9±0.9	25.8±1.3	27.9±1.6	1.25E-02	4.03E-02	ns
MV Decel Time (msec)	17.7±1.1	18.2±1.2	20.4±1.6	nd	nd	ns
MV E/A	1.14±0.04	1.15±0.04	1.17±0.05	ns	ns	ns
E'/A'	0.74±0.14	0.63±0.05	0.65±0.06	ns	ns	ns
E/E'	29.4±3.3	48.3±5.9	39.7±2.7	ns	ns	ns
IVRT (msec)	13.7±1.1	20.2±1.4	19.7±1.3	ns	ns	ns
VTI (msec)	25.1±0.7	20.9±0.8	24.8±1.5	5.70E-03	ns	4.63E-02
LVM (mg)	103.1±8.2	151.5±11.2	166.5±7.0	1.70E-03	<1.00E-4	ns
LVM/BW (mg/g)	5.7±0.5	9.3±0.7	9.4±0.3	<1.00E-4	<1.00E-4	ns
EF (%)	60.5±2.2	36.7±1.8	38.7±3.3	<1.00E-4	<1.00E-4	ns
SV (µl)	33.2±1.6	18.1±1.9	18.2±1.4	<1.00E-4	<1.00E-4	ns
CO (ml/min)	18.1±0.8	9.8±1.1	9.5±0.9	<1.00E-4	<1.00E-4	ns

4 weeks treatment / 7-week-old				One-way ANOVA and Tukey's multiple comparisons test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	12	11			
F/M	7/5	12/2	6/5			
BW (g)	20.6±0.9	17.8±0.5	20.0±0.5	1.43E-02	ns	ns
IVSd (mm)	0.53±0.03	0.62±0.04	0.70±0.04	ns	5.00E-03	ns
LVPWd (mm)	0.52±0.03	0.62±0.04	0.65±0.04	ns	5.56E-02	ns
LVEDD (mm)	3.92±0.07	4.26±0.07	4.50±0.07	3.50E-03	<1.00E-4	6.86E-02
LVESD (mm)	3.04±0.09	3.71±0.07	3.85±0.09	<1.00E-4	<1.00E-4	ns
FAC (%)	41.6±2.3	25.6±2.4	27.1±1.6	<1.00E-4	<1.00E-4	ns
AET (msec)	43.0±1.5	27.8±1.1	32±2.0	<1.00E-4	<1.00E-4	ns
MV Decel Time (msec)	15.4±1.2	21.0±1.8	25.7±2.8	ns	2.20E-03	ns
MV E/A	1.20±0.05	1.16±0.03	1.19±0.07	ns	ns	ns
E'/A'	0.87±0.14	0.54±0.02	0.61±0.03	2.71E-02	ns	ns
E/E'	32.7±2.9	45.3±2.4	42.6±3.5	4.50E-03	3.43E-02	ns
IVRT (msec)	15.5±1.2	22.8±0.9	21.7±1.0	<1.00E-4	4.00E-04	ns
VTI (msec)	22.9±0.9	20.8±0.9	22.7±1.7	ns	ns	ns
LVM (mg)	128.2±6.4	160.2±11.7	190.3±13.9	ns	1.10E-03	ns
LVM/BW (mg/g)	6.3±0.3	8.9±0.5	9.6±0.7	2.50E-03	3.00E-04	ns
EF (%)	50.7±3.7	32.7±1.9	40.8±2.5	2.00E-04	4.89E-02	ns
SV (µl)	34.5±3.0	17.1±1.7	22.8±2.0	<1.00E-4	3.70E-03	ns
CO (ml/min)	17.7±1.6	8.8±1.1	11.3±1.2	<1.00E-4	5.30E-03	ns

Table S2…. Continued on next page

8 weeks treatment / 11-week-old			One-way ANOVA and Tukey's multiple comparisons test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	11	11			
F/M	7/5	12/2	6/5			
BW (g)	23.6±1.1	21.1±0.5	23.0±0.7	9.10E-02	ns	ns
IVSd (mm)	0.58±0.02	0.71±0.05	0.76±0.05	5.04E-02	4.48E-03	ns
LVPWd (mm)	0.38±0.02	0.46±0.03	0.50±0.02	3.40E-03	ns	ns
LVEDD (mm)	4.09±0.07	4.37±0.09	4.71±0.11	7.64E-02	<1.00E-4	2.71E-02
LVESD (mm)	3.21±0.08	3.88±0.07	4.11±0.09	<1.00E-4	<1.00E-4	ns
FAC (%)	44.2±1.8	24.41±1.8	23.9±2.0	<1.00E-4	<1.00E-4	ns
AET (msec)	44.8±2.2	31.1±1.2	29.5±1.6	<1.00E-4	<1.00E-4	ns
MV Decel Time (msec)	19.7±1.6	20.3±1.0	23.1±1.1	ns	ns	ns
MV E/A	1.20±0.06	1.13±0.06	1.24±0.03	ns	ns	ns
E'/A'	0.65±0.08	0.66±0.10	0.56±0.06	ns	ns	ns
E/E'	35.8±2.7	42.2±3.3	48.1±3.6	ns	2.87E-02	ns
IVRT (msec)	16.3±0.4	22.8±1.0	23.1±0.7	<1.00E-4	<1.00E-4	ns
VTI (msec)	24.1±1.2	23.0±1.2	27.8±1.9	ns	ns	ns
LVM (mg)	133.2±6.6	220.2±9.8	242.4±14.1	<1.00E-4	<1.00E-4	ns
LVM/BW (mg/g)	5.7±0.3	10.5±0.5	10.5±0.5	<1.00E-4	<1.00E-4	ns
EF (%)	53.1±1.7	28.3±2.4	38.5±1.3	<1.00E-4	<1.00E-4	1.70E-03
SV (µl)	39.7±1.7	17.0±2.3	25.4±2.0	<1.00E-4	<1.00E-4	1.72E-02
CO (ml/min)	20.3±1.0	8.6±1.2	12.4±1.1	<1.00E-4	<1.00E-4	ns

12 weeks treatment - 15-week-old			One-way ANOVA and Tukey's multiple comparisons test		
	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	12	11	11			
F/M	7/5	9/2	6/5			
BW (g)	25.6±1.0	22.6±0.6	24.0±0.7	3.01E-02	ns	ns
IVSd (mm)	0.64±0.03	0.69±0.05	0.63±0.04	ns	ns	ns
LVPWd (mm)	0.63±0.03	0.74±0.05	0.67±0.05	ns	ns	ns
LVEDD (mm)	3.93±0.07	4.33±0.10	4.85±0.22	1.57E-02	<1.00E-4	1.90E-03
LVESD (mm)	2.76±0.10	3.82±0.06	4.16±0.12	<1.00E-4	<1.00E-4	ns
FAC (%)	44.3±2.9	26.4±2.2	24.4±2.3	<1.00E-4	1.20E-03	ns
AET (msec)	37.9±1.5	26.8±1.2	29.8±1.5	<1.00E-4	1.20E-03	ns
MV Decel Time (msec)	20.5±1.8	18.0±1.5	17.6±1.6	ns	ns	ns
MV E/A	1.10±0.05	1.10±0.08	1.29±0.11	ns	ns	ns
E'/A'	0.79±0.12	0.75±0.15	0.76±0.17	ns	ns	ns
E/E'	32.0±3.0	33.4±5.6	45.9±3.6	ns	ns	ns
IVRT (msec)	12.3±0.7	21.5±1.3	23.3±0.6	<1.00E-4	<1.00E-4	ns
VTI (msec)	24.0±1.0	19.8±1.4	22.4±2.1	ns	ns	ns
LVM (mg)	134.9±4.3	231.7±8.9	247.4±12.9	<1.00E-4	<1.00E-4	ns
LVM/BW (mg/g)	5.3±0.2	10.3±0.4	10.3±0.3	<1.00E-4	<1.00E-4	ns
EF (%)	60.7±2.8	28.0±2.0	40.1±3.2	<1.00E-4	<1.00E-4	1.09E-02
SV (µl)	40.6±2.7	16.2±1.6	28.7±2.7	<1.00E-4	3.90E-03	3.20E-03
CO (ml/min)	22.8±1.5	8.8±0.9	14.5±1.4	<1.00E-4	2.00E-04	1.22E-02

Data are expressed as mean±SEM. Statistical significance was assessed with one-way ANOVA and followed by Tukey's multiple comparisons test. Abbreviations: **AET,** aortic ejection time; **BW,** body weight; **CO,** cardiac output; **FAC,** fractional area change; **E'/A',** peak velocity blood flow from LV relaxation in early diastole (E´ wave) to peak velocity flow in late diastole caused by atrial contraction (A' wave) obtained by tissue Doppler of the mitral annulus; **E/E',** calculated ratio of peak velocity in early diastole; **EF,** ejection fraction; **F/M,** number of females/males; **IVRT,** isovolumic relaxation time; **IVSd,** interventricular septum thickness in diastole; **LVM,** left ventricular mass; **LVM/BW,** left ventricular mass to body weight ratio; **N,** number of mice; **nd,** not determined; **LVEDD,** left ventricular end-diastolic diameter; **LVESD,** left ventricular end-systolic diameter; **LVPWd,** left ventricular posterior wall thickness in diastole; **MV E/A,** peak velocity blood flow from LV relaxation in early diastole (E wave) to peak velocity flow in late diastole caused by atrial contraction (A wave) obtained by Tissue Doppler through the mitral valve; **MV Decel Time,** mitral valve deceleration time; **nd,** not determined; **ns,** non-significant; **SV,** stroke volume; **VTI,** velocity time interval.

Table S3. Hemodynamic parameters in *Mybpc3*-targeted knock-in (KI) and wild-type (WT) mice treated with AAV9-TTL and/or AAV9-Emtpy.
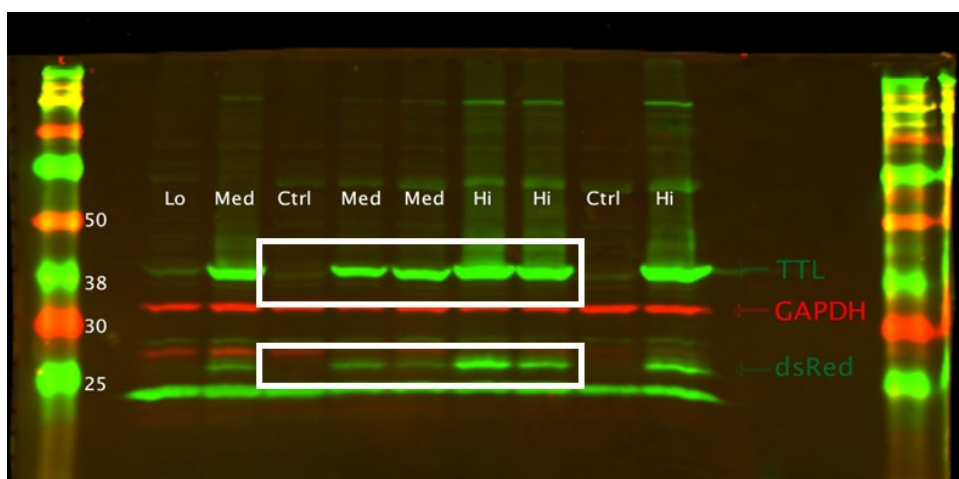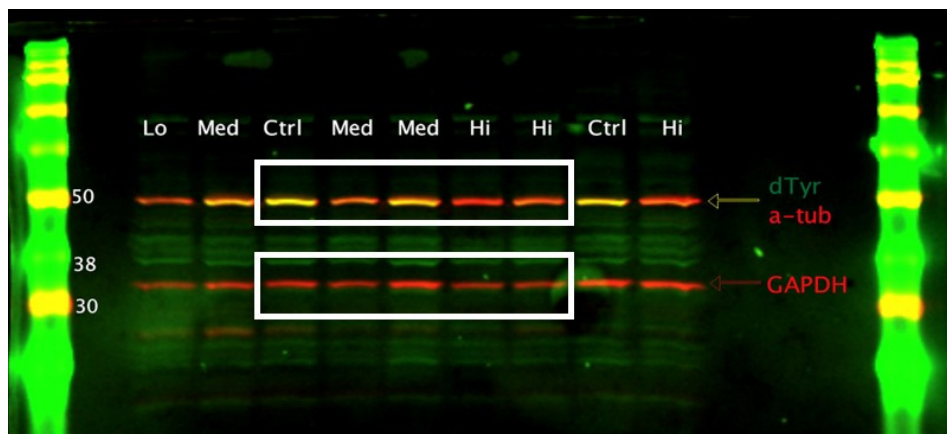
	12 weeks treatment / 15-wk-old			Unpaired Student's t-test		
Parameters	WT-Empty	KI-Empty	KI-TTL	KI vs WT	KI-TTL vs WT	KI-TTL vs KI
N number	9	9	11			
F/M	5/4	7/2	6/5			
BW (g)	24.7±1.2	22.5±0.5	23.7±0.8	ns	ns	ns
Global function						
Heart rate (bpm)	581±12	564±10	572±11	ns	ns	ns
Cardiac output (ml/min)	16.37±1.9	10.71±1.09	15.29±1.52	1.84E-02	ns	3.06E-02
Stroke volume (µl)	28.3±3.3	18.9±1.9	26.9±2.7	2.51E-02	ns	3.31E-02
Stroke work (mmHg*µl)	2107±261	1250±194	1986±249	1.79E-02	ns	3.69E-02
Systolic function						
Ejection fraction (%)	80±6	49±4	51±5	8.00E-04	1.80E-03	ns
LVESP (Pes) (mmHg)	68±4	78±2	81±4	ns	ns	ns
dP/dt_{max} (mmHg/s)	10778±908	9351±778	11422±829	ns	ns	ns
LVESV (µl)	9.7±4.0	25.7±2.6	35.5±6.1	3.90E-03	3.60E-03	ns
dV/dt_{max} (µl/s)	1922±222	1564±210	1867±230	ns	ns	ns
PRSW (mmHg)	87±7.5	65.2±8.2	79.5±4.9	ns	ns	ns
Diastolic function						
LVEDP (Ped) (mmHg)	2.37±0.42	3.64±0.54	2.95±0.52	ns	ns	ns
dP/dt_{min} (mmHg/s)	-8448±652	-4738±216	-5372±322	<1.00E-4	3.00E-04	ns
LVEDV (µl)	32.7±5.5	36.8±3.1	54.8±6.3	ns	1.93E-02	2.87E-02
dV/dt_{min} (µl/s)	-2208±218	-2158±260	-3024±313	ns	ns	ns
Tau (ms)	4.62±0.18	7.28±0.31	6.48±0.32	<1.00E-4	2.00E-04	ns
EDPVR (mmHg/µl)	0.29±0.07	0.34±0.04	0.18±0.04	ns	ns	1.92E-02

Data are expressed as mean ± SEM. Statistical significance was assessed with the unpaired Student's t-test. Abbreviations: **BW,** body weight; **dP/dt_{max},** maximal rate of left ventricular pressure development in systole; **dP/dt_{min},** maximal rate of left ventricular pressure development in diastole; **dV/dt_{max},** point of maximum volume increase; **dV/dt_{min},** point of maximum volume decrease; **EDPVR,** end-diastolic pressure-volume relation; **F/M,** number of females/males; **LVEDV,** left ventricular end-diastolic volume; **LVESP,** left ventricular end-systolic pressure; **N,** number of mice; **ns**, non-significant; **PRSW,** preload recruitable stroke work; **Tau,** time constant of active relaxation.
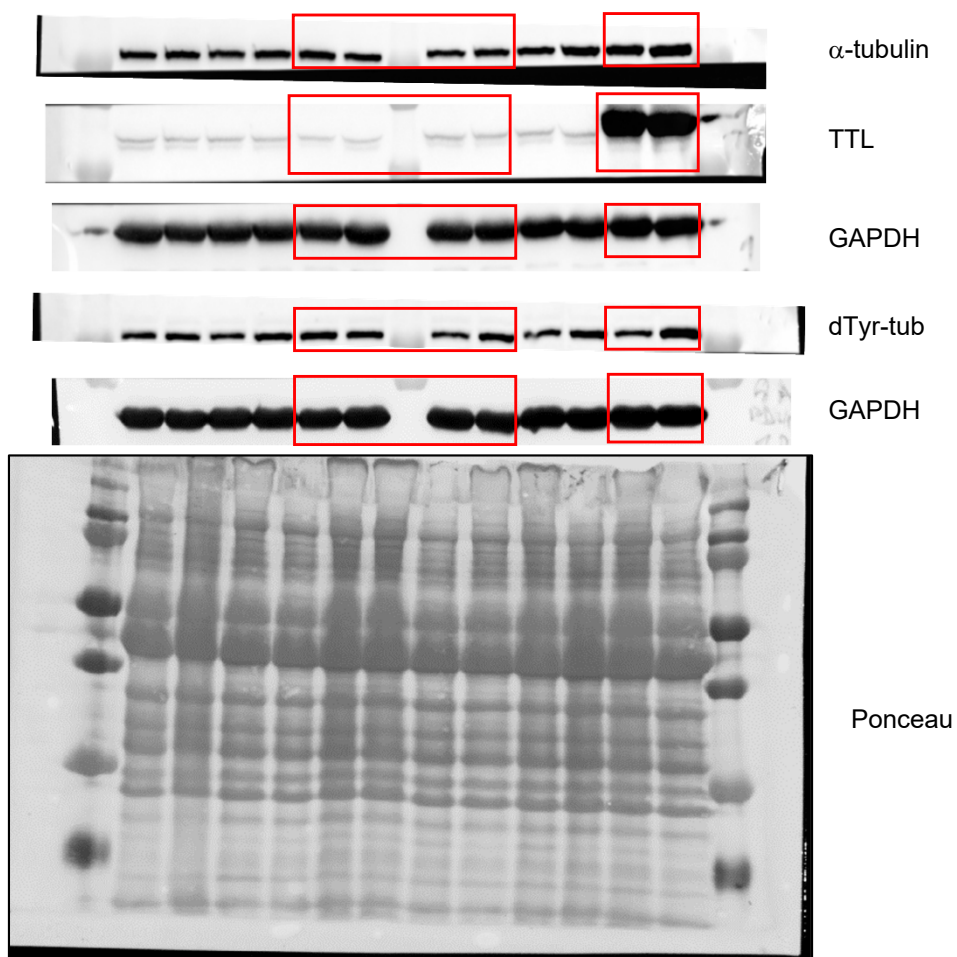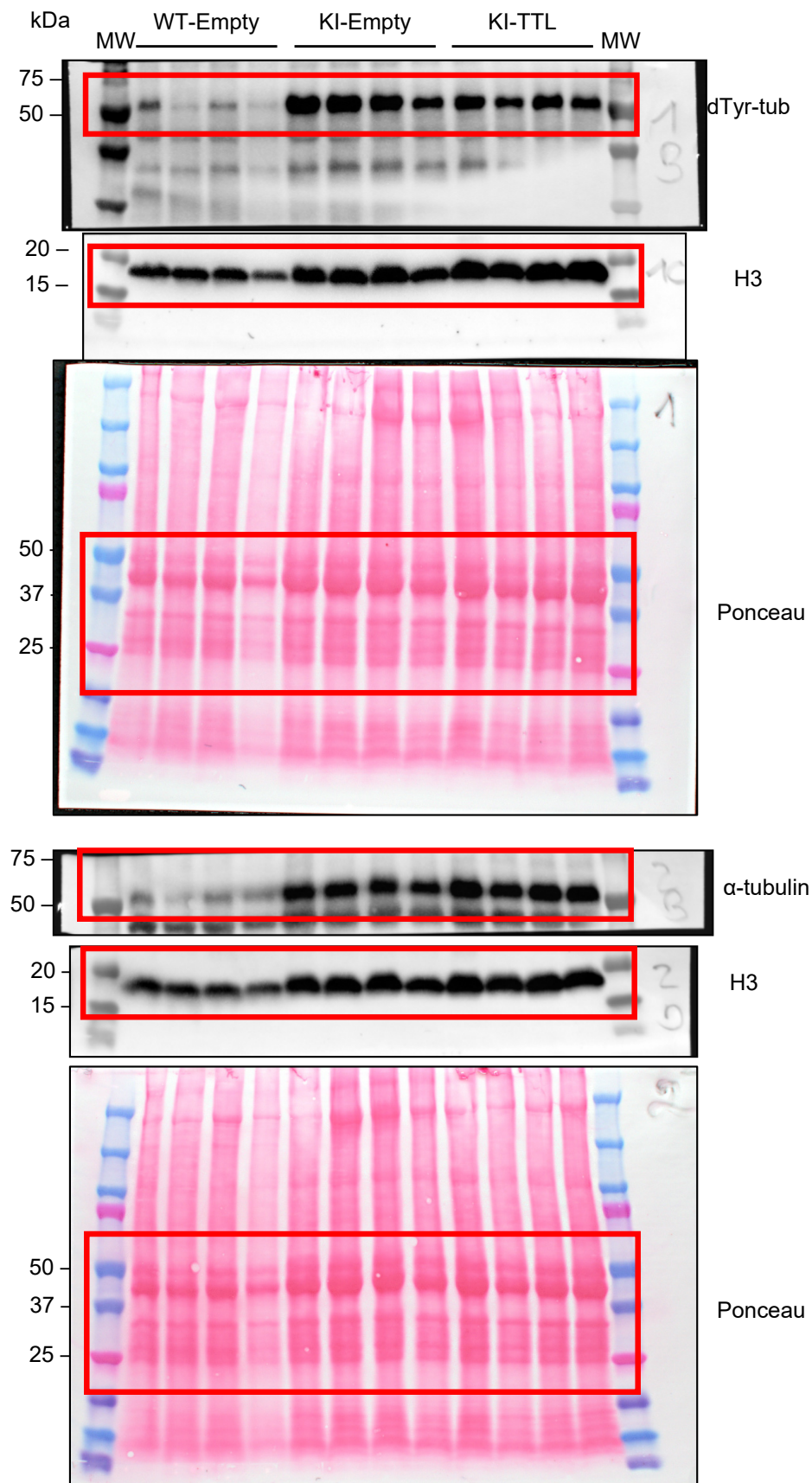
Uncut Gels, Ponceau and Blots

Uncut immunoblots (Figure 1B)
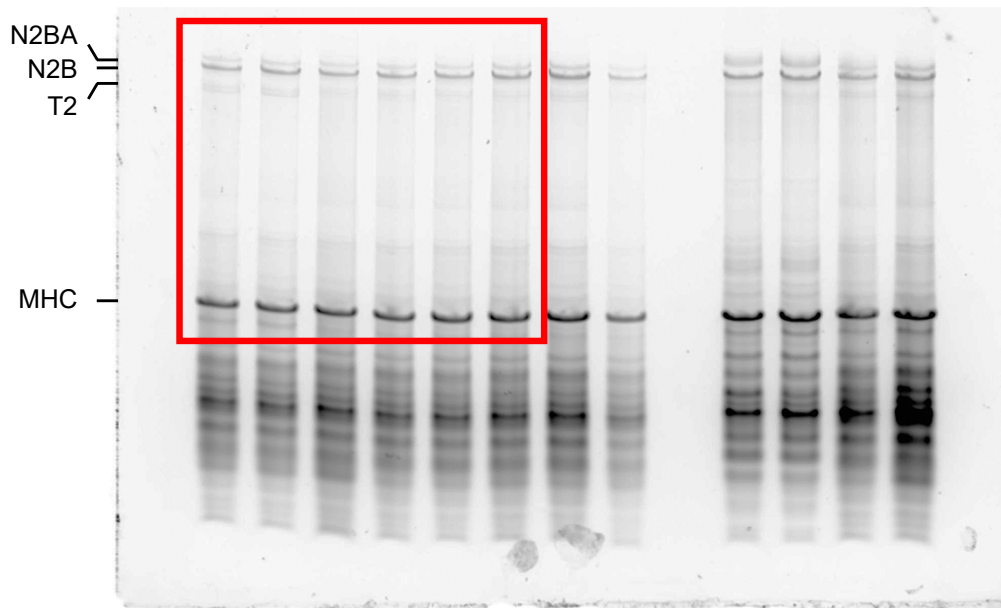

Uncut immunoblots and Ponceau (Figure 4A)
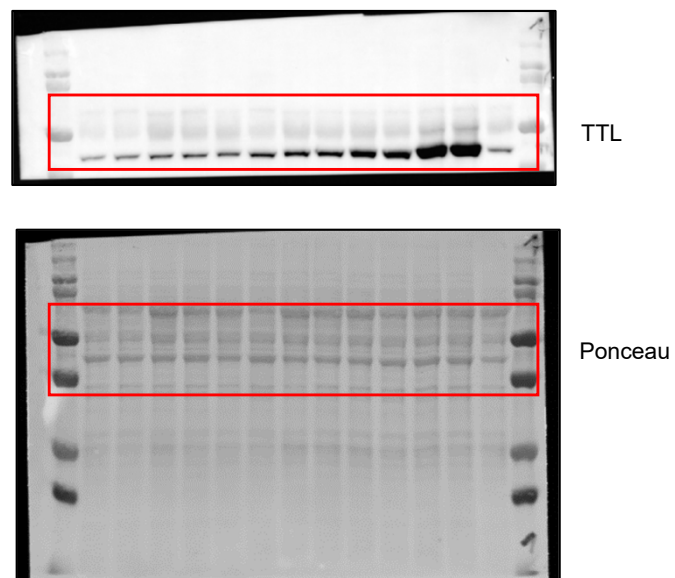
Uncut immunoblots and Ponceau (Figure 4B)

Uncut SYPRO Ruby stained gel (Figure 4E)
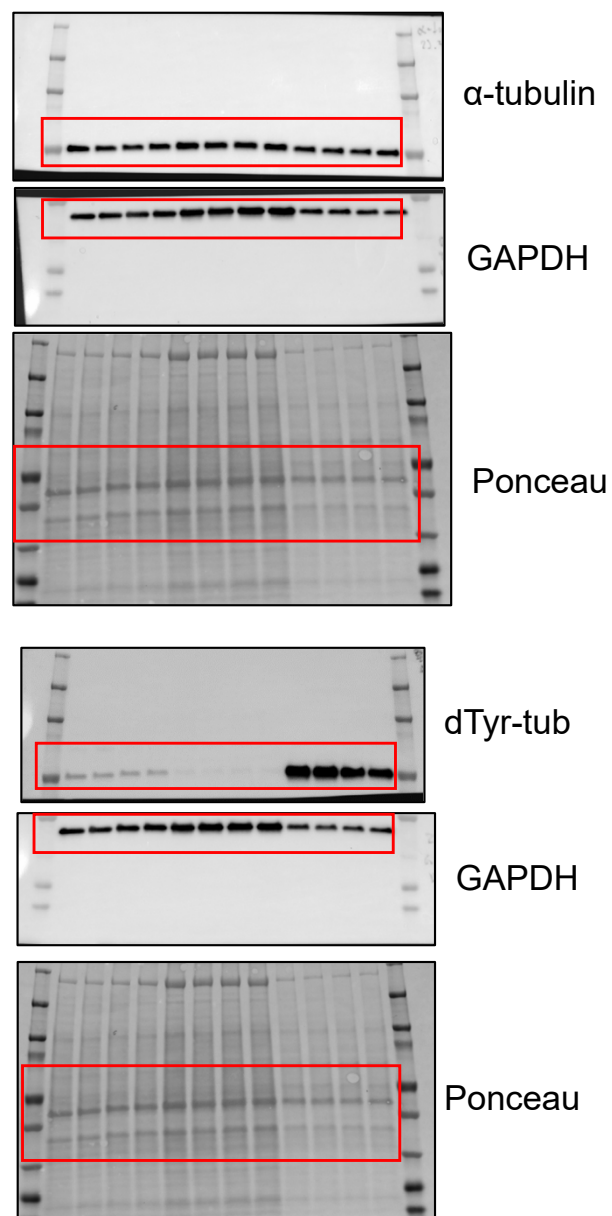
N2BA
N2B
T2

MHC

Uncut immunoblot and Ponceau (Figure 6A)

TTL

Ponceau

Uncut immunoblots and Ponceau (Figure 7B)

α-tubulin

GAPDH

Ponceau

dTyr-tub

GAPDH

Ponceau

Uncut immunoblots (Figure S1C)