Supplemental Materials

Wang et al., AAV gene therapy prevents and reverses heart failure in a murine knockout model of Barth syndrome, Circ. Res. 2020.

Expanded Materials and Methods

Animal

Animal experiments were performed under protocols approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. We studied male mice because BTHS is an X-linked disease that primarily affects males. Mice harboring the Taz^{fl} and Taz^{Δ} allele were described previously[21]. These alleles have been backcrossed onto C57BL/6J for over 5 generations. Myh6-Cre[35] mice were purchased from Jackson Laboratory and have been backcrossed to C57BL/6J for over 10 generations. Echocardiography of awake mice was performed using a VEVO 2100. Echocardiography was performed blinded to genotype and treatment group.

Animals were enrolled into studies based on inclusion criteria described in the experimental outline at the start of each figure. No animals were excluded. Number of each mice in each study was determined by power calculation (80% power, α=0.05). For survival studies, we powered studies to detect a 6-fold improvement in survival compared to control-treated mutant mice. For cardiac function studies, we powered studies to detect a 12% change in FS%, assuming a standard deviation of 5%.

Genotyping

Genomic DNA was isolated using KAPA Express Extraction Kit (KK7102). Genotyping PCR of Taz WT allele vs. KO allele was carried out using the primers WT-U1 (5'- CTTGCCCACTGCTCACAAAC- 3'), WT-D1 (5'- CAGGCACATGGTCCTGTTTC- 3') and KO-U1 (5'- CCAAGTTGCTAGCCCACAAG- 3'), which generates products of 383bp and 280bp, representing WT allele and KO allele, respectively. Differentiation of Taz-floxed allele against WT allele was detected by PCR using primers WT-U1 and WT-D1, which generates a 451 bp product for the identification of floxed allele. All the genotyping PCR was performed using Go-Taq Mastermix (Promega, M7122) and shares the same thermo-program: 95˚C for 2min; 35 cycles of 95˚C for 30sec, 60˚C for 1min, 72˚C for 1min; and a final extension step of 72˚C for 5min. Amplicon sizes were analyzed with electrophoresis using 1% agarose gel with ethidium bromide.

AAV production

AAV-TAZ and AAV-Luciferase vectors were constructed from AAV-CAG-GFP (Addgene plasmid #37825) by replacing GFP cDNA with luciferase or codon optimized human full-length Tafazzin cDNA (hTAZ), synthesized by Genewiz. Constructs have been deposited at Addgene.

AAV was produced according to previously published protocol[36]. Briefly, AAV was produced by HEK293T cells transfected with three plasmids carrying AAV9-Rep/Cap, target gene flanked by ITRs, and necessary adenovirus helper genes. 72 hours later HEK293T cells were lysed and the AAV-containing cell medium and lysate were both collected. AAV particles were purified by ultracentrifugation on an iodixanol-based density gradient. Purified AAV was stored at -80˚C until needed. AAV plasmids were obtained from the Penn Vector Core.

QPCR-based AAV titration

Purified AAV (5 µl) was first treated with DNase I for digesting residual plasmid carried over from transfected HEK293 cells and then incubated with proteinase K for digesting the viral capsid. The viral genome (VG) was quantified using SYBR Green PCR Master Mix (Applied Biosystems, Cat# 4367659) with two primers flanking a 170 bp region in the CAG promoter. Standard curve was established using serial dilution of the amplicon DNA with known concentration as the input of QPCR.

RNA extraction and Q-RT-PCR

Total RNA was isolated using TRIzol reagent (ThermoFisher, 15596026) according to the manufacturer guidelines. RNA was treated with DNase I (ThermoFisher, 12185010) and then stored at -80℃. 1µg of DNase-pretreated RNA was used as input for reverse transcription using SuperScript™ III First-strand Synthesis SuperMix (Invitrogen, 11752-050). Gene expression was analyzed by Q-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) and Bio-Rad thermocycler, using qPCR primers listed in Online Table I.

Protein quantification by Wes

The *TAZ* transgene expression was evaluated by automated capillary western blotting (Protein Simple, model: Wes) according to manufacturer's protocol. Primary antibody against TAZ was used to recognize both human and mouse isoforms of TAZ (Santa Cruz Bio., Cat # sc-365810). Full gel images are provided in attached supplement.

Cardiolipin analysis

CL was extracted into chloroform/methanol and analyzed by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry(MALDI-TOF MS)[37] with minor modifications[9].

Blood cell count

Automated complete blood count was performed using Drew Scientific Hemavet 950FS Hematology Analyzer (Cat# HV950FS). Mice were euthanized with CO_2 and blood samples were collected from the heart and stored in K2EDTA spray coated tubes until analysis.

Histology and immunostaining

Mouse samples were collected and fixed overnight in 4% paraformaldehyde solution at 4℃. For histology analyses, samples were dehydrated through ethanol and sectioned at 7 μm. Sections were dewaxed, rehydrated and post-fixed in Bouin's Fixative for 1 hour at 55℃, followed by 10 min of washing under tap water. Fixed slides were then incubated in 0.1% Fast Green solution for 10 min, washed in 1% acetic acid for 2 min and stained with 0.1% Sirius Red staining solution for another 30 min before dehydration and mounting with coverslip. Images were taken under brightfield microscope (Keyence) at both 4x and 10x magnification for the analysis and quantification of fibrotic area.

For immunofluorescent staining, fixed samples were dehydrated in 30% sucrose/PBS overnight at 4℃, embedded in freezing medium, and sectioned by cryostat at 10 μm. Sections were permeablized with 0.2% TritonX-100/PBS, blocked with 10% normal donkey serum (Fisher, Cat# NC9624464) for 1 hour, and incubated with primary antibodies at 4℃ overnight. After washing with PBS, sections were incubated with the secondary antibody at room temperature for 1 hour and subsequently mounted with coverslip. Primary antibodies were: GFP (Rockland, Cat# 600-101-215) and TNNI3 (Abcam, Cat# 56357). Fluorescent samples were imaged with an epifluorescent microscope (Keyence) or a laser scanning confocal (Olympus FV3000RS). Negative controls were performed by omitting the primary antibody.

Where images were quantitatively analyzed, we selected representative images that displayed quantities that were close to the mean value for the group. For images that were not quantitated, we selected images that exemplified consistent findings that are reported in the text.

RNA *in situ* hybridization

RNAscope *in situ* hybridization was carried out according to manufacturer's protocol using RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat# 323100). In situ probes were from Advanced Cell Diagnostics (*hTAZ* Cat# 828651-C2; *Actn2* Cat# 569061). Briefly, hearts or skeletal muscle samples were fixed with 4% PFA, embedded in O.C.T. and sectioned at -20℃. Sections were pretreated with protease and incubated with hybridization probes and amplification solutions as directed by the manufacturer's protocol. After developing *in situ* hybridization signal by incubation with fluorescent dyes, an additional staining with fluorophore-conjugated wheat germ agglutinin (Invitrogen Cat# W32464) was performed to visualize the cell membrane.

Detection of apoptotic CMs

After dewaxing and rehydration, paraffin-embedded sections were pretreated with 10 μg/ml proteinase K at room temperature for 15 min. After washing, the TUNEL labeling mixture (In Situ Cell Death Detection Kit; Sigma, Cat# 11684795910) was applied to sections and incubated at 37℃ for one hour. Sections were then washed in PBS and immunofluorescent staining with

anti-TNNI3 antibody was performed. Sections were imaged with an epifluorescent microscope (Keyence) or a laser scanning confocal (Olympus FV3000RS).

Electron Microscopy

Sample were fixed in EM fixative (2.5% Glutaraldehyde 2.5% Paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) overnight at 4℃. After fixation, tissue is washed in 0.1M cacodylate buffer and postfixed with 1% Osmium tetroxide (OsO_4)/1.5% Potassium ferrocyanide ($KFeCN_6$) for 1 hour, washed in water for 3 times and incubated in 1% aqueous uranyl acetate for 1hr followed by 2 washes in water and subsequent dehydration in grades of alcohol (10 min each; 50%, 70%, 90%, 2x10 min 100%). Subsequently, tissue samples were incubated in propyleneoxide for 1 hr and infiltrated ON in a 1:1 mixture of propyleneoxide and TAAB Epon (Marivac Canada Inc. St. Laurent, Canada). On the next day, samples were embedded in TAAB Epon and polymerized at 60℃ for 2 additional days.

Ultrathin sections (about 60nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope. Images were recorded with an AMT 2k CCD camera.

Behavior studies

To study the locomotor function and fatigue phenotype of TAZ-KO mice, we performed two different assays. First, we measured spontaneous activity after sub-maximal exercise. Mice were individually placed in open field chambers to allow free exploration for 6 minutes. These chambers are equipped with monitors and software that measure spontaneous mouse activity (movement, distance traveled, rearing behavior, resting time, as well as time spent at center vs. peripheral of the chamber; Ethovision XT 9.0, Noldus, Netherlands). After a baseline recording of resting motor activity, mice were put on a treadmill and acclimated by setting the treadmill at 3 m/min for 1 minute. Then the treadmill was set to 3 m/min and increased to 8 m/min over 4 minutes. The 8 m/min pace was maintained for an additional 10 minutes. Immediately after the exercise, mice were placed back in their original open field chamber and their spontaneous activity was again monitored for 6 minutes.

Second, we measured the maximal exercise capacity of mice using an exhaustion assay, as described previously[22]. The treadmill is equipped with an electrified metal grid at the end of the moving belt to provide motivation for mice to run rather than rest on the grid. Animals were first trained to use treadmill and then run at 10 m/min for a total of 14 minutes. Mice were removed from the treadmill for exhaustion if 1) they stayed on the shock grid for over 5 seconds and won't get back to running or 2) the third time willing to sustain 2 sec or more of electric shocking rather than return to the treadmill. The time mice spent on treadmill running was recorded and the shorter running time reflects more severe exercise intolerance.

Human myocardial samples

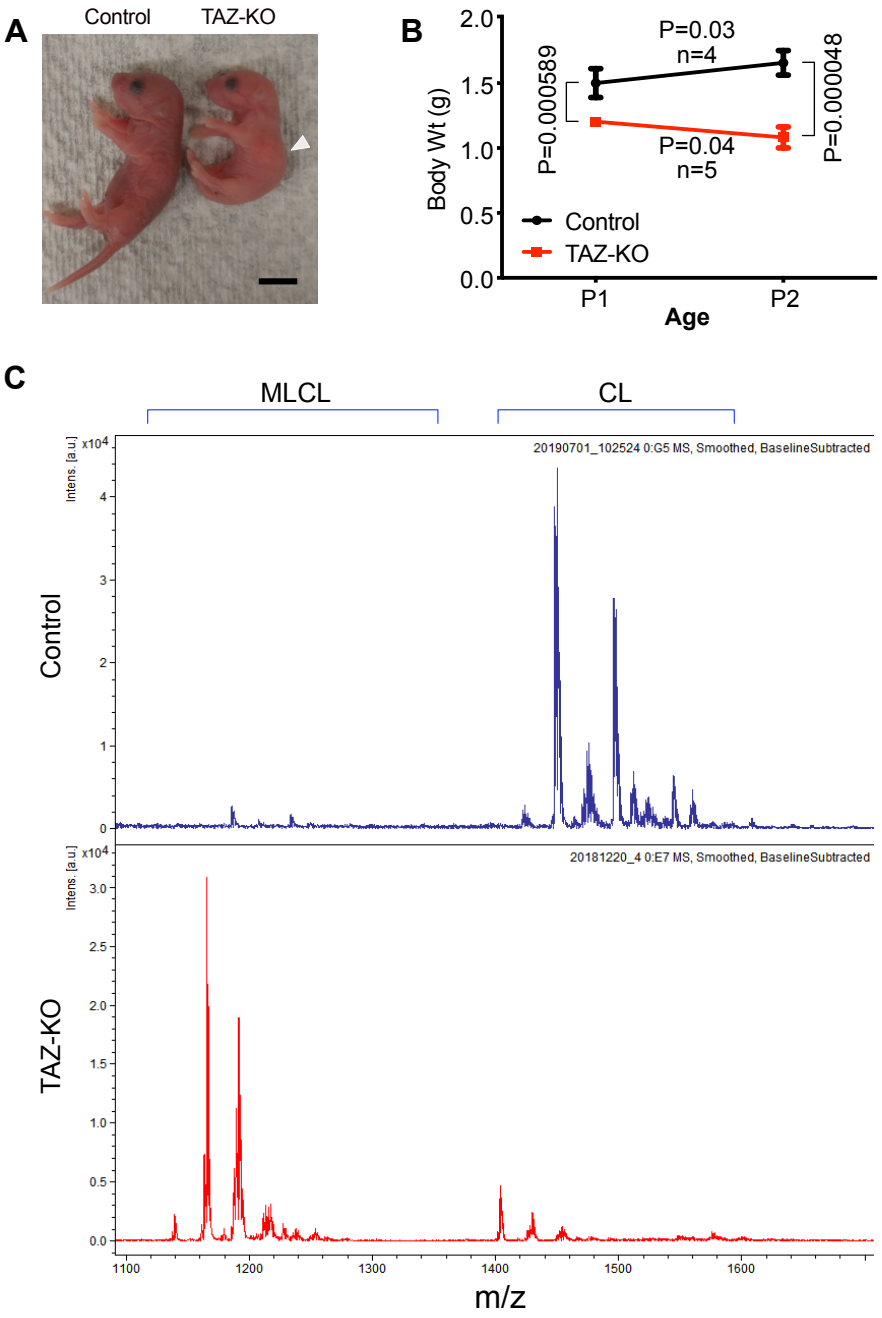
Deidentified BTHS human heart samples were obtained from the Barth Syndrome Registry or from archival specimens from Boston Children's Hospital under protocols approved by the Boston Children's Hospital Institutional Review Board. Control healthy adult samples were obtained from Biochain Institute Inc. (Cat# 50180957).

Statistics

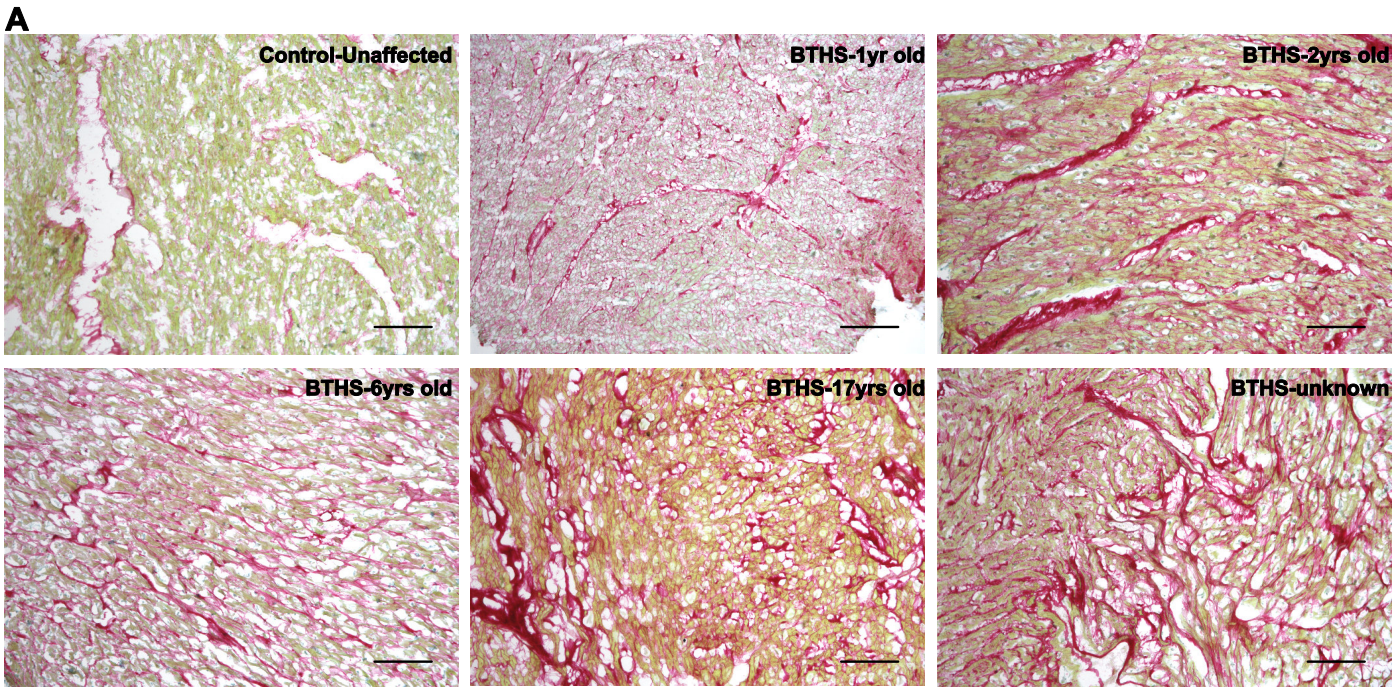
Violin plots were used in some figures. shapes represent sample distribution. Dashed line, median. Dotted lines, quartiles. Numbers next to violin plots indicate number of mitochondria analyzed from at least 3 different mice per group.

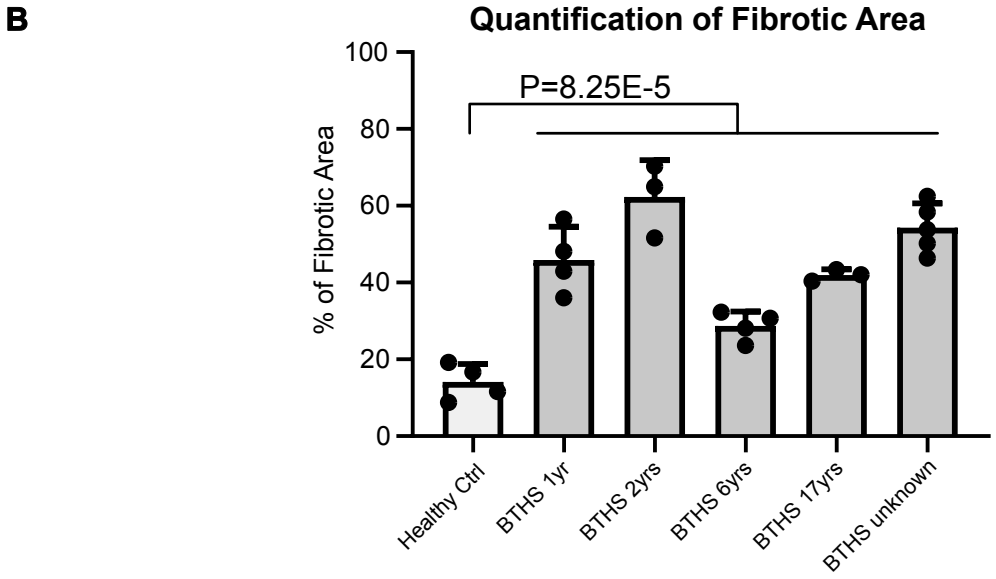
We used QQ plots and the Shapiro-Wilk test to assess normality. Where these metrics indicated that data was normally distributed, we used Student's t-test for two samples or ANOVA for three or more samples. Where data was not normally distributed, we used non-parametric tests. Permutation testing was performed in R, other statistics were performed using GraphPad Prism 8. Multiple testing correction was performed to correct for multiple testing within each experiment, but correction for multiple testing across the entire manuscript was not performed. Where we p-values were below the threshold to show exact values in GraphPad Prism, we provided the raw data in Online Data II.

Online Table 1. Primers used in this study.			
Target		**Sequence (5'-3')**	**Application**
TAZ	F	CCATGGGGACTGGGTGCAC	cloning
	R	TCTGCCTGCATCTTCAGCCG	cloning
mTaz	F	ATTGGACGGCTGATTGCTGAGTGT	QPCR
	R	AGTCTGTGAGGGCTTTCCGCATCT	QPCR
hTaz	F	GAGAACAAGTCGGCTGTG	QPCR
	R	GGCTGGAGGTGGTTGTGG	QPCR
Myh6	F	AACCAGAGTTTGAGTGACAGAATG	QPCR
	R	ACTCCGTGCGGATGTCAA	QPCR
Myh7	F	GCGACTCAAAAAGAAGGACTTTG	QPCR
	R	GGCTTGCTCATCCTCAATCC	QPCR
Nppa	F	GCTTCCAGGCCATATTGGAG	QPCR
	R	GGGGGCATGACCTCATCTT	QPCR
Nppb	F	GAGGTCACTCCTATCCTCTGG	QPCR
	R	GCCATTTCCTCCGACTTTTCTC	QPCR
Il1a	F	CGAAGACTACAGTTCTGCCATT	QPCR
	R	GACGTTTCAGAGGTTCTCAGAG	QPCR
Co-1	F	TGAAACCCCCAGCCATAAC	QPCR
	R	GGGTGCCCAAAGAATCAGA	QPCR
Nd-1	F	GCCCCCTTCGACCTGACA	QPCR
	R	CGGAAGCGTGGATAAGATGC	QPCR
Apt6	F	CTCAAAACGCCTAATCAACAAC	QPCR
	R	TACGGCTCCAGCTCATAGTG	QPCR
Mcu	F	AAAGGAGCCAAAAAGTCACG	QPCR
	R	AACGGCGTGAGTTACAAACA	QPCR
Apool	F	ATGGCGGCCTTTAGGATGG	QPCR
	R	TCCGGTCTCACTAGCTGCT	QPCR
Opa1	F	TGGAAAATGGTTCGAGAGTCAG	QPCR
	R	CATTCCGTCTCTAGGTTAAAGCG	QPCR
Mfn2	F	AGAACTGGACCCGGTTACCA	QPCR
	R	CACTTCGCTGATACCCCTGA	QPCR
Dnm1	F	TTACGGTTCCCTAAACTTCACG	QPCR
	R	GTCACGGGCAACCTTTTACGA	QPCR
Col1a1	F	GCTCCTCTTAGGGGCCACT	QPCR
	R	CCACGTCTCACCATTGGGG	QPCR
Col3a1	F	CTGTAACATGGAAACTGGGGAAA	QPCR
	R	CCATAGCTGAACTGAAAACCACC	QPCR
Taz-WT-F1	F	CTTGCCCACTGCTCACAAAC	Genotyping
Taz-WT-R1	R	CAGGCACATGGTCCTGTTTC	Genotyping
Taz-KO-R1	R	CCAAGTTGCTAGCCCACAAG	Genotyping
Myh6-Cre	F	ATGACAGACAGATCCCTCCTATCTCC	Genotyping
	R	CTCATCACTCGTTGCATCATCGAC	Genotyping
AAV-Titer	F	CTCAAGGCTTTCACGCAGCCAC	AAV titration
	R	GGCATGAACATGGTTAGCAGAGGCTCTAG	AAV titration

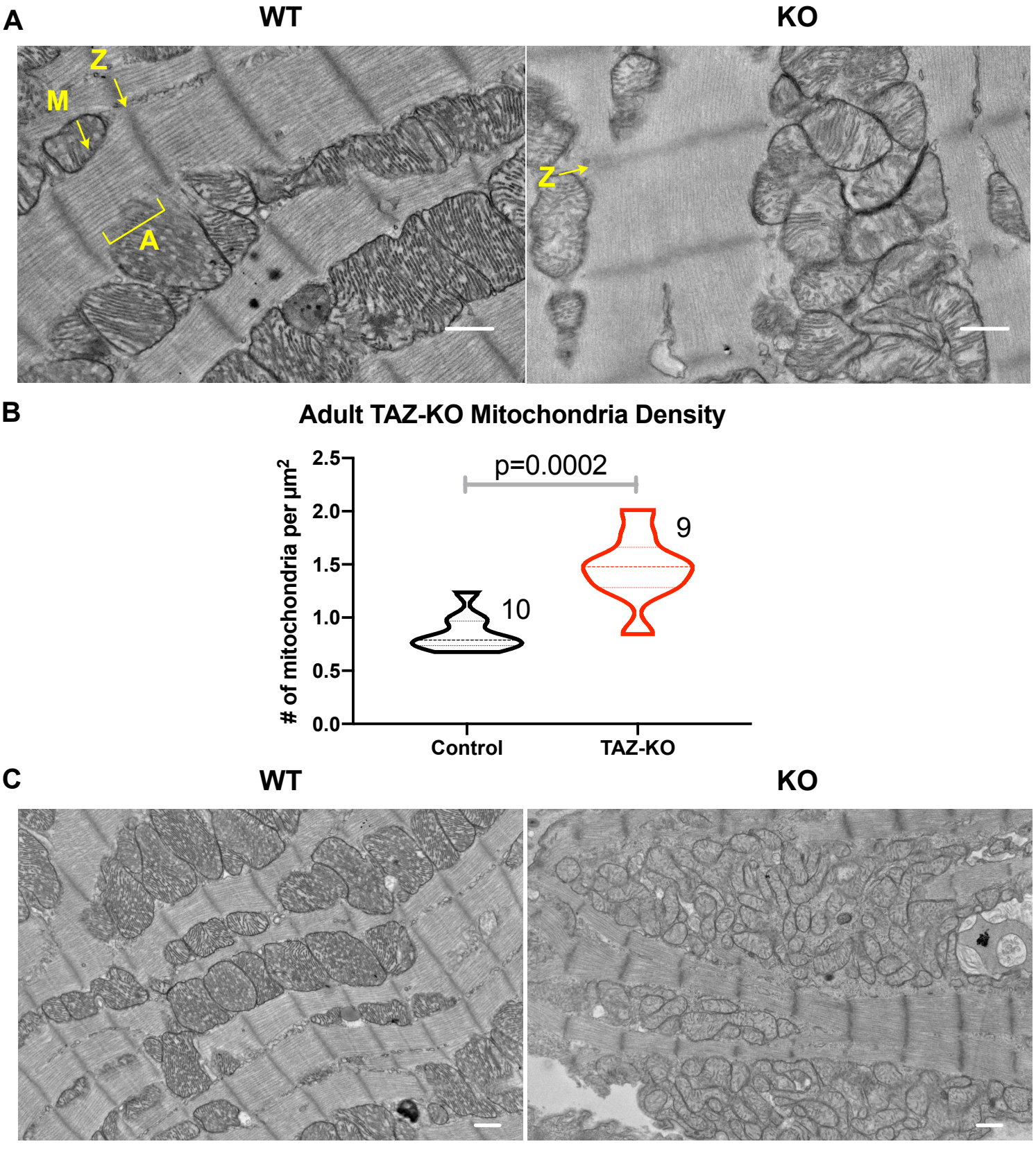


Online Fig. I. Weight of mice at P1 and P2. A. Representative control and TAZ-KO mice at P1. TAZ-KO mice were smaller compared to control littermates and typically had a hunchback (arrowhead) and more pale skin tone. Bar=1 cm. **B.** Body weight of P1 and P2 mice. Between genotypes: Unpaired t-test. Within genotype: Paired t-test. **C.** Representative spectra of cardiac lipids extracted from control and TAZ-KO mice at 2 months of age. Regions of the spectra corresponding to MLCL or CL, containing acyl chains of differing lengths and saturation, are labeled.

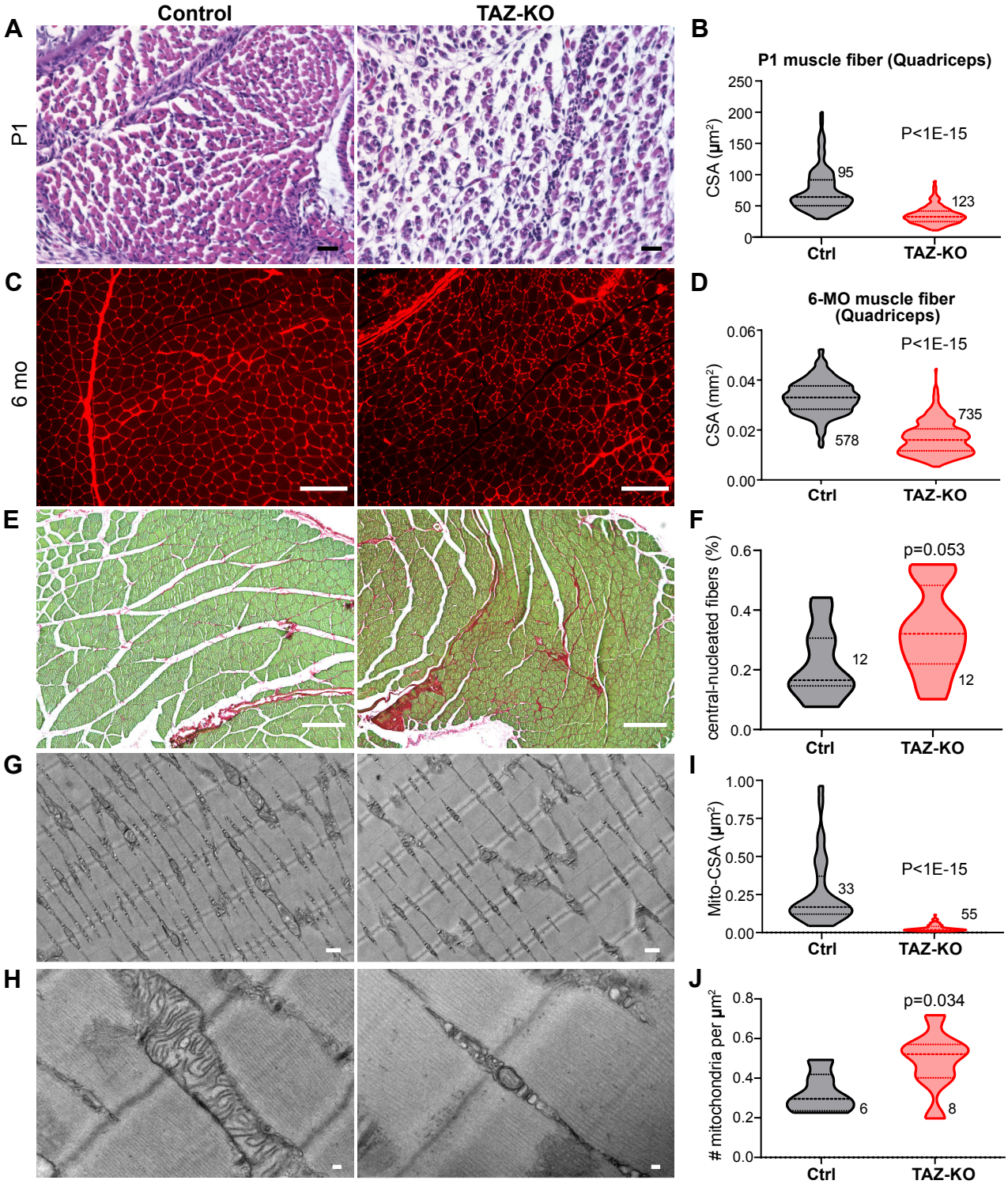




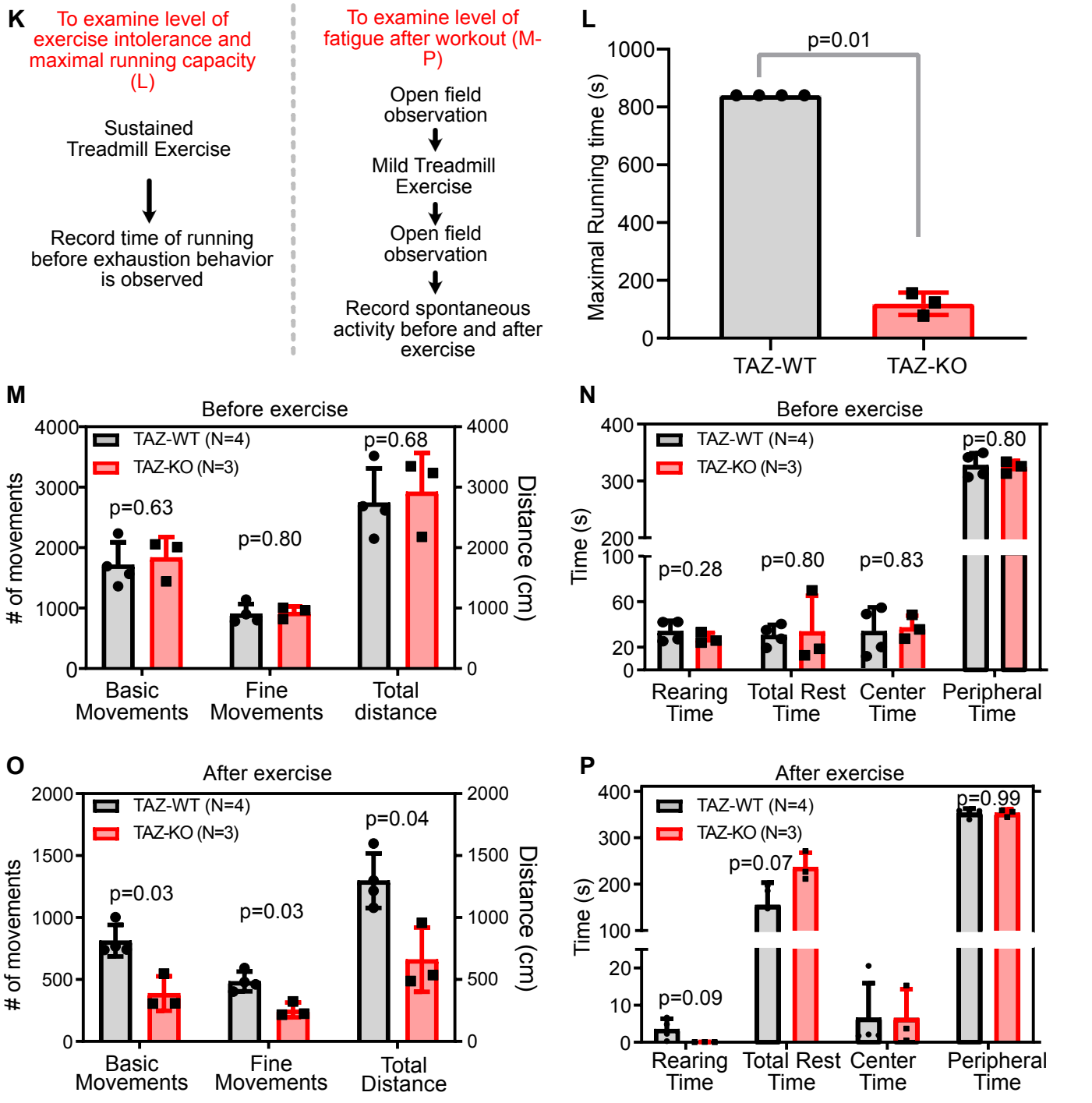
Online Fig. II. **Cardiac fibrosis in human BTHS patients. A**. Human cardiac samples were stained with picrosirius red/fast green. **B**. Percentage of myocardial area (green) occupied by red staining of fibrotic tissue was quantified in at lease 3 sections per patient sample. The experiment was repeated twice. Unpaired t-test: P=8.25E-5 of all BTHS sections compared to the healthy control. Bar=200 μm.



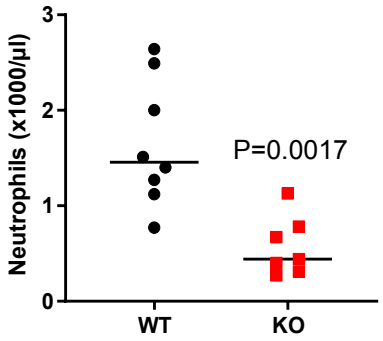
Online Fig. III. Ultrastructure of control and TAZ-KO cardiomyocytes. A. Abnormal sarcomere morphology in TAZ-KO cardiomyocytes. M and Z lines and the A band are labeled. M line and A band were not obvious in TAZ-KO. Bar = 500 nm. **B.** Reduced mitochondrial density in TAZ-KO cardiomyocytes. Numbers of section examined are labled next to the plot. Mann-Whitney U test. **C.** Abnormal mitochondrial organization in TAZ-KO cardiomyocytes. Bar=500 nm.



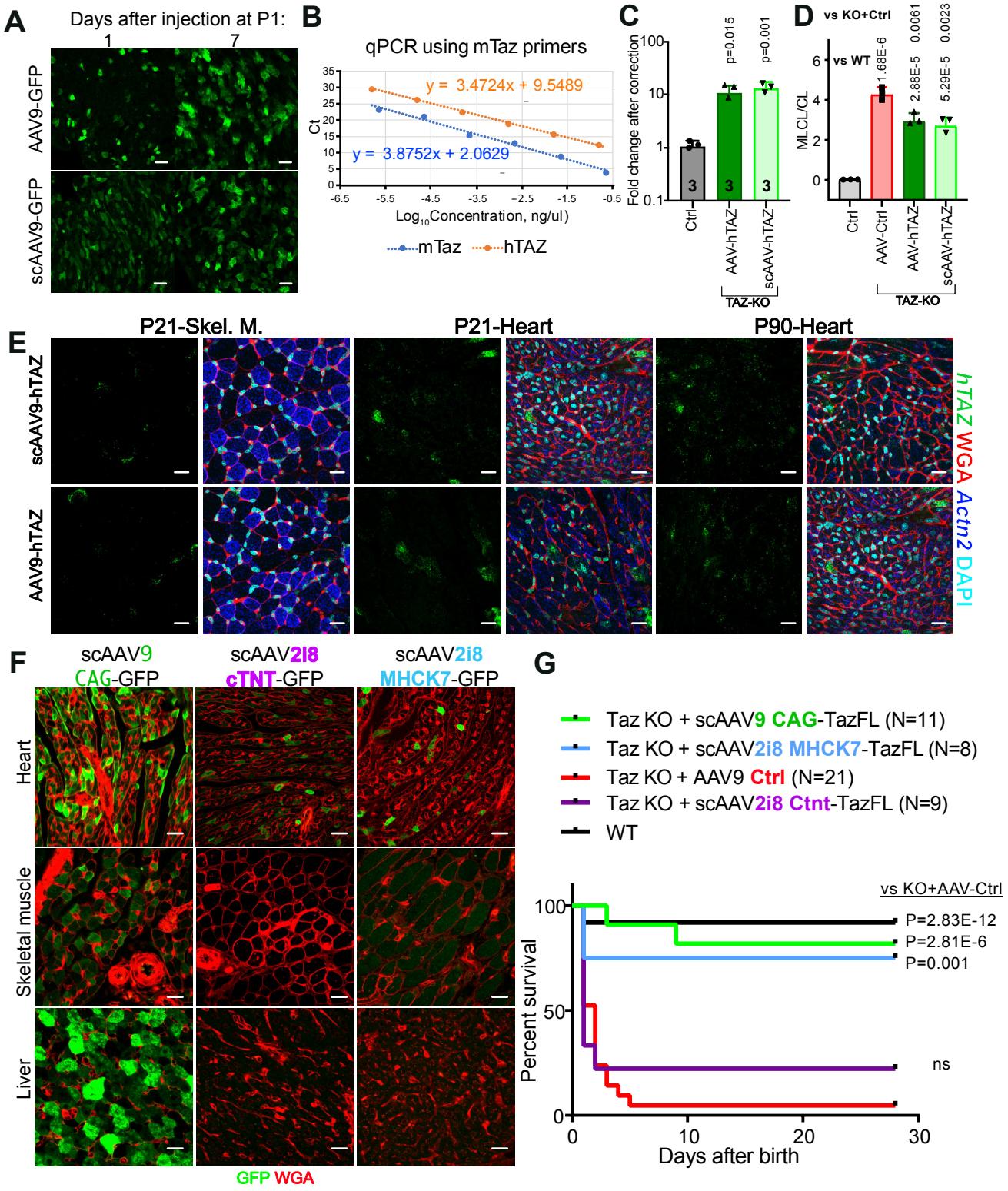
Online Fig. IV. Skeletal muscle defects in TAZ-KO mice. Quadriceps muscle sections were examined by light and electron microscopy. **A-B.** H&E sections at P1. Muscle fiber cross sectional area (CSA) was quantified. Numbers by violin shapes indicates number of muscle fibers analyzed, from 3 mice per group. **C-D.** WGA-stained sections at 6-months-old. Cross sectional area (CSA) of indicated number of muscle fibers from 3 mice per group were analyzed. **E.** Muscle fibrosis. Sections were stained with picrosirus red/fast green. **F.** Fraction of muscle fibers with centrally located nuclei. Number of sections analyzed for each genotype is shown next to violin shapes. A total of 35376 fibers were examined. n=4 animals per group. **G-H.** Muscle ultrastructure as imaged by transmission electron microscopy. Higher magnification images (H) shows mitochondrial morphology. **I.** Quantification of mitochondrial CSA. Number of mitochondria analyzed is indicated by numbers next to violin shapes. **J.** Mitochondrial area density. Number of EM images quantified is indicated by numbers next to violin shapes. Scale bars: 20 μm (A), 200 μm (C, E), 500 nm (G), 100 nm (H). F, J: t-test. B, D, I: Mann-Whitney U test.



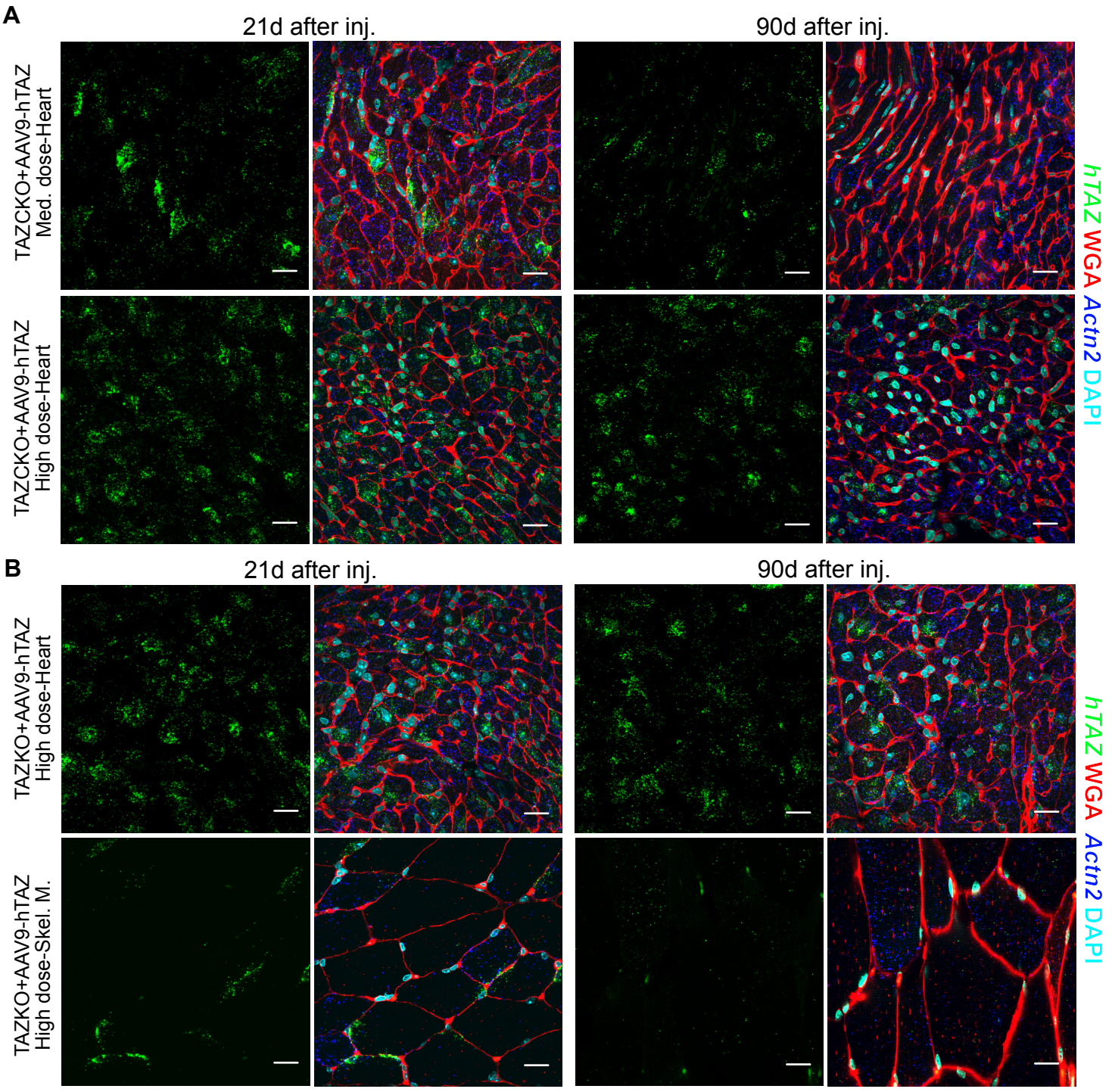
Online Fig. IV (continued). Skeletal muscle defects in TAZ-KO mice. K. Schematic of behavior assays. **L.** Maximal running time before exhaustion (N=4 TAZ-WT vs. 3 TAZ-KO). TAZ-KO had severely compromised endurance. **M-P.** Open field test before and after exercise. TAZ-KO mice were comparable to control mice before exercise, but had reduced voluntary movement after exercise. Number of spontaneous movements and distance traveled, as well as resting time were recorded from TAZ-WT and TAZ-KO mice before (M-N) and after exercise (running on treadmill) (O-P). Time spent exploring the center or peripheral of the open field chamber was also recorded to reveal levels of anxiety and stress. This did not significantly differ between gentoypes. L: Mantel-Cox test. M-P: permutation test.



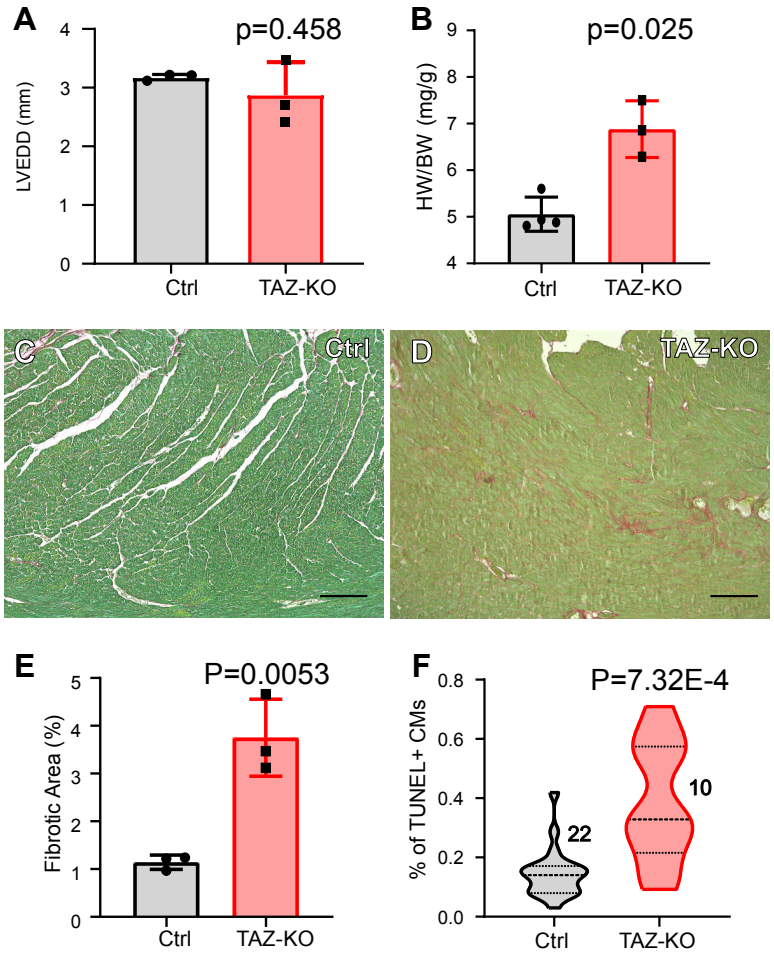
Online Fig. V. Circulating neutrophil count in TAZ-KO mice. Absolute circulating neutrophil count was measured at 6 months old. Unpaired t-test.



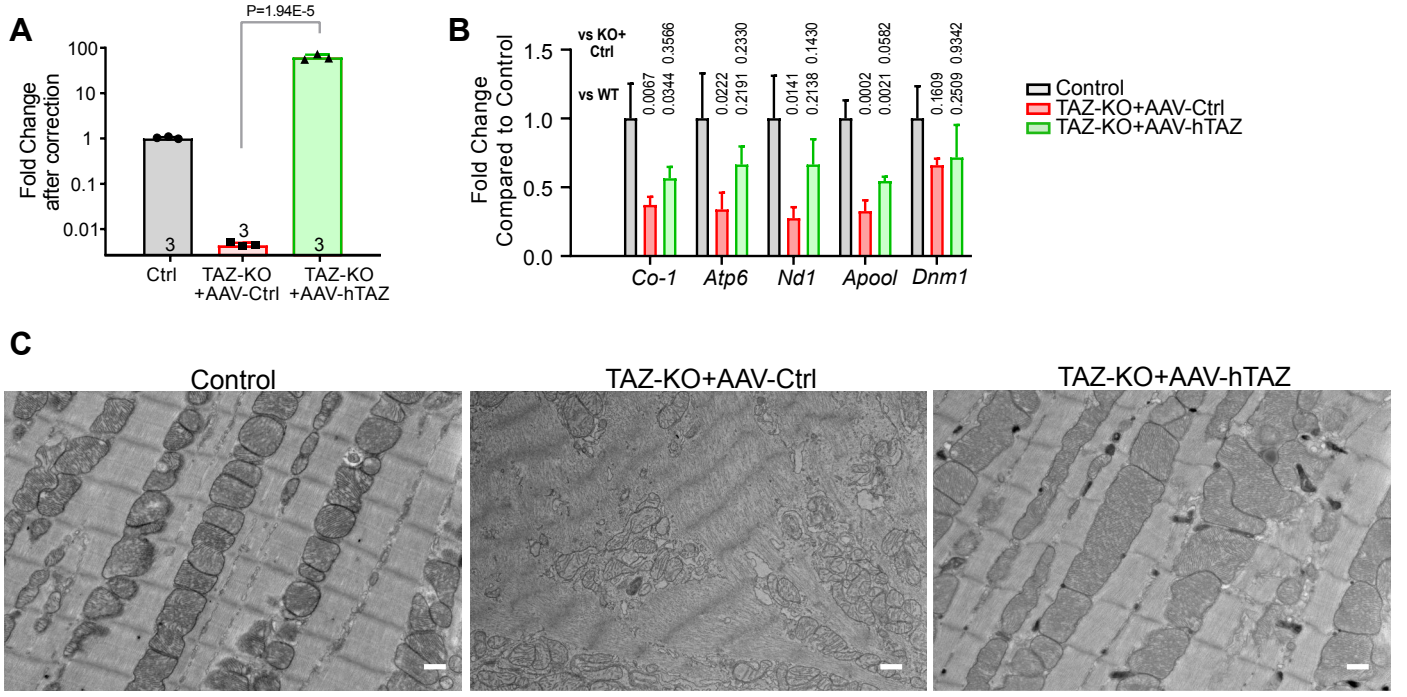
Online Fig. VI. Neonatal treatment of TAZ-KO mice with gene therapy. A. Transduction of neonatal cardiomyocytes by equivalent doses of AAV-GFP and scAAV-GFP. scAAV-GFP showed slightly higher fraction of transduced cardiac cells at one day after injection, but differences were small thereafter. **B.** Primers specific to m*Taz* were used to amplify human *TAZ* (orange) or mouse *Taz* (blue). Standard curves were established using DNA fragment cloned from mouse cDNA or the coding region of hTAZ. One-way ANOVA with Tukey post-hoc test. **C.** Comparison of *hTAZ* expression after treatment of AAV-hTAZ and scAAV-hTAZ. Equivalent doses were given at P1 and expression levels in heart were measured at 4 months after treatment, using *mTAZ* primers and the expression of *hTAZ* was normalized according to B. **D.** Cardiac cardiolipin was analyzed by mass spectrometry at 4 months after treatment. **E.** Visualization of hTAZ-positive myocytes after AAV injection at P1. Sections were examined at 21 and 90 days after injection. hTAZ and *Actn2* transcripts were visualized via RNA probes that specifically hybridized to human *TAZ or Actn2*, respectively. **F.** Tissue tropism of scAAV9-CAG-GFP/scAAV2i8-cTNT-GFP/scAAV2i8-MHCK7-GFP. Bar= 20 μm. **G.** Survival curves of mice receiving different AAV treatment. Mantel-Cox compared to KO+AAV-Ctrl. n.s., not significant.



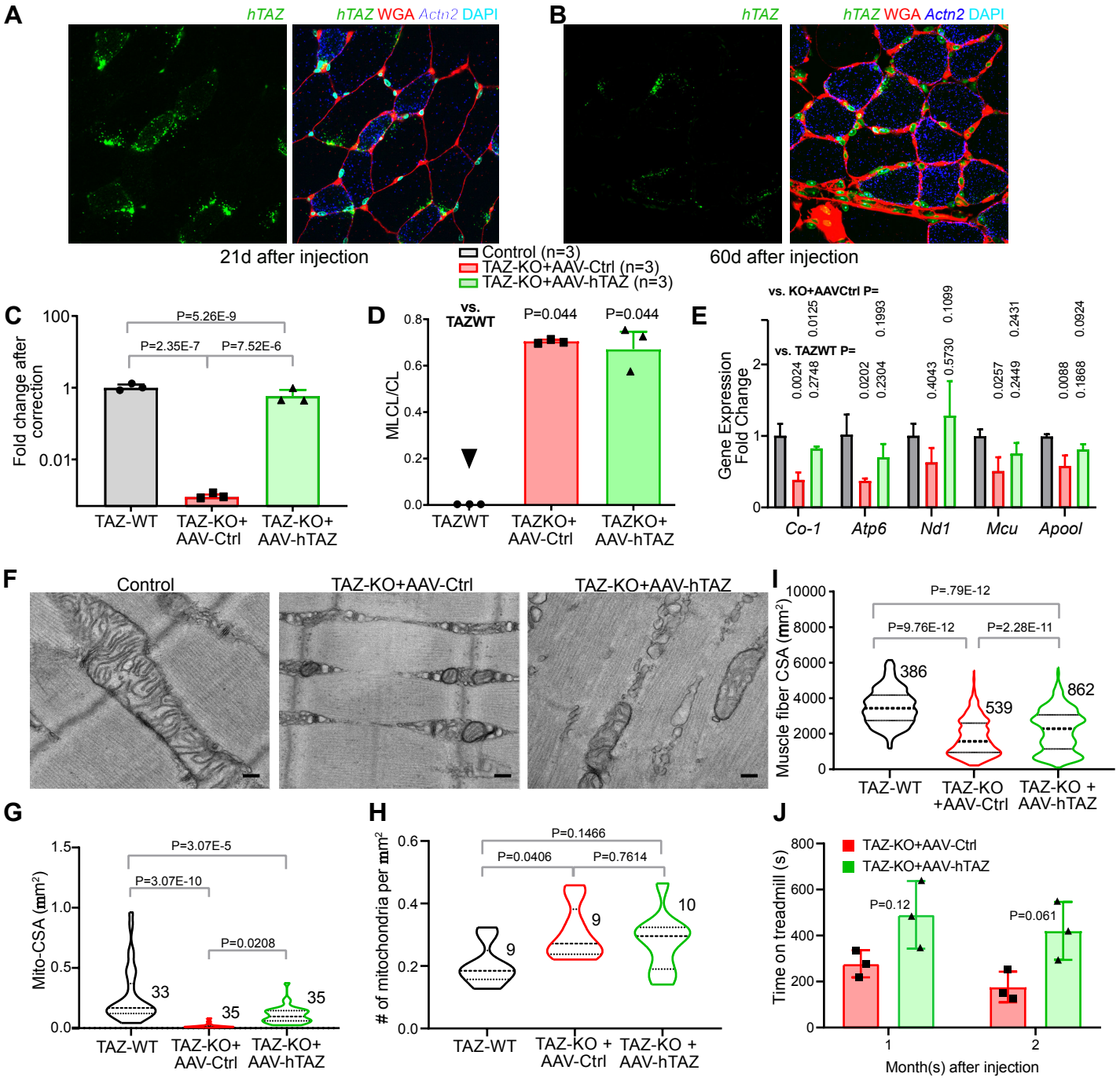
Online Fig. VII. AAV9 CAG-hTAZ skeletal muscle cell transduction. A. Visualization of AAV9-CAG-hTAZ transduced cells in TAZ-CKO mice using an RNA probe against *hTAZ*. *hTAZ* transcripts are shown as green fluorescent puncta in the images. Cardiomyocyte marker *Actn2* was stained blue using a specific RNA probe. AAV was administered at P20. Two time points (21 days and 90 days after injection) were examined. Cardiomyocytes were identified by double-labeling with *Actn2* (blue) and cell membrane was stained with WGA (red). At 21 days after viral injection, high dose of AAV-hTAZ transduced 74% of CMs and medium dose transduced 33% of of CMs in the heart. Over time transduction efficiency remained constant in the heart. **B.** AAV-hTAZ was injected to TAZ-KO mice at 3 months-of-age with the high dose defined in TAZ-CKO mice. AAV-hTAZ reached similar transduction of CMs in TAZ-KO mice as in TAZ-CKO mice but transduced only 27% of skeletal muscle fibers in quadriceps 21 days after injection and even less (11%) when examined at 90 days after the treatment. Transduction efficiency remained constant in the heart between 21 and 90 days yet decreased in the skeletal muscle between these time points. Bar= 20 μm. Two biological replicates were examined and at least 5 images were quantified per each dose.



Online VIII. Cardiac defects in adult (3 month-old) TAZ-KO mice before AAV treatment. A. LV end diastolic diameter evaluated by echocardiography. **B.** TAZ-KO hearts showed elevated ratio of heart weight vs. body weight. **C-E.** Heart sections stained with picrosirius red and fast green to evaluate levels of fibrosis. Percentage of fibrotic myocardium is quantified in E. Bar=200 μm. **F.** CM cell death was evaluated by TUNEL staining. The percentage of apoptotic CMs was quantified. Numbers of sections examined are shown next to the violin shapes. A, E: t-test. B, permutation test. F: Mann Whitney U test.



Online Fig. IX. AAV-hTAZ improved expression mitochondrial genes and corrected mitochondrial morphology. A. Transgene expression evaluated by mouse-specific *Taz* primers via qRT-PCR. Expression in AAV-hTAZ treated group was corrected for different mTAZ versus hTAZ amplification efficiency using standard curves shown in Suppl. Fig. 6B. Relative expression in all groups was normalized to *Gapdh*. Statistical difference was analyzed by one-way ANOVA followed by Tukey's multiple test correction. Sample size is indicated in the bar graph. **B.** Expression of genes that are critical for mitochondrial function and morphology were evaluated by qRT-PCR. Relative expression was normalized to *Gapdh*. Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple test correction. N=3 per group. **C.** Morphology and sub-cellular distribution of mitochondria were improved by AAV-hTAZ treatment in the heart. Scale bar=500 nm.



Online Fig. X. AAV-hTAZ improved skeletal muscle defects in TAZ-KO mice. A-B. AAV CAG-hTAZ was administered at 3 months-of-age. Skeletal muscle (quadriceps) transduction was evaluated by human-specific *TAZ* RNA probe. Percent of *hTAZ*-positive fibers were quantified at 3 wks (A, 27%) and 2 months (B, 17%) after treatment. **C.** Expression of *hTAZ* measured by qRT-PCR using primers specific to *mTaz*. Relative expression of *hTAZ* was corrected for different amplification efficiency of *mTaz* and *hTAZ* as shown in Suppl. Fig. 6B. Relative expression was normalized to *Gapdh*. n=3. **D.** Levels of MLCL/CL in skeletal muscles were examined by mass-spectrometry. n=3. **E.** Expression of genes that are critical for mitochondrial function and morphology was evaluated by qRT-PCR. Relative expression was normalized to *Gapdh*. n=3. **F.** Electron microscopy showing mitochondrial morphology. Bar=200nm. n=3. **G-H.** Quantification of cross sectional area (G) and density of mitochondria (H) in three groups. Numbers of mitochondria (G) and sections (H) examined are shown as numbers next to the plot. **I.** Quantification of cross sectional area of muscle fibers in quadriceps. **J.** Exhaustion test was performed to evaluate the maximal running capacity by measuring the maximal time mice running on treadmill before exhaustion behavior was observed. The test was stopped at 840 sec. C, E: one-way ANOVA followed by Tukey's test. D: permutation test with Benjamini-Hochberg multiple testing correction. J: Welch's t-test. G-I: One way ANOVA followed by Tukey's multiple comparison correction. C-E, I, J, were analyzed 60 days post injection; F-H were analyzed 90 days post injection.