Fine-tuning cardiac IGF1 receptor signaling to promote health and longevity

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Mouse lifespan evaluation

An independent cohort of mice (Table S3) was used for survival analysis and cardiac assessments in aging. This lifespan cohort was generated through a few cycles of breeding in our animal facility by crossing heterozygous males or females (IGF1R^{tg} or dnPI3K) with WT mice. Mice were randomly assigned to the lifespan/aging cohort at the age of 2 months, and animal husbandry staff checked daily for their general health status. A subset of these mice underwent *in vivo* cardiac phenotyping at the age of 20 months, and was subsequently euthanized for organ/tissue collection and gross pathology (tumor incidence, % of mice with tumors). These mice along with those euthanized due to unnatural causes, such as those sacrificed because of severe bites, were censored in the lifespan analysis. Mice were deemed to have severe bites, if their wounds covered over 10% of the skin or were bleeding or infected. All other mice were left undisturbed, except for daily check, until they were found dead in cage. Animals deemed severely moribund were euthanized because of imminent death estimated to occur within less than 24 hours, on the basis of previously established humane endpoints.[14] In such cases, the day of sacrifice was considered the time of death and counted as events in the survival analysis.

Kaplan-Meier analysis was used to compare survival data between different genotypes. Log rank test was applied to compare median survival rates, whereas Student's *t*-test was applied to compare maximum lifespan, defined as the longest-lived decile in a given group. Finally, the χ^2 test was used to compare daily risk of mortality, with censored mice considered only if still alive. Of note, the overall survival rates of WT mice within this study are consistent with published data on the lifespan of males with similar genetic background.[42]

Echocardiography

Cardiac function and dimensions were evaluated in lightly anesthetized mice (0.5-1% isoflurane for maintenance; 4-5% for induction) by transthoracic echocardiography using a micro-imaging system Vevo770 (Fujifilm VisualSonics Inc., Canada). Animal temperature was kept at 37°C using a temperature-controlled heating platform. Mice were placed in a supine position with their limbs in direct contact with non-invasive electrocardiogram leads for heart rate assessment. Pre-warmed ultrasound transmission gel was spread on a shaved chest to obtain cardiac tracings in the parasternal long axis using high-resolution 30 MHz linear-array probe. M-mode tracings were used to evaluate cardiac walls thickness and internal left ventricular dimensions at the level of the papillary muscles during systole and diastole, as previously described.[43] Ventricular volumes and myocardial mass were estimated using Teichholtz and Troy formulas, respectively. Fractional shortening and ejection fraction were determined to assess systolic function. Left atrial area was measured using 2D tracings and indexed to body surface area. Pulsed-wave Doppler tracing of mitral flow were used to measure the myocardial performance index (MPI), which was calculated as the sum of isovolumic contraction and relaxation times divided by ejection time. Generally, 3 stable cardiac cycles were averaged to obtain the reported parameters.

Hemodynamics

Invasive hemodynamic assessment was performed in mice subjected to halogenate anesthesia with isoflurane (induction: 4-5%; maintenance: 1-2%), and intubated for mechanical ventilation (Harvard Mini-Vent 845, Harvard Apparatus, USA). Animal body temperature was kept at 37°C by a temperature-controlled warming pad (TC-1000, CWE Inc., USA). Three-lead electrocardiogram (Animal Bio Amp, FE136; ADInstruments, Sydney, Australia) was used for continuous monitoring of heart rate. Pressure-conductance measurements were obtained in an open-chest setting after inserting a mouse pressure-conductance catheter (1.4F, Cat. No. SPR-839; Millar, ADInstruments, Sydney,

Australia) into the left ventricle through the apex following left thoracotomy and pericardium dissection.[44] Upon stabilization, maximal ventricular pressure as well as maximum and minimum rates of pressure change (dP/dt_{max} and dP/dt_{min}, respectively) were recorded. At least 10 stable consecutive cardiac cycles were analyzed. Subsequently, the inferior vena cava was transiently occluded to derive preload recruitable stoke work (PRSW), as a load-independent measure of contractility. To minimize the signal noise introduced by lung motion, analyzed cardiac cycles were obtained upon brief suspension of ventilation at end-expiration. The analysis was done offline using LabChart V.8 Pro (ADInstruments, Sydney, Australia). At the end of each experiment, animals were sacrificed and different organs (e.g., heart and lungs) were collected for gravimetric analysis and gross pathology. Harvested tissues were immediately processed, flash-frozen in liquid nitrogen, and stored at -80°C for *in vitro* experiments.

Exercise tolerance testing and indirect calorimetry

Peak effort testing was performed on a motorized treadmill housed in an air-tight chamber coupled to a gas analyzer (CaloTreadmill, TSE Systems, Bad Homburg vor der Hoehe, Germany). Mice were subjected to a ramp running protocol with an initial adaptation velocity of 3 m/min for 60 seconds, followed by a constant acceleration of 3 m/min without inclination as described elsewhere.[45] The exercise session ended at maximal exhaustion, defined as the animal's inability to maintain running speed despite being in contact with the electrical grid for more than 5 seconds. Maximum workload was calculated as the final running distance multiplied by body weight$^{(3/4)}$. Prior to oxygen measurements, sampled air from the chamber was continuously dried and cooled down to 5°C, and maximum oxygen consumption (VO_{2max}) was measured at the level where oxygen uptake plateaued during maximal exhaustion.

Mitochondrial respiration and ATP synthesis assays

Oxygen consumption in isolated cardiac mitochondria from 20 months old IGF1R^{tg} and WT mice was measured using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria), and a substrate, uncoupler, inhibitor titration ("SUIT") protocol at 37°C following the manufacturer's instructions. Briefly, mice were euthanized by cervical dislocation under isoflurane anesthesia and hearts were immediately excised. All subsequent steps for the isolation of mitochondria were performed at 4°C using ice-cold reagents. Hearts were washed in STE1 buffer consisting of (in mmol/L): 250 sucrose, 5 Tris/HCl, 2 EGTA at pH 7.4 to remove excess blood. Hearts were then transferred into 4 ml fresh STE1 buffer, and minced into small pieces. Tissue was rinsed twice with STE1 buffer and incubated for 4 minutes in ice-cold 2.5 ml STE2 buffer composed of (in mmol/L): 250 sucrose, 5 Tris/HCl, 2 EGTA, 0.5% BSA (fatty acid free), 5 $MgCl_2$, 1 ATP, 2.5 U/ml bacterial proteinase (Cat. No. P8038, Sigma-Aldrich, Vienna, Austria). After adding an equal amount of STE1 buffer, the tissue was homogenized by hand using a Potter-homogenizer equipped with a Teflon pestle (10 strokes). STE1 buffer (2 ml) was added to homogenates prior to centrifugation at 8000 g, 4°C for 10 min. The pellet was resuspended in STE1 buffer (4 ml) followed by centrifugation (10 min, 700 g, 4°C). Mitochondria were pelleted from the supernatant (10 min, 8000 g, 4°C), washed in 4 ml of STE1-Buffer (10 min, 8000 g, 4°C) and resuspended completely in 100 µl of STE1 buffer. Protein concentration of isolated mitochondria (3-7 µg/µl) was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

High-resolution respirometry was performed simultaneously in mitochondria isolated from WT and IGF1R^{tg} mice (for direct pairwise comparisons) in the closed and calibrated chamber system with constant stirring at 37°C. Mitochondrial preparations, substrates, and inhibitors were kept on ice between the measurements. Equal amounts of mitochondria (equivalent to 16 µg total protein) were transferred into a chamber, containing 2 ml of equilibrated assay buffer composed of (in mmol/L): 100 sucrose, 20 K^+-TES at pH 7.2, 50 KCl, 1 EDTA, 2 $MgCl_2$, 40 KH_2PO_3, 30 malate, and 0.2% fatty acid-free BSA. Respiratory activity measurements started upon addition of 10 mmol/L pyruvate, which resulted in leak respiration. The respiratory capacity of mitochondria in an ADP-activated state of oxidative phosphorylation (OXPHOS) was measured in the presence of 0.6 mmol/L ADP-Mg until a steady-state oxygen consumption rate was reached. Cytochrome (10 µmol/L; CI+CytC) was added to test the outer mitochondrial membrane integrity. Respiration signals were analyzed using the O2k-

software DatLab. Parallel measurements of ATP generation were conducted with another O2k system using the same settings as those used for mitochondrial respiration. Briefly, 2 ml assay buffer was equilibrated in an open-chamber into which ADP was injected. Thirty seconds after ADP injection, 6 samples (10 µl each) were collected in 10-second intervals. The samples were immediately transferred directly into 190 µl of pre-cooled DMSO at -20°C, and rapidly frozen.

As for the ATP measurements, a luminescence-based reaction using the ENLITEN rLuciferase/Luciferin Reagent (Promega) was used. Samples and ATP standards (covering a range of 0 to 60 nmol/L final concentration) were diluted in a reaction buffer composed of (in mmol/L): 100 Tris-acetate and 2.5 EDTA at pH 7.75. For each reaction, 25 µl reagent were added to 100 µl standard or sample dilution, and the reading was performed on LUMIstar (BMG LABTECH, Ortenberg, Germany).

In vivo autophagic flux assessment

Autophagic flux was determined using leupeptin-based inhibition of LC3-II turnover and following published protocols.[22] Mice were subjected to an intraperitoneal injection of leupeptin (40 mg/kg body weight, Sigma-Aldrich, Vienna, Austria) or vehicle (sterile 0.9% NaCl solution). One hour after the i.p. injection, animals were euthanized using cervical dislocation after short isoflurane anesthesia and the hearts were rapidly excised and flash-frozen. The tissue was subsequently subjected to immunoblot analysis of the autophagy-substrate p62/SQSTM1 and measurements of LC3 lipidation (LC3-II to GAPDH ratio), which were performed on tissue extracts using lysis buffer containing 50 mmol/L Tris-HCl at pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100 supplemented with 1x cOmplete Protease Inhibitor Cocktail (Roche, Vienna, Austria) and 1x PhosSTOP Phosphatase Inhibitor (Roche, Vienna, Austria). Immunoblotting onto polyvinylidenfluorid (PVDF) membranes was performed using standard procedures and probed with primary antibodies recognizing p62/SQSTM1 used at 1:1000 (Cat. No. PM045, MBL), LC3B used at 1:1000 (Cat. No. 2775, Cell Signaling Technology), GAPDH used at 1:1000 (Cat. No. 2118, Cell Signaling Technology), and horseradish peroxidase (HRP)-linked anti–rabbit IgG used at 1:3000 (Cat. No. 7074, Cell Signaling Technology).

Immunoblot analysis

For Figures S3 (panels A, C, D), S5, S11, S12 and S13, where phospho- and total protein blots were done in parallel instances, mice were sacrificed and protein lysates of cardiac tissue were mixed with sample buffer (10% glycerol, 1% beta-mercaptoethanol, 1.7% SDS, 62.5 mmol/L TRIS base at pH 6.8, and 0.05% bromophenol blue), and heated for 5 min at 95°C. Lysates were then loaded on a discontinuous SDS polyacrylamide gel electrophoresis (PAGE) stacking and separation gel with a concentration of 8%, 10% or 14% polyacrylamide. The separation gel contained polyacrylamide with the respective percentage diluted in 375 mmol/L TRIS base at pH 8.8. The stacking gel contained polyacrylamide mixed with 0.125 mol/L TRIS base at pH 6.8 to a final concentration of 13%. Electrophoresis was performed using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell system filled with electrophoresis buffer (0.2 mol/L glycine, 25 mmol/L TRIS base, and 0.1% SDS), and an applied voltage of 90-170 V. Next, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the same electrophoresis system containing blotting buffer (0.1 mol/L glycine, 50 mmol/L TRIS base, 0.01% SDS at pH 8.3, and 10% methanol), and an applied voltage of 45 V for 2 hours. Subsequently, membranes were blocked in 5% bovine serum albumin (BSA) in TRIS-buffered saline tween (TBST) buffer (150 mmol/L NaCl, 60 mmol/L TRIS base, 3 mmol/L KCl, and 0.1% Tween-20 at pH 7.4). Membranes were incubated overnight with primary antibodies at 4°C. The following antibodies were obtained from Cell Signaling Technology and used in a dilution 1:1000, 5% BSA in TBST: p70 S6K (Cat. No. 2708), p70 S6K-pT389 (Cat. No. 9234), 4E-BP1 (Cat. No. 9644), 4E-BP1-pT37/46 (Cat. No. 2855), PRAS40 (Cat. No. 2691), PRAS40-pS183 (Cat. No. 5936), AMPK (Cat. No. 2603), ULK1 (Cat. No. 8054), ULK1-pS757 (Cat. No. 6888), ULK1-pS317 (Cat. No. 37762), TFEB (Cat. No. 83010). GAPDH obtained from Abcam (Cat. No. AB8245) was used in a dilution of 1:10000, 5% BSA in TBST. After overnight incubation, membranes were washed in TBST buffer and incubated for a minimum of 2 hours with the corresponding horseradish peroxidase (HRP) coupled secondary antibodies (Goat anti-Mouse, Cat. No. 31430; Goat anti-Rabbit, Cat. No. 31460; Thermo Fisher Scientific). Detection was performed using a Fusion Fx camera system with PURECL Dura (Cat. No. PU4400125, Vilber, Eberhardzell, Germany) or SuperSignal West FEMTO (Cat. No. 34096, Thermo Fisher Scientific, Vienna, Austria) as substrates. Signal intensities were quantified using ImageQuant TL Version 8.1. Background subtraction was performed using the rolling ball method with a defined radius of

200 for all images. As for the rest of figures, where total protein blots were stripped and reprobed with phospho-specific antibodies, protein lysates were separated on 4-12% Bis-Tris gels (Criterion, Bio-Rad, Vienna, Austria) at 70 or 120 V, and electrotransferred on nitrocellulose or PVDF membranes (Amersham, GE, Vienna, Austria) for 2 hours using a Criterion Blotter (Bio-Rad, Hercules, CA, USA) at 400 mA and 4°C. Membranes were stained with Ponceau S solution, and non-specific binding sites were saturated by incubating membranes in TBST composed of 10 mmol/L Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% Tween-20, and supplemented with 5% w/v non-fat dry milk. Overnight incubation at 4°C was then performed with primary antibodies recognizing IGF1R (dilution 1:500; Cat. No. sc-713, Santa Cruz Biotechnology), AKT (dilution 1:1000; Cat. No. 9272, Cell Signaling Technology), phospho(Ser473)-AKT (dilution 1:750; Cat. No. 9271, Cell Signaling Technology), phospho(Thr308)-AKT (dilution 1:750; Cat. No. 9275, Cell Signaling Technology), phosphor(Ser434)-p70 S6K (dilution 1:500; Cat. No. sc-8416, Santa Cruz Biotechnology), p70 S6K (dilution 1:750; Cat. No. 9202, Cell Signaling Technology), phospho(Ser757)-ULK1 (dilution 1:750; Cat. No. 14202, Cell Signaling Technology), ULK1 (dilution 1:750; Cat. No. 8054, Cell Signaling Technology). GAPDH (dilution 1:5000; Cat. No. 5174, Cell Signaling Technology) served as loading control. Secondary anti-rabbit IgG (dilution 1:5000; NA934, GE Healthcare) and anti-mouse IgG (dilution 1:5000; NA931, GE Healthcare) antibodies were incubated for 30 min at room temperature. Detection was performed using Clarity Western ECL Substrate and the ChemiDoc Image System (both Bio-Rad, Hercules, CA, USA). Signal intensities were quantified by densitometry of indicated immunoblot signals using the ImageLab V5.2 software (Bio-Rad, Hercules, CA, USA) after subtracting the in-lane background. Generally, mice used for these experiments were sacrificed in the morning following *ad libitum* feeding overnight.

qPCR analysis for TFEB target gene expression

Heart tissue was lysed in TRIzol reagent (Ambion/Life Technologies, Naugatuck, CT) and homogenized with a rotor-stator homogenizator (Polytron MR 2100 set at power 27. Kinematika AG, Switzerland). RNA was isolated according to TRIzol protocol and 500 ng were used for reverse transcription (RT) by using SCRIPT (Jena Bioscience, Germany). qPCR was performed on a StepOne RealTime PCR System (Thermo-Fisher Scientific, Walthan, MA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). TFEB target genes were selected based on Alesi *et al.*[29] Primer sequences were obtained from the Harvard

PrimerBank.[46] The ribosomal gene 18S was used for sample normalization. Heatmaps were generated with the package Heatmap.2 {gplots} in RStudio with the option "scale" set per gene.

	Forward	Reverse
Ppargc1a	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Hexa	TGGCCCCAGTACATCCAAAC	GGTTACGGTAGCGTCGAAAGG
Mcoln1	GCTGGGTTACTCTGATGGGTC	CCACCACGGACATAGGCATAC
Ctsa	CCCTCTTTCCGGCAATACTCC	CGGGGCTGTTCTTTGGGTC
Rragc	AGATGTCACCCAATGAGACTCT	AGTCGTCCTGTGCATCAATGA
Rragd	CTGTTTGACGTGGTCAGTAAGAT	GTTGAGTCCTTGTCATACGGG
Gabarap	AAGAGGAGCATCCGTTCGAGA	GCTTTGGGGGCTTTTTCCAC
Asah1	CGTGGACAGAAGATTGCAGAA	TGGTGCCTTTTGAGCCAATAAT
Ctsk	GAAGAAGACTCACCAGAAGCAG	TCCAGGTTATGGGCAGAGATT
Flcn	AACGCCATAGTCGCCCTCT	CTGCTCATCTGAATGCCACC
Gpnmb	TGCCAAGCGATTTCGTGATGT	GCCACGTAATTGGTTGTGCTC
Npc1	TGTTTGGTATGGAGAGTGTGGA	GTCACAGCAGAGACTGACATTG
Npc2	AGGACTGCGGCTCTAAGGT	AGGCTCAGGAATAGGGAAGGG
Rn18s	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

Metabolites profiling

Frozen cardiac samples weighing around 30 mg were solubilized into 1.5 ml polypropylene microtubes with ceramic beads with 1 ml of cold lysate buffer with ISTD (MeOH/Water/Chloroform, 9/1/1, -20°C). Next, they were homogenized three times for 20 s at 5500 rpm using Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), followed by centrifugation (10 min at 15000 g, 4°C). Then the upper phase of supernatant was split in two parts: (i) 300 µl were used for Gas Chromatography coupled to Mass Spectrometry (GC/MS) measurements, and (ii) 300 µl were used for Ultra High-Pressure Liquid Chromatography coupled to Mass Spectrometry (UHPLC/MS) measurements. For the GC-MS measurements, the aliquots were dried in a glass tube before adding 50 µl of methoxyamine (20 mg/ml in pyridine), then they were stored at room temperature for 16 hours

under dark conditions. The following day, 80 µl of MSTFA were added and final derivatization was performed for 30 minutes at 40°C. The final aliquots were then transferred in vials and directly injected into the GC-MS. As for UHPLC-MS, the aliquot was evaporated in microtubes at 40°C in a pneumatically-assisted concentrator (Techne DB3, Staffordshire, UK). The dried extracts were then solubilized with 200 µl of MilliQ water, and transferred in LC vials to be injected into UHPLC-MS or kept at -80°C until injection. Finally, targeted metabolites were profiled applying established protocols to measure TCA and glycolytic intermediates (GC-triple quadrupole MS) as well as NADPH/NADP ratio (UHPLC-triple quadrupole MS), as detailed previously.[43,47]

Morphometric analysis of hypertrophy and fibrosis

Hearts were fixed in 4% formaldehyde, processed and embedded in paraffin to assess cardiac myocyte cross-sectional area and evaluate fibrosis using wheat germ agglutinin (WGA) and picrosirius staining, respectively. Serial 4 µm transversal sections were produced using a sliding microtome (Microm HM 360, Apogent Technologies, UK). Paraffin-embedded sections of mouse myocardial tissue, which were de-paraffinized using 100% xylene (Sigma-Aldrich, Cat. No. 1330-20-7) for 10 minutes, followed by hydration in 100%, 95%, 70%, and 50% ethanol solutions (Decon labs, Cat. No. 2701) for 3 minutes in each solution. The slides were then washed in Tris-buffered saline (TBS; Bio-Rad, Cat. No. 1706435) for 5 minutes, and blocked using a mixture of 1% BSA (Sigma-Aldrich, Cat. No. A9647-100G) and TBS solution (200 mg BSA in 20 ml TBS) for 60 minutes in the dark. One percent WGA solution (Invitrogen, Cat. No. W11261) was freshly prepared in blocking solution with addition of $CaCl_2$ (Sigma-Aldrich, Cat. No. C3306) to reach the final concentration of 13 mmol/L. WGA solution was added to the tissue sections, which were incubated for 60 minutes at room temperature and protected from light. Subsequently, the slides were washed thrice in TBS for 5 minutes while protected from light, and tissues were mounted using DAPI (Vector Labs, Cat. No. H1200) to stain the nuclei. The slides were covered with glass cover slips and sealed with nail polish. Imaging was done using Zeiss

700 LSM confocal microscope using 20x objective lens magnification (Zeiss, Jena, Germany). Two or more fields-of-view were imaged per sample for determining the area of the cardiac myocytes and collagen amount in the left ventricle using ImageJ analysis software (NIH, USA), as described elsewhere.[48] All quantifications were performed blinded to the sample origin.

In vitro assessment of autophagy

Cell lines and cell culture: Embryonic myocardium H9c2(2-1) cells (CRL-1446™) were purchased from ATCC. GFP-LC3-tagged H9c2(2-1) cells were constructed from the aforementioned cell line by transduction using LentiBrite™ GFP-LC3 Lentiviral Biosensor viral particles (Merck Millipore, Burlington, MA, USA) according to the manufacturer's instructions. H9c2(2-1) GFP-LC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco® FBS), 1% nonessential amino acids, 1% HEPES in a humidified incubator with 5% CO_2 at 37°C. All cell lines were regularly tested for the absence of mycoplasma contamination. DMEM and all supplements were purchased from Thermo Fisher Scientific (Vienna, Austria).

Compounds and reagents: spermidine (Cat. No. S2626), insulin-like growth factor 1 (Cat. No. SRP4121), and formaldehyde (Cat. No. F8775) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342 (H3570) was purchased from Thermo Fisher Scientific.

Fluorescence microscopy, image acquisition and analysis: One day before treatment, H9c2(2-1) cells stably expressing GFP-LC3 were seeded onto 384-well μClear imaging plates (Greiner Bio-One) and allowed to adhere for 24 hours. To assess the effect of IGF1, cells were pre-treated for 24 hours with increasing IGF1 concentrations, and after one day, the treatment pursued in the presence or absence of spermidine for additional 6 hours. Cells were then fixed in the presence of 3.7% formaldehyde containing 1 μg/ml Hoechst 33342 for 1 hour at room temperature. The fixative was replaced with PBS and the plates were analyzed by automated microscopy. Four view fields per well

were acquired with a robot-assisted Molecular Devices IXM-C (Molecular Devices, Sunnyvale, CA, USA) equipped with an Aura II light source (Lumencor, Beaverton, OR, USA), appropriate excitation and emission filters (Semrock, Rochester, NY, USA), an Andor Zyla camera (Belfast, Northern Ireland), and a 20x PlanAPO objective (Nikon, Tokyo, Japan). The images were segmented and analyzed with the freely available software R (https://www.r-project.org), integrated with the *EBImage* package from the Bioconductor repository (https://www.bioconductor.org), the *MetaxpR* package (https://github.com/kroemerlab/MetaxpR), the *RBioFormats* package (https://github.com/aoles/RBioFormats), as well as the *MorphR* package (https://github.com/kroemerlab/MorphR). The primary region of interest (ROI) was defined by a polygon mask around the nucleus allowing for the enumeration of cells. To quantify GFP-LC3 aggregation, a segmentation mask of high intensity dots was generated in the cytoplasm of cells. After exclusion of cellular debris and dead cells, the relative difference (%) in LC3 dots count was calculated as follows: $(x - c^{-}) \times 100/c^{-}$, where x is test and c^{-} negative control.

Assessment of IGF1 levels

Plasma and cardiac IGF1 concentrations were measured using a mouse IGF1 ELISA kit (Cat. No. ab100695, Abcam, Cambridge, UK) according to the manufacturer's instructions.

Supplemental Tables

Table S1. Echocardiographic parameters of young, middle-aged and old IGF1R^{tg} mice and their WT littermates.
Abbreviations: BW, body weight (g); CI, cardiac index (calculated as the cardiac output divided by body surface area); HR, heart rate; IVSd, interventricular septum thickness during diastole; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; SV, stroke volume.

Data show means ± SEM of the indicated number of mice per group.

	Young		Middle-aged		Old				
	WT (N=10)	IGF1R^{tg} (N=10)	WT (N=10)	IGF1R^{tg} (N=10)	WT (N=10)	IGF1R^{tg} (N=10)	*P* values§		
							Genotype	Age	Interaction
BW (g)	35.2±0.7	35.4±0.5	42.4±1.3	43.5±1.8	41.1±0.8	40.7±1.0	0.823	**<0.001**	0.835
HR (bpm)	517±16	514±19	458±21	434±10	464±13	489±24	0.971	**0.001**	0.393
IVSd (mm)	1.04±0.03	1.25±0.02***	1.11±0.05	1.27±0.04**	1.24±0.04	1.45±0.05***	**<0.001**	**<0.001**	0.804
LVIDd (mm)	4.00±0.04	3.83±0.04	4.28±0.09	4.23±0.07	4.23±0.10	4.00±0.14	0.056	**0.001**	0.670
LVIDs (mm)	2.35±0.05	2.0±0.06**	2.61±0.07	2.51±0.05	2.76±0.12	2.86±0.10	0.086	**0.001**	**0.029**
LVPWd (mm)	0.84±0.02	0.92±0.02*	0.84±0.03	0.95±0.03**	0.98±0.03	1.20±0.07**	**<0.001**	**<0.001**	0.664
LVEDV (µl)	70±2	63±1	83±4	80±3	81±5	71±6	**0.049**	**0.001**	**0.677**
SV (µl)	51±1	50±1	58±3	57±3	51±2	40±4*	0.124	**0.002**	0.110
LVEF (%)	73±1	80±1**	70±1	72±1	64±2	55±2***	0.987	**<0.001**	**<0.001**
LVFS (%)	41±1	48±1**	39±1	41±1	35±2	29±1**	0.639	**<0.001**	**<0.001**
CI (µl/min/cm^2)	269±11	263±8	235±9	221±9	218±8	177±15**	**0.019**	**<0.001**	0.205
LV mass (mg)	147±5	168±4*	172±9	203±10*	204±10	243±12**	**<0.001**	**<0.001**	0.908
LV mass/BW (mg/g)	4.17±.0.16	4.74±0.11**	4.04±0.14	4.67±0.11**	4.93±0.16	6.04±0.40**	**<0.001**	**<0.001**	0.980

§Two-way ANOVA including Age (young/middle-aged/old) and Genotype (IGF1R^{tg}/WT) as fixed factors.
***P<0.001, **P<0.01, *P<0.05 denote the following pairwise comparisons *vs.* age-matched WT.

Table S2. Invasive hemodynamic parameters of old IGF1R^{tg} mice and their WT littermates.

Abbreviations: BW, body weight (g); dP/dt_{max}, maximal rate of pressure rise; dP/dt_{min}, maximal rate of pressure decay; EDP, end-diastolic pressure; ESP, end-systolic pressure; P_{max}, maximum left ventricular pressure; PRSW, preload recruitable stroke work; SPD, spermidine; τ, time constant of isovolumic relaxation (Weiss Method).

Data show means ± SEM of the indicated number of mice per group.

	WT (N=10)	**IGF1R^{tg} (N=7)**	**IGF1R^{tg}+SPD (N=7)**	*P value*[§]
BW (g)	38.8±0.7	39.8±2.1	36.7±1.7	0.4205
P_{max} (mmHg)	63±2	52±1***	59±2$^{\#}$	**0.0014**
ESP (mmHg)	57±2	47±2**	51±3	**0.0090**
EDP (mmHg)	3.9±0.4	4.6±0.7	3.7±0.5	0.5007
dP/dt_{max} (mmHg/s)	4415±186	3578±188*	4596±297$^{\#}$	**0.0131**
dP/dt_{min} (mmHg/s)	-3194±132	-2150±139***	-2874±172$^{\#\#}$	**0.0002**
τ (ms)	7.2±0.4	8.3±0.6	7.3±0.5	0.2600
PRSW (mmHg)	78.2±2.6	59.0±5.0***	72.6±1.7$^{\#}$	**0.0014**

[§]*P* value calculated using ANOVA or Welch's test, as appropriate, and the following pairwise comparisons (adjusted using the Dunnett post hoc test or the Dunnett T3 post hoc test, respectively) are indicated as follows:

***P<0.001, **P<0.01, *P<0.05 (IGF1R^{tg} *vs.* WT)

$^{\#\#}P$<0.01, $^{\#}P$<0.05 (IGF1R^{tg}+SPD *vs.* IGF1R^{tg})

Table S3. Lifespan estimates in WT, IGF1R^{tg} and dnPI3K mice.

	Lifespan estimates[§]								NUMBER OF MICE				*P* VALUE (LOG RANK TEST)	
	Mean	SEM	**25%**	SEM	**50%**	SEM	**75%**	SEM	**Total**	**Natural death**	**Estimated death**	**Censored**	*vs.* **WT**	*vs.* **IGF1R^{tg}**
WT	521	28	282	41	569	76	768	44	**90**	46	11	33		
IGF1R^{tg}	511	31	358	87	610	24	674	33	**48**	31	3	14	0.270	
dnPI3K	528	37	209	37	591	130	807	23	**80**	45	4	31	0.640	0.268

[§]Kaplan-Meier analysis of mean, median (50%) as well as first (25%) and third (75%) quartiles of survival.

Table S4. Echocardiographic parameters of young and old dnPI3K mice and their WT littermates.

Abbreviations: BW, body weight; CI, cardiac index (calculated as the cardiac output divided by body surface area); HR, heart rate; IVSd, interventricular septum thickness during diastole; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; SV, stroke volume.

Data show means ± SEM of the indicated number of mice per group.

	Young		**Old**				
	WT (N=5)	**dnPI3K (N=5)**	**WT (N=12)**	**dnPI3K (N=15)**	***P* values[§]**		
					Genotype	**Age**	**Interaction**
BW (g)	28.6±1.6	29.5±1.8	40.0±2.0	38.6±1.0	0.854	**<0.001**	0.463
HR (bpm)	485±17	470±14	485±12	470±12	0.275	0.990	0.973
IVSd (mm)	1.05±0.05	0.92±0.04*	1.12±0.05	1.06±0.03	**0.029**	**0.019**	0.395
LVIDd (mm)	3.57±0.14	3.70±0.11	4.36±0.09	4.23±0.11	0.987	**<0.001**	0.261
LVIDs (mm)	1.98±0.14	2.25±0.10	2.93±0.11	2.66±0.11	0.996	**<0.001**	**0.026**
LVPWd (mm)	0.75±0.02	0.66±0.02*	0.87±0.03	0.78±0.03*	**0.002**	**<0.001**	0.964
LVEDV (µl)	55±5	59±4	87±4	81±4	0.915	**<0.001**	0.296
SV (µl)	41±3	41±3	53±3	54±2	0.785	**<0.001**	0.816
LVEF (%)	77±2	70±2*	61±2	67±2*	0.915	**<0.001**	**0.005**
LVFS (%)	45±2	39±1*	33±2	37±1*	0.642	**<0.001**	**0.002**
CI (µl/min/cm^2)	232±12	222±7	241±11	244±13	0.780	0.197	0.562
LV mass (mg)	116±8	103±7	184±11	155±5**	**0.014**	**<0.001**	0.319
LV mass/BW (mg/g)	4.06±.0.19	3.48±0.09**	4.58±0.12	4.02±0.11**	**<0.001**	**<0.001**	0.952

[§]Two-way ANOVA including Age (young/old) and Genotype (dnPI3K/WT) as fixed factors.

***P*<0.01, **P*<0.05 denote the following pairwise comparisons *vs.* age-matched WT.

Table S5. Characteristics of the failing and non-failing heart donors.

Please refer to the Methods section for selection criteria.

Abbreviations: BMI, body mass index; EF, ejection fraction; N/A, not available.

Data are presented as means ± SEM for continuous variables, or as a count and percentage for categorical variables of the included probands per group. Unless indicated otherwise, N=10/9/10 in nonfailing (NF) without hypertrophy, NF with hypertrophy and heart failure (HF), respectively.

	Nonfailing (NF)		**Failing (HF)**	*P value*[§]
	(-) hypertrophy	(+) hypertrophy		
Demographics				
Mean Age, years (±SEM)	64(±2)	59(±4)	60(±2)	0.381
Mean BMI, kg/m^2 (±SEM)	25.3(±0.6) (N=10)	28.9(±2.2) (N=9)	23.7(±1.1) (N=9)	0.075
Female, n (%)	4(40%)	5(56%)	2(20%)	0.277
Mean EF, % (±SEM)	61(±1) (N=10)	60(±2) (N=9)	19(±2)$^{***,###}$ (N=8)	**<0.001**
Mean IVS, mm (±SEM)	10.7(±0.5) (N=9)	14.4(±0.4)‡‡‡ (N=9)	N/A	N/A
Comorbidities				
Hypertension, n (%)	3(30%)	7(78%)	6(60%)	0.105
Diabetes Mellitus, n (%)	1(10%)	1(11%)	5(50)	0.062
Renal insufficiency, n (%)	0(0%)	0(0%)	4(40%)	**0.012**
Coronary artery disease, n (%)	0(0%)	3(33%)	3(30%)	0.134
Arrhythmia, n (%)	0(0%)	0(0%)	5(50%)$^{*,\#}$	**0.003**
Laboratory				
Mean NTpro-BNP, pg/ml (±SEM)	88(±23) (N=6)	121(±19) (N=7)	19114(±6793)$^{***,###}$ (N=4)	**0.003**

[§]*P* value calculated using ANOVA or Welch's test (continuous variables) and χ^2 test (categorical variables), as appropriate, with the following Bonferroni-adjusted pairwise comparisons indicated as follows:

****P*<0.001, ***P*<0.01, **P*<0.05 (Failing *vs.* nonfailing without cardiac hypertrophy)

$^{\#\#}P$<0.01, $^{\#}P$<0.05 (Failing *vs.* nonfailing with cardiac hypertrophy)

$^{‡‡‡}P$<0.001 denotes the comparison between nonfailing donors with cardiac hypertrophy versus those without hypertrophy using Student's *t*-test.

Supplemental Figures

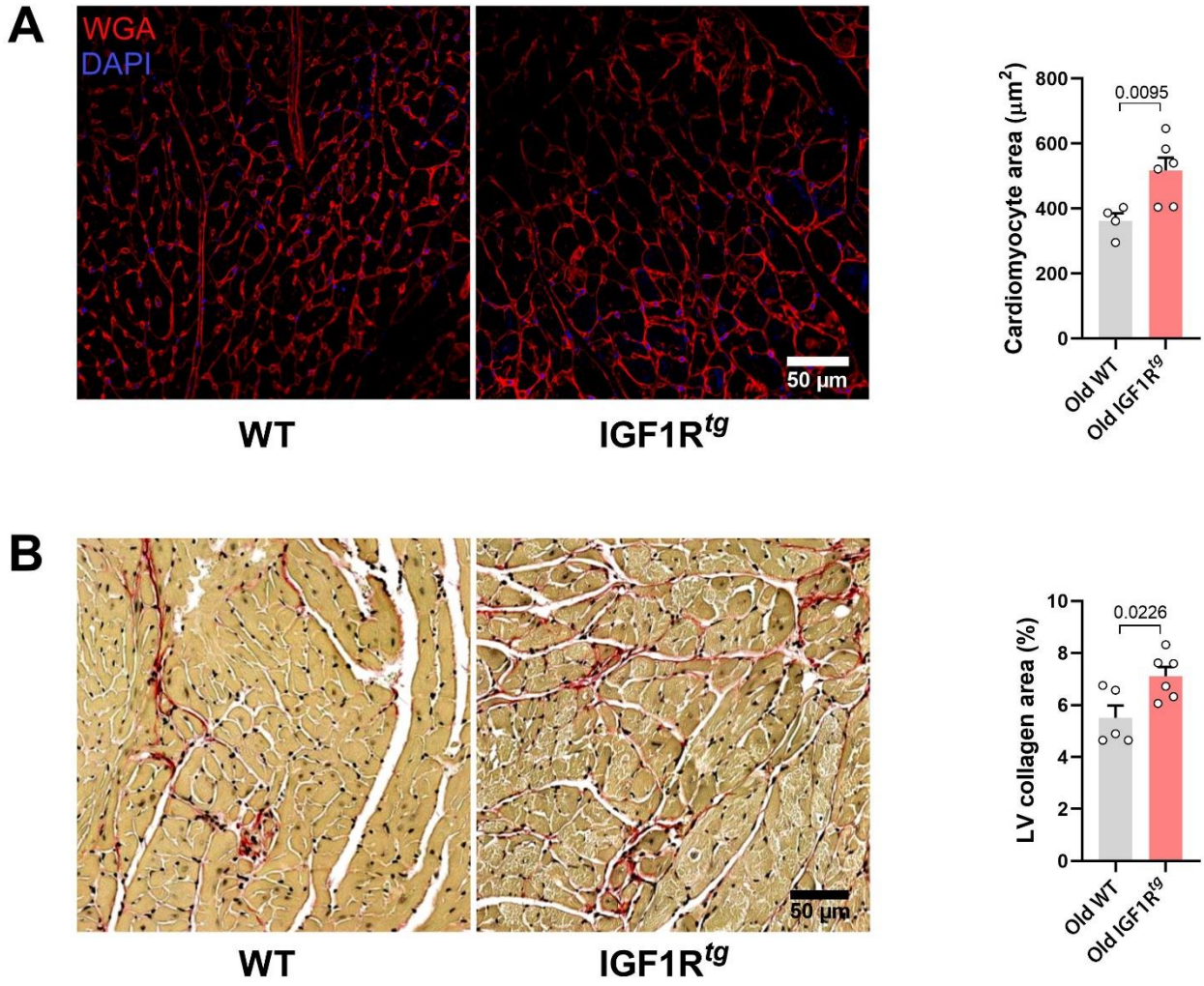

Figure S1. Increased hypertrophy and fibrosis in IGF1R^{tg} hearts.
(A) Representative confocal images (left) and quantification (right) of cardiac myocyte cross-sectional area, as evaluated by wheat germ agglutinin (WGA) staining in 20-month-old IGF1R^{tg} mice. DAPI was used as the nuclear stain. **(B)** Representative microphotographs (left) and quantification (right) of the left ventricular fibrotic area indicative of collagen deposition, as evaluated by picrosirius red staining in 20-month-old IGF1R^{tg} mice. (n=4-6 mice per group). *P* values were calculated by Mann-Whitney test (A) or unpaired Student's *t*-test (B). Bars and error bars show means and SEM, respectively, with individual data points superimposed.

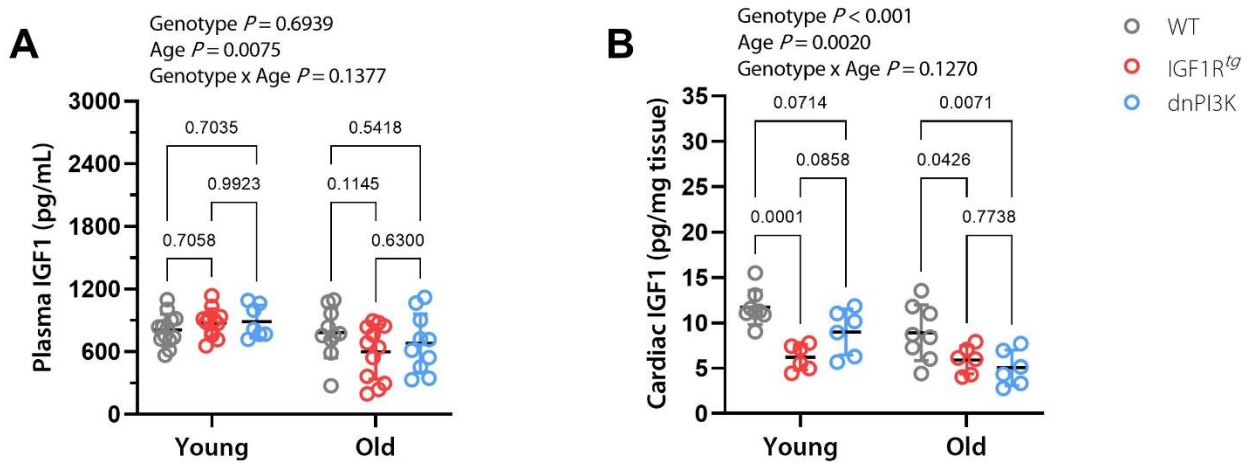

Figure S2. IGF1 concentration in the plasma and hearts of young and old WT, IGF1R^{tg} and dnPI3K mice.

(A) Plasma and **(B)** cardiac concentrations of IGF1 in young (3-6 months old) and old (20 months old) WT, IGF1R^{tg} and dnPI3K mice (n=6-13 mice per group). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by Tukey's post hoc test comparing different genotypes. Bars and error bars show means and SEM, respectively, with individual data points superimposed.

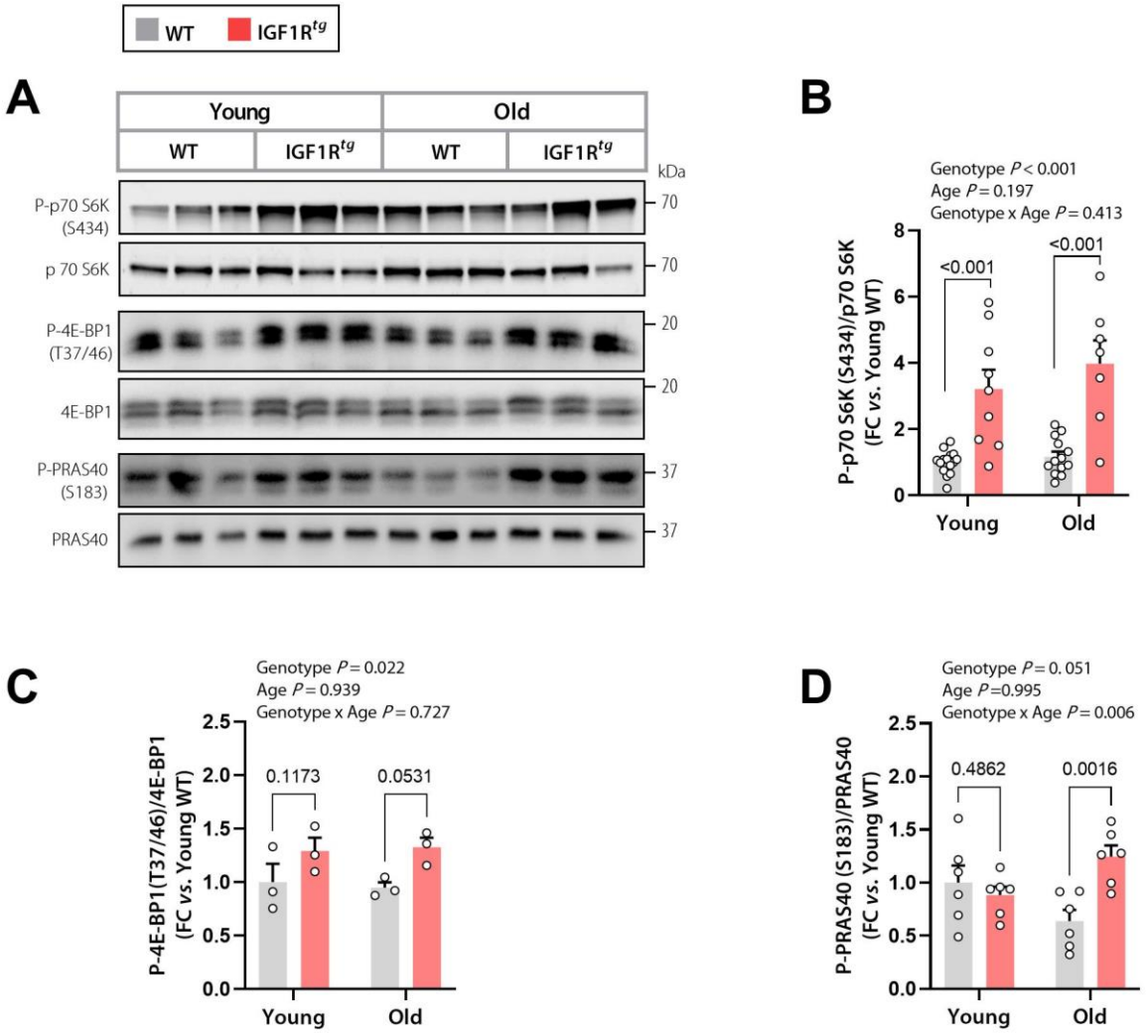

Figure S3. Phosphorylation of p70 S6K, 4E-BP1 and PRAS40 in young and old IGF1R^{tg} mice. Representative Western blot images **(A)** and densitometry-based quantification of cardiac **(B)** p70 S6K phosphorylation (n=9/15 and 7/13 mice per genotype in young and old, respectively), **(C)** 4E-BP1 (n=3 mice per group), and **(D)** PRAS40 (n=6 mice per group) normalized to the respective protein expression in young (3-6 months old) and old (20 months old) IGF1R^{tg} and WT mice. *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between IGF1R^{tg} mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

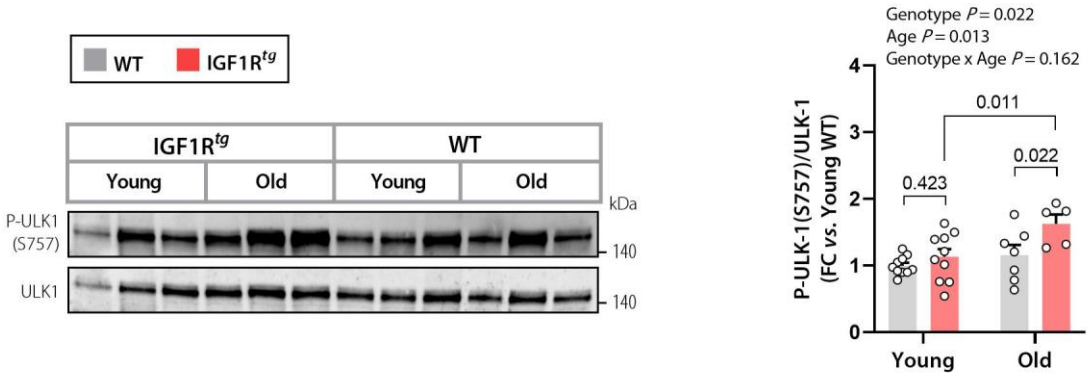

Figure S4. ULK-1 phosphorylation in young and old IGF1R^{tg} mice.
Representative Western blot images (left) and densitometry-based quantification (right) of cardiac ULK-1 phosphorylation (normalized to total ULK-1 expression) in young (6-month-old) and old (20-month-old) IGF1R^{tg} and WT mice (n=10/5-7 mice per genotype in young and old, respectively). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between IGF1R^{tg} mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

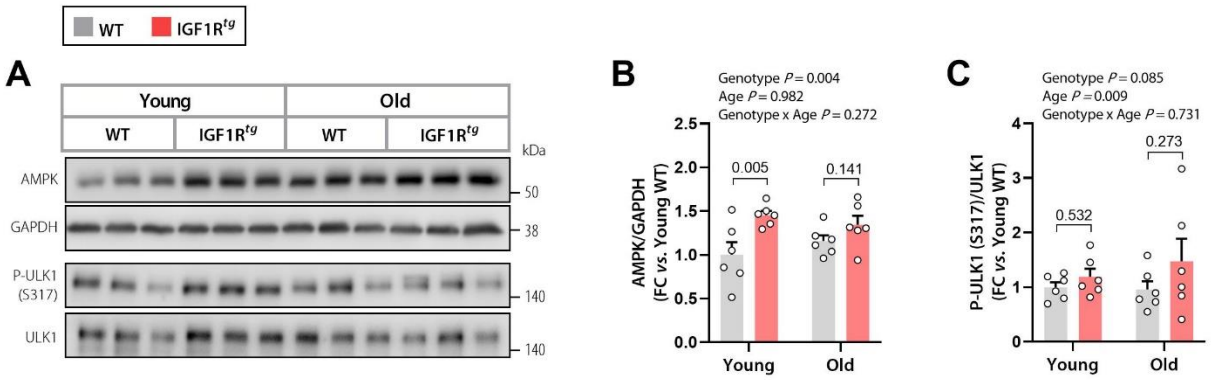

Figure S5. AMPK-dependent ULK-1 phosphorylation in young and old IGF1R^{tg} mice.
Representative Western blots **(A)** and quantification of cardiac **(B)** AMPK expression (normalized to GAPDH) and **(C)** ULK-1 (S317) phosphorylation (normalized to ULK-1 protein expression) in young (3-6 months old) and old (20 months old) IGF1R^{tg} and WT mice (n=6 mice per group). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between IGF1R^{tg} mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

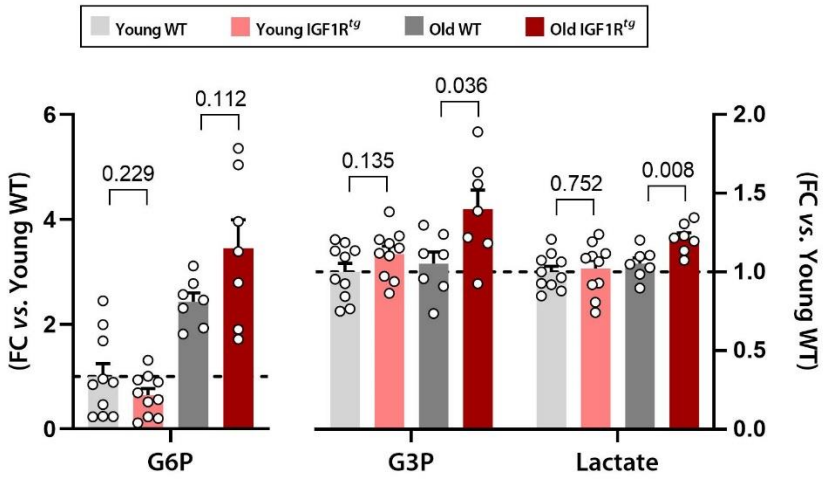

Figure S6. Glycolytic intermediates in young and old IGF1R^{tg} mice.
Relative abundance of the glycolytic intermediates, glucose 6-phosphate (G6P) and glycerol 3-phosphate (G3P), as well as lactate in young (3 months old) and old (20 months old) IGF1R^{tg} and WT mice, (n=7-10 mice per group). *P* values were calculated by Welch's *t*-test or Mann-Whitney test, as appropriate. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

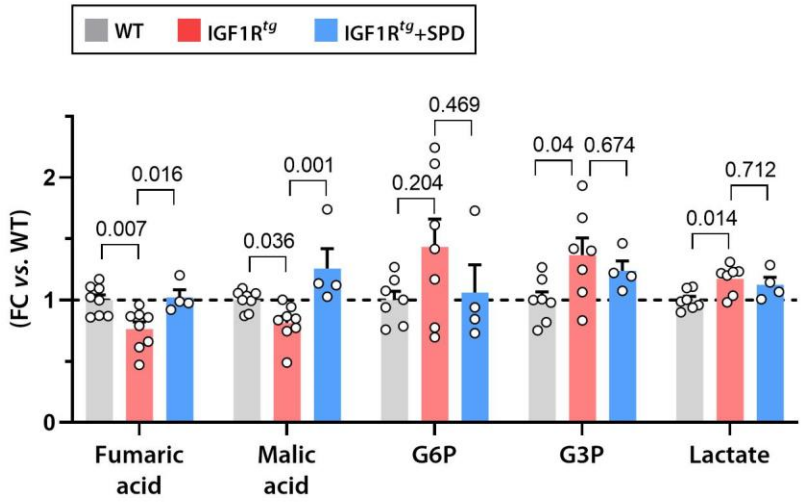

Figure S7. The effect of spermidine on tricarboxylic acid cycle and glycolytic intermediates in aged IGF1R^{tg} mice.

Relative difference in the tricarboxylic acid cycle (TCA) intermediates, fumarate and malate, as well as the glycolytic intermediates, glucose 6-phosphate (G6P) and glycerol 3-phosphate (G3P) and lactate in 20-month-old WT and IGF1R^{tg} mice treated or not with spermidine (SPD), (n=4-8 mice per group). Of note, WT and control IGF1R^{tg} mice are the same as those shown in Figure 2. Indicated *P* values were calculated by ANOVA with Dunnett's post hoc, Welch's test with Dunnett's T3 post hoc or Kruskal-Wallis with Dunn's post hoc test, as appropriate. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

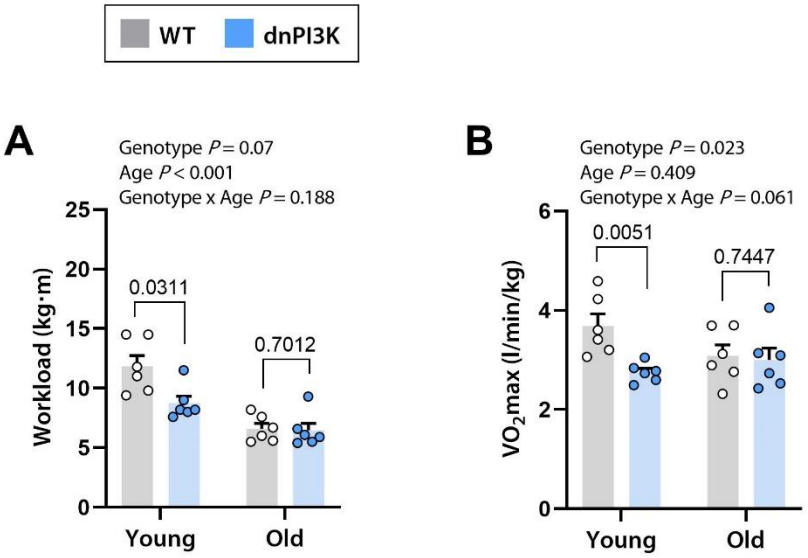

Figure S8. Exercise tolerance testing in dnPI3K mice.
(A) Maximum workload and **(B)** peak oxygen consumption (VO_2max) during exercise tolerance testing in young (3 months old) and old (20 months old) dnPI3K and WT mice, (n=6 mice per group). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between dnPI3K mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed.

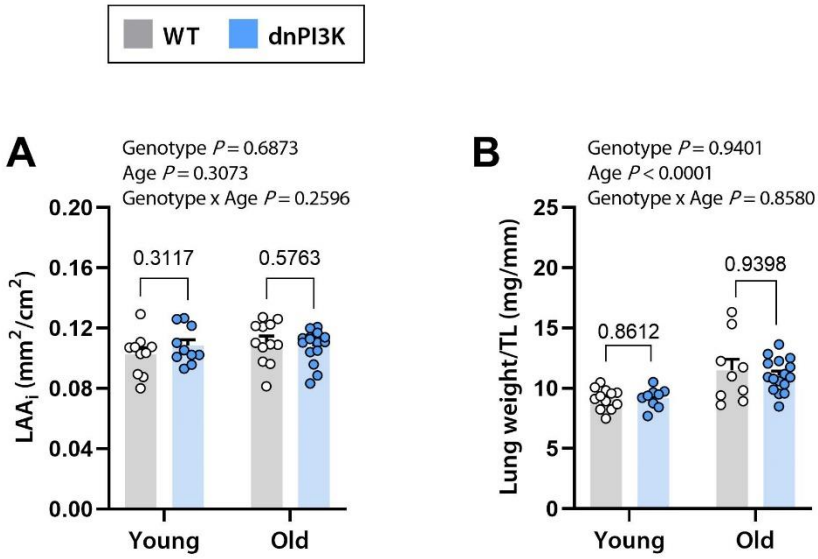

Figure S9. Left atrial area and lung weight-to-tibia length in dnPI3K mice.
(A) Body surface area-normalized left atrial area (LAA_i) and **(B)** tibia length (TL)-normalized lung weight in young (3 months old) and old (20 months old) dnPI3K and WT mice, (n=9-16 mice per group). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between dnPI3K mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed.

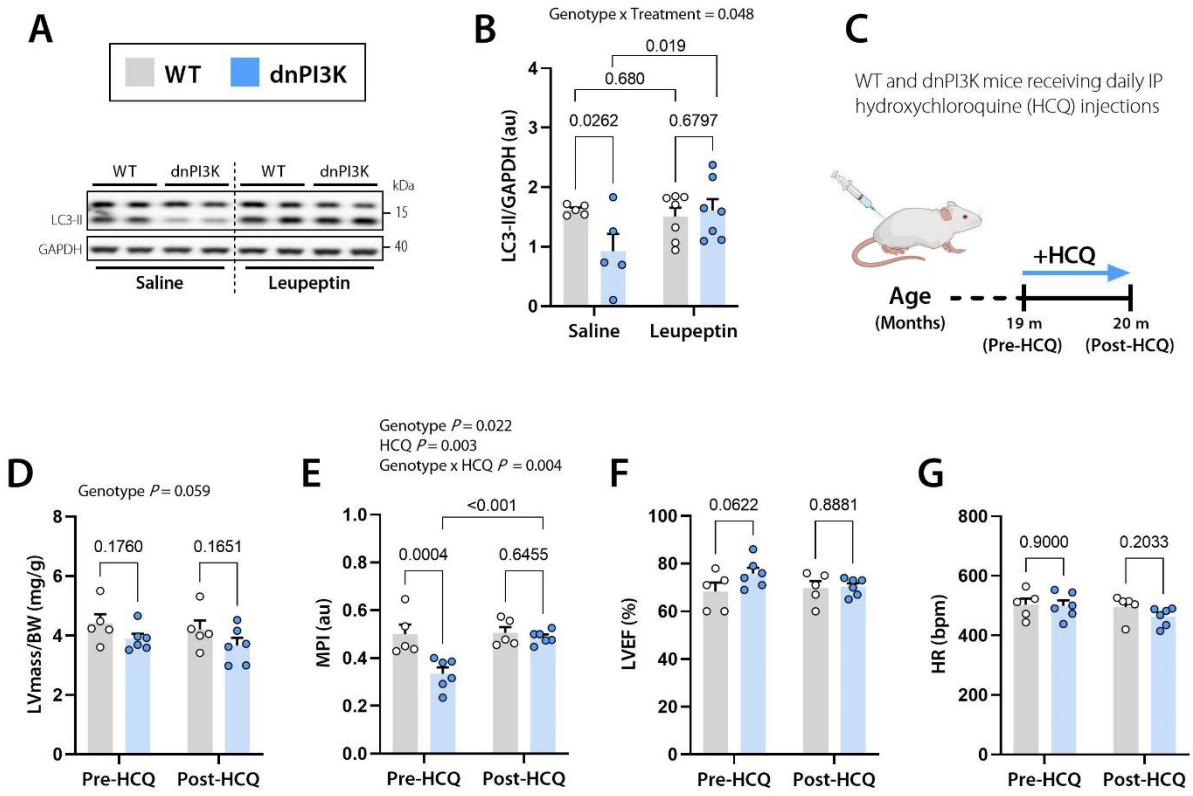

Figure S10. Attenuated IGF1R signaling in dnPI3K mice extends cardiac healthspan in an autophagy-dependent manner.

(A-B) Representative Western blots (A) and quantification (B) of the lipidated autophagy marker LC3-II in the hearts of 9-month-old dnPI3K and WT mice that were treated with the protease inhibitor leupeptin (n=7 mice per group) or saline (n=5 mice per group).

(C) Schematic representation of hydroxychloroquine (HCQ) administration protocol to dnPI3K mice. HCQ (60 mg/kg, IP) was injected daily starting at the age of 19 months, and cardiac parameters were assessed before and after 4 weeks of injections.

(D) Echocardiography-derived left ventricular (LV) mass normalized to body weight (BW).

(E) Myocardial performance index (MPI), calculated as the sum of isovolumic contraction and relaxation times over the ejection time.

(F) LV ejection fraction (LVEF) and **(G)** heart rate (HR) in aged dnPI3K and WT mice before (Pre-HCQ) and after hydroxychloroquine (Post-HCQ) administration, (n=6/5 mice per genotype, respectively).

Indicated *P* values on top of the panels represent factor comparisons by independent two-way ANOVA (B) or repeated measures two-way ANOVA (D-G), including genotype and treatment as fixed factors, followed by pairwise comparisons between dnPI3K and WT mice. Bars and error bars show means and SEM, respectively, with individual data points superimposed.

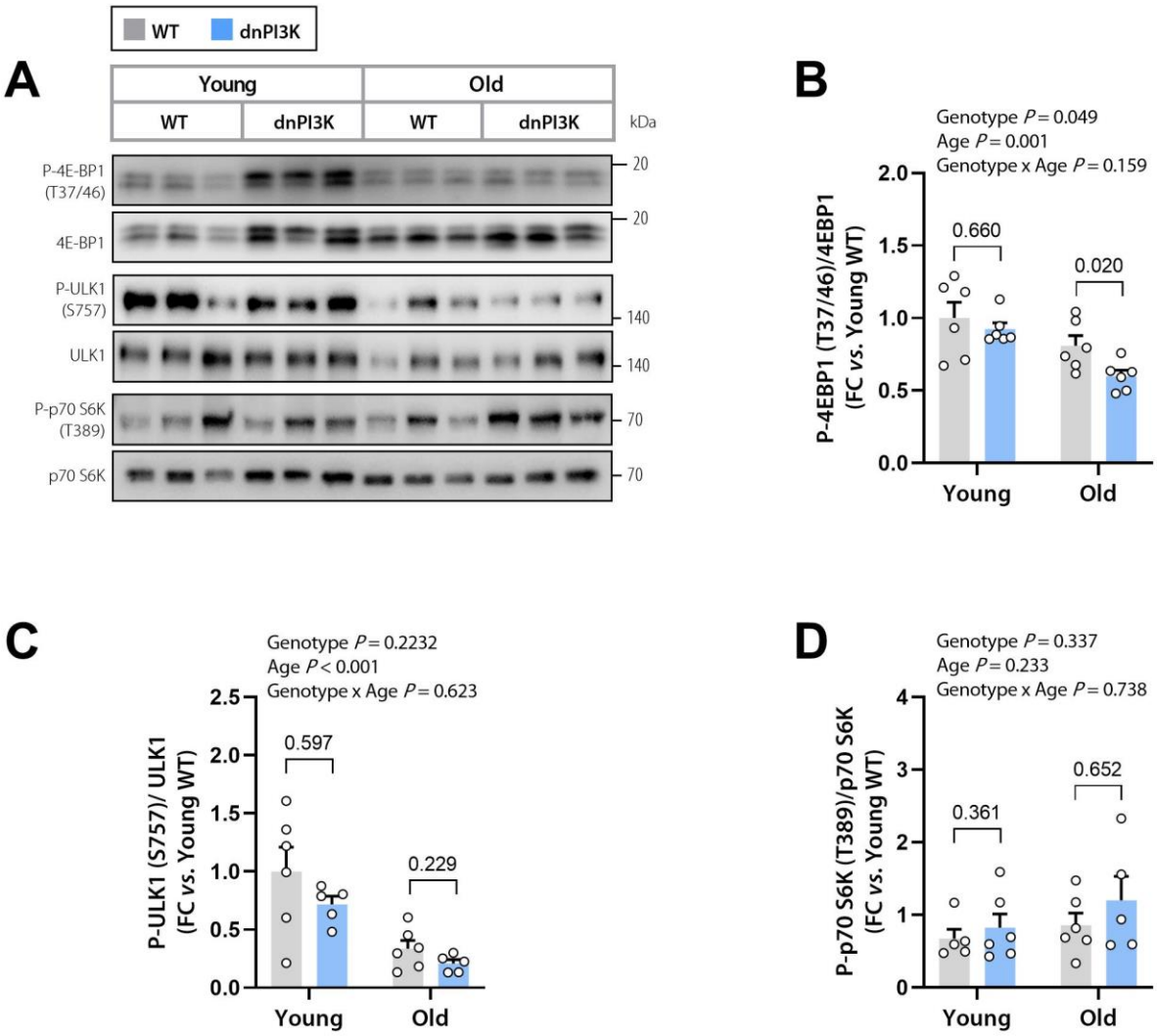

Figure S11. Phosphorylation of 4E-BP1, ULK-1 and p70 S6K in young and old dnPI3K mice.
Representative Western blot images **(A)** and densitometry-based quantification of cardiac **(B)** 4E-BP1, **(C)** ULK-1 and **(D)** p70 S6K phosphorylation (n=5-6 mice per group, depending on whether an outlier was identified by the 1.5 IQR rule) normalized to the respective protein expression in young (3-6 months old) and old (20 months old) dnPI3K and WT mice. Of note, *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between dnPI3K mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

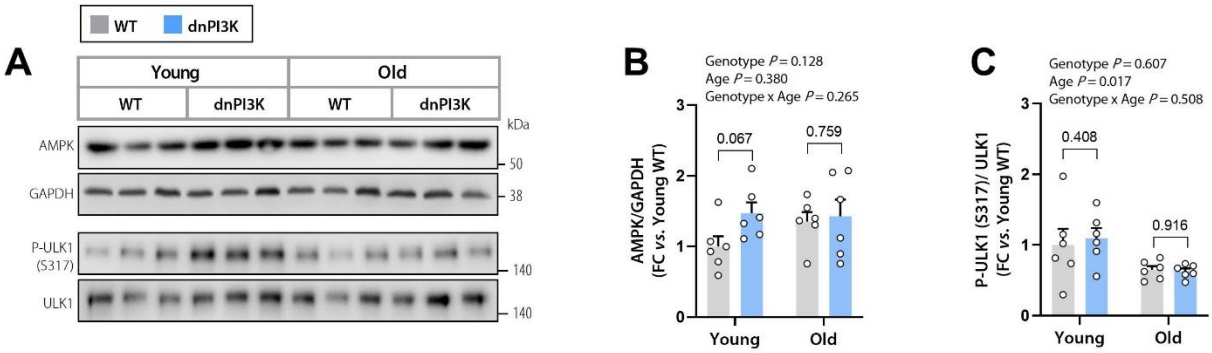

Figure S12. AMPK-dependent ULK-1 phosphorylation in young and old dnPI3K mice.
Representative Western blots **(A)** and quantification of cardiac **(B)** AMPK expression (normalized to GAPDH) and, **(C)** ULK-1 (S317) phosphorylation normalized to ULK-1 protein expression in young (3-6 months old) and old (20 months old) dnPI3K and WT mice (n=6 mice per group). *P* values were calculated by two-way ANOVA, including genotype and age as fixed factors, followed by pairwise comparisons between dnPI3K mice and their age-matched WT controls. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

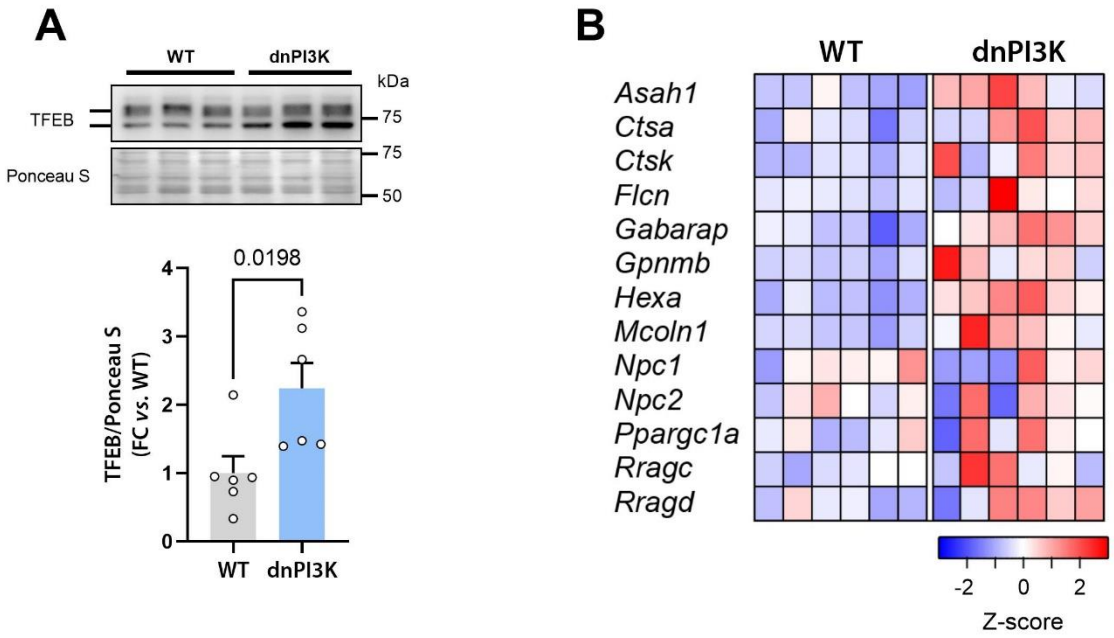

Figure S13. TFEB activity in dnPI3K mice.
(A) Representative Western blots (above) and quantification (below) of cardiac TFEB expression (lower band normalized to Ponceau S) as well as **(B)** TFEB target genes in young dnPI3K and WT mice (n=6 mice per group). *P* value was calculated by Student's *t*-test. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.

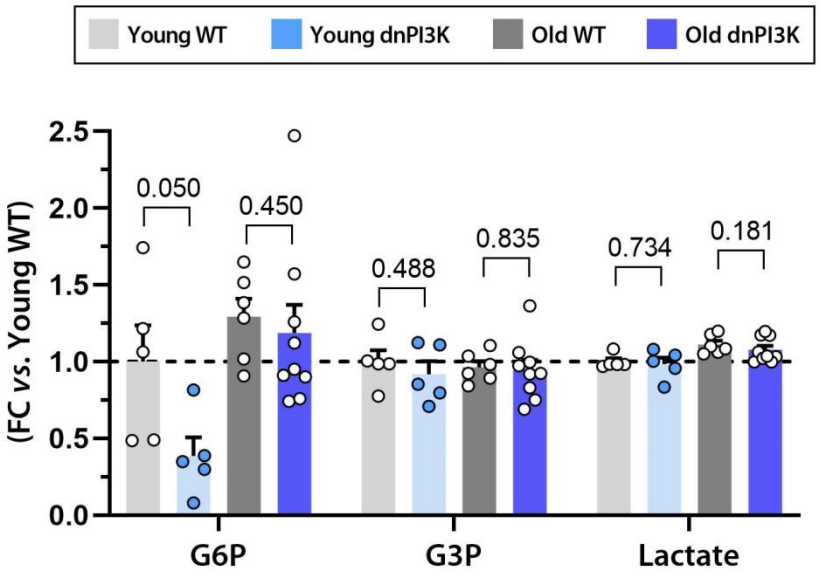

Figure S14. Glycolytic intermediates in young and old dnPI3K mice.
Relative abundance of the glycolytic intermediates, glucose 6-phosphate (G6P) and glycerol 3-phosphate (G3P), as well as lactate in young (3 months old) and old (20 months old) dnPI3K and WT mice, (n=5-9 mice per group). *P* values were calculated by Welch's *t*-test or Mann-Whitney test, as appropriate. Bars and error bars show means and SEM, respectively, with individual data points superimposed. FC, fold change.