Supplemental Material

Metabolic Dysfunction And Premature Aging Are Caused By Pim Deletion

Shabana Din BS[1]; Mathias H Konstandin MD[1]; Bevan Johnson BS[1]; Jacqueline Emathinger BS[1]; Mirko Völkers MD[1]; Haruhiro Toko MD[1]; Brett Collins MS[1]; Lucy Ormachea BS[1]; Kaitlen Samse BS[1]; Dieter A Kubli PhD[2]; Andrea De La Torre BS[1]; Andrew S Kraft MD[3]; Asa B Gustafsson PhD[2]; Daniel P Kelly MD[4]; and Mark A Sussman PhD[1]

Supplemental Methods

Mitochondrial respiration assay

Mitochondrial isolation procedures were followed as previously published [1]. Hearts were removed while still contracting from mice anesthetized with ketamine/xylazine (50mg/kg and 10mg/kg, respectively) via intraperitoneal injection. Individual mouse hearts were rapidly minced in ice-cold isolation buffer (100 mmol/L KCl, 50 mmol/L MOPS pH 7.4, 1 mmol/L EGTA, 5 mmol/L MgSO4, 1 mmol/L ATP, 0.2% essentially fatty acid free BSA). Henceforth, all steps were performed at 0˚C on wet ice. Tissue was homogenized in isolation buffer with a Polytron tissue grinder at 11,000 RPM for 2.5 s, followed by 3 strokes at 500 RPM with a Potter-Elvehjem PTFE tissue grinder. The homogenate was centrifuged at 600×g twice for 5 min and the supernatant was saved. Mitochondria were pelleted from the supernatant by centrifugation at 3,000×g twice, and the pellet was rinsed with isolation buffer. The final mitochondrial pellet was resuspended in 100 µl of resuspension buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 2 mmol/L Tris base, and 20 mmol/L HEPES pH 7.4). Protein concentration was determined by Bradford assay using BSA standards. Oxygen consumption measurements were performed with an Oxygraph Clark type electrode (Hansatech Instruments) in respiration buffer (10 mmol/L MgCl2, 100 mmol/L KCl, 50 mmol/L MOPS pH 7.0, 1 mmol/L EGTA, 5 mmol/L KH_2PO_4, 0.2% essentially fatty acid free BSA). 200 µg of mitochondria were added to a final volume of 1 ml respiration buffer at 30˚C. Complex I activity was measured using 2 mmol/L pyruvate and 2 mmol/L malate as substrates. Complex II activity was measured using 5 µmol/L rotenone with 5 mmol/L succinate as substrate.

Quantitative Fluorescence In Situ Hybridization (QFISH) Telomere Measurements

Telomere length was labeled and analyzed by quantitative in situ hybridization (QFISH) using DAKO's PNA probe (K5325) and Leica confocal microscopy. Results were obtained by altering manufacturer's protocol as follows. Slides were deparffinized, rehydrated and treated with 3.7% Formaldehyde for 2 minutes, then subsequently washed with TBS. Next, followed antigen retrieval using 10mM Citrate pH 6.0 for 15 minutes. Slides were washed again with TBS and underwent porteolytic treatment using Proteinase K (Dako, S3004) diluted 1:10 in TBS, for 8 minutes. Preceding another wash, slides were dehydrated in cold ethanol. PNA probe was applied to dried sections, coversliped using a 2mm round coverslip, and incubated on a 85°C hot plate for 5 minutes then allowed to hybridize at 37°C overnight. The following day, slides were immersed in Rinse Solution to remove coverslips and washed with 70% Formamide pH 7.2. Next, slides were washed in pre-heated Wash Solution at 65°C and quenched with 3% H_2O_2 for 20 minutes at room temperature. Slides were then washed, incubated with Sheep anti-FITC/HRP 1:200 in TNB for 1 hour at room temperature, washed again, amplified with Tyr/FITC 1:50 for 10 minutes, then dehydrated in cold ethanol. Lastly, slides were counter-stained and coversliped using Topro 3 1:200 in Vectashield. Telomere signal in each nucleus was acquired using Leica software and divided by nuclear size followed by normalization to NTG controls. Additionally, signals were scanned using identical setting to control experimental variation.

Telomere Length measurement (RT-PCR)

Telomere length was measured by real time PCR using a modified monochrome multiplex quantitative PCR method previously described[2]. Albumin and the telomere template were amplified at the same time to account for the differences in DNA concentration per well and sample. To account for difference in reaction samples, 12

replicates of each sample were prepared using 10ng of DNA, 1X syber green, albumin, and telomere primers, bringing the reaction volume to 15uL. The thermal cycle protocol used is as follows, stage 1 (2 cycles of 15 mine at 95°C, 15 seconds at 94°C and 15 seconds at 49°C), stage 2 (15 seconds at 94°C, 10 seconds at 62°C and 15 seconds at 73°C with plate acquisition), stage 3 (32 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 73°C with signal acquisition, 10 seconds at 84°C, 15 seconds at 87°C with signal acquisition), and stage 4 (1 cycles of 0.05 seconds at 65°C with signal acquisition).

Sample preparation, Immunoblotting, RT-PCR, Mitochondrial DNA Content

Immunoblotting was performed using protein samples separated on 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) by electrophoresis. Protein contents of gels were then transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked in iBLOCK (Life Technologies), and exposed to primary antibodies over night. Alkaline phosphatase (AP), horseradish peroxidase (HRP), Fitc-, Cy5, or Cy3-conjugated IgGs (Jackson ImmunoResearch) were used as secondary antibodies. Fluorescence signal was detected and quantified by using a Typhoon 9400 fluorescence scanner together with ImageQuant 5.0 software (Amersham Biosciences).

mRNA was isolated using the Quick RNA Mini Prep kit (Zymo Research) according to the manufacturer protocol. cDNA was transcribed using the cDNA preparation kit (Biora). For RT-PCR sybr green (Biorad) was employed using the manufacturer protocol. Data were analyzed with the ΔΔC(t) method. A complete list of primers used for qPCR is provided (Table 2). Mitochondrial DNA (mtDNA) copy number was quantified by qPCR from isolated total DNA derived from heart tissue of PTKO mice (n=4) and wildtype mice (n=4) according to the manufacturer protocol of NucleoSpin tissue kit (Macherey-Nagel). 50 ng of total heart DNA was used and each sample was measured in duplicates. Two different primer pairs were used to quantify and confirm relative mtDNA copy number: COXI and Cytochrome b (mitochondrial) and β-Globin/H-19 for genomic DNA. All sequences are listed inTable 2. Data obtained by qPCR were analyzed by the ΔΔCT method.

Transmission Electron Microscopy and Mitochondrial Area

Mitochondria were examined by transmission electron microscopy. Animals were euthanized by cervical dislocation before dissection. The heart was excised and submerged immediately in 2% glutaraldehyde in 0.1M cacodylate buffer with 1% sucrose. Cardiac tissue was cut with a razor blade into 1 mm^2 pieces and incubated in fixative solution on ice for 10 minutes. All of the following steps, through the 100% ethanol dehydration step, were performed on ice. After incubation, each sample received a fresh replacement of fixative solution and was fixed for 1 hour. Following fixation, the samples were washed three times (10 minutes each wash) in 0.1M cacodylate buffer with 1% sucrose. The samples were then postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer with 1% sucrose for 1 hour in the dark and washed three times (10 minutes each wash) in water. The samples were dehydrated through a graded series of ethanol solutions from 30% to 50%, 70%, 85%, 95% and 100% (10 minutes each). Two successive 10 minute incubations in 100% ethanol were performed before the samples were transferred into 100% acetone at room temperature followed by an additional 10 minute incubation step in 100% acetone. Tissue samples were infiltrated with 1:2 EPON/acetone resin followed by 2:1 EPON/acetone resin for 8 hours each. Infiltration with pure EPON was completed overnight. Tissue samples were transferred into a flat embedding mold in pure EPON and polymerized at 60°C for 48

hours. Thin sections (60nm thick) from the embedded samples were cut, collected on 100 mesh copper grids, and stained with uranyl acetate and lead citrate. Images were captured using a FEI Tecnai 12 transmission electron microscope operated at 120kV. Images were acquired at 11000X. Measurements of mitochondrial area per section were calculated as follows using ImageJ software: mitochondrial area was measured followed by normalization to area of section. Sample size was n=3 for each group analyzed.

Sample preparation, Immunoblotting, qPCR, Mitochondrial DNA Content, Microarray Analysis

Immunoblotting was performed using protein samples separated on 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) by electrophoresis.

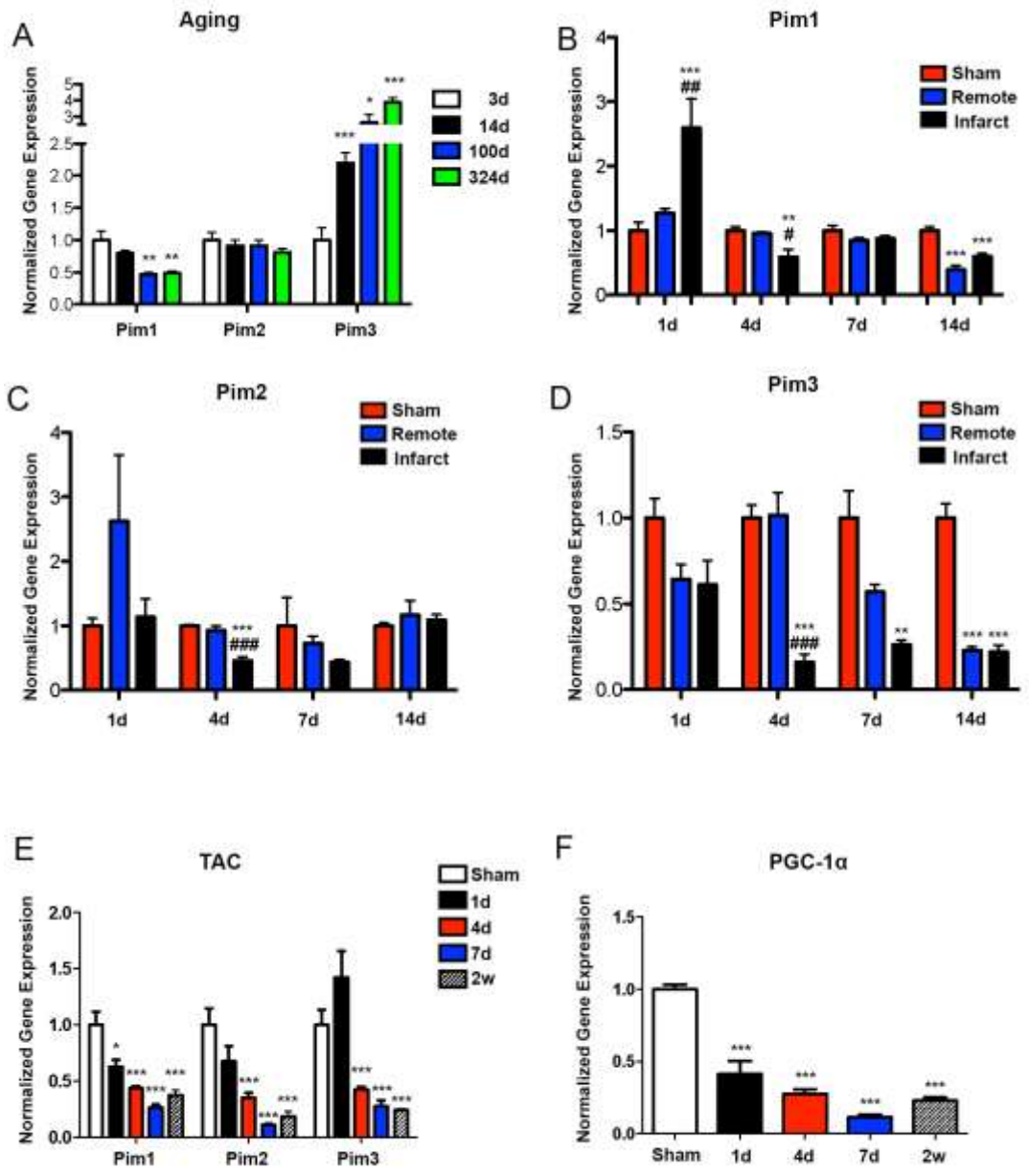
mRNA was isolated using the Quick RNA Mini Prep kit (Zymo Research) according to the manufacturer protocol. cDNA was transcribed using the cDNA preparation kit (Bio-Rad). For RT-PCR sybr green (Biorad) was employed using the manufacturer protocol. Data were analyzed with the ΔΔC(t) method. A complete list of primers used for qPCR is provided (Table II). Further details are provided in supplemental information

Microarray analysis was performed according to the manufacturer protocol using PPAR signaling array from Bar Harbor technologies (catalog number: 00198247) on CFX Connect thermal cycler (Bio-Rad).

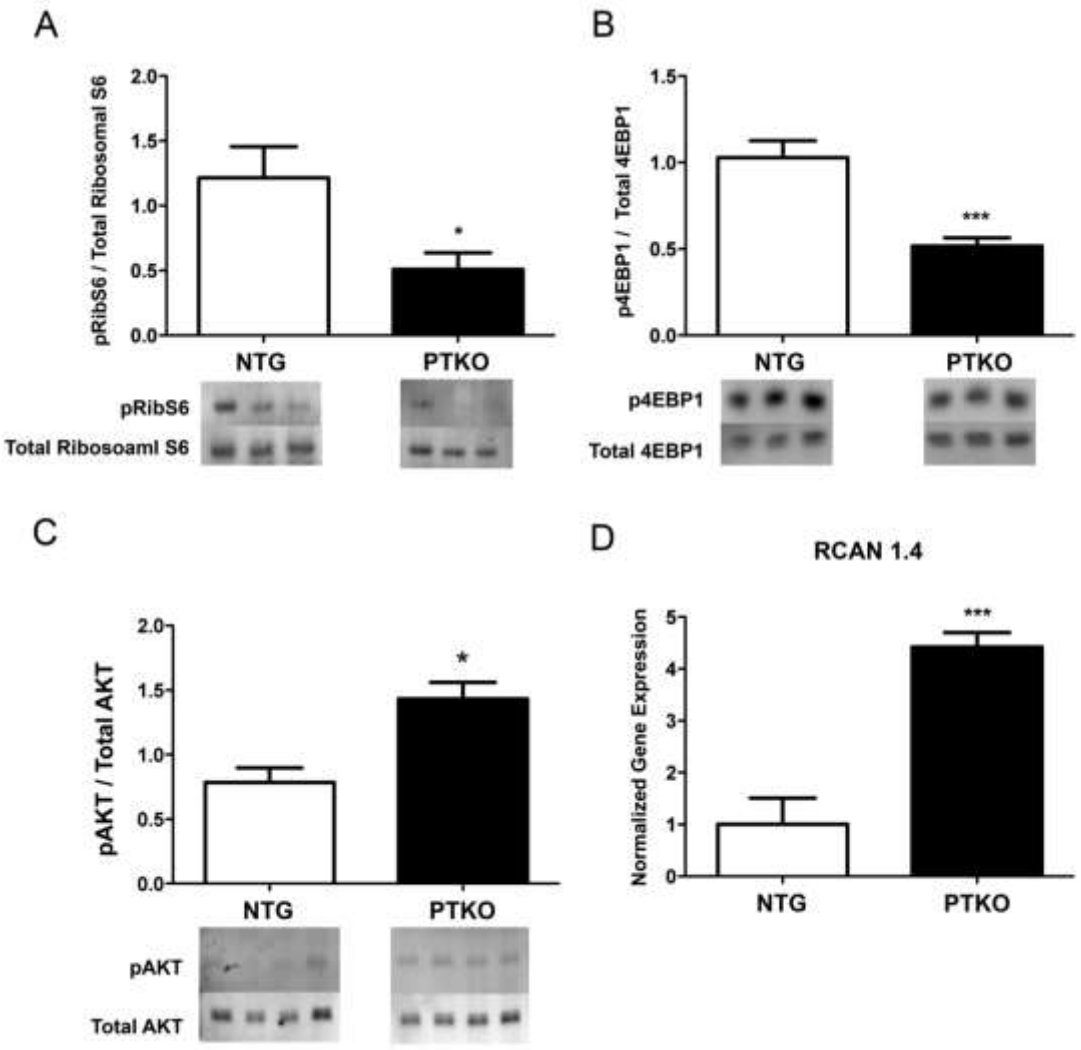
Immunohistochemistry and Cell Counts

Heart sections were deparaffinized followed by antigen retrieval in citrate (10 mM, pH 6.0). One-hour block in TNB buffer was followed by incubation in primary antibody overnight. Primary antibodies and concentration used are listed (Table I). Three washes to remove unbound primary antibody in Tris/NaCl were followed by secondary antibody incubation for 1.5 hours at room temperature. Specimens were mounted with Vectashield (Vector Laboratories). Topro to label nuclei was added in the last wash step after incubation with secondary antibodies at 1:5000 for 20 minutes. Images were obtained on a Leica DMRE confocal microscope. Area assessments for myocyte size were performed using the outlining tool from ImageJ after images of cardiac specimens stained with wheat germ agglutinin. Sections were stained with sarcomeric actin to visualize cardiomyocytes and myocardial area. Infarct size was calculated as proportion of infarcted area relative to total area of the respective section with at least 6 high power fields were analyzed per heart for non-transgenic (NTG) or Pim Triple KnockOut (PTKO) mouse heart samples. Image acquisition and size measurements were performed under sample-blinded conditions.

Masson Trichrome staining was performed as previously described[3] and micrographs were acquired using Leica DMRE confocal microscope.

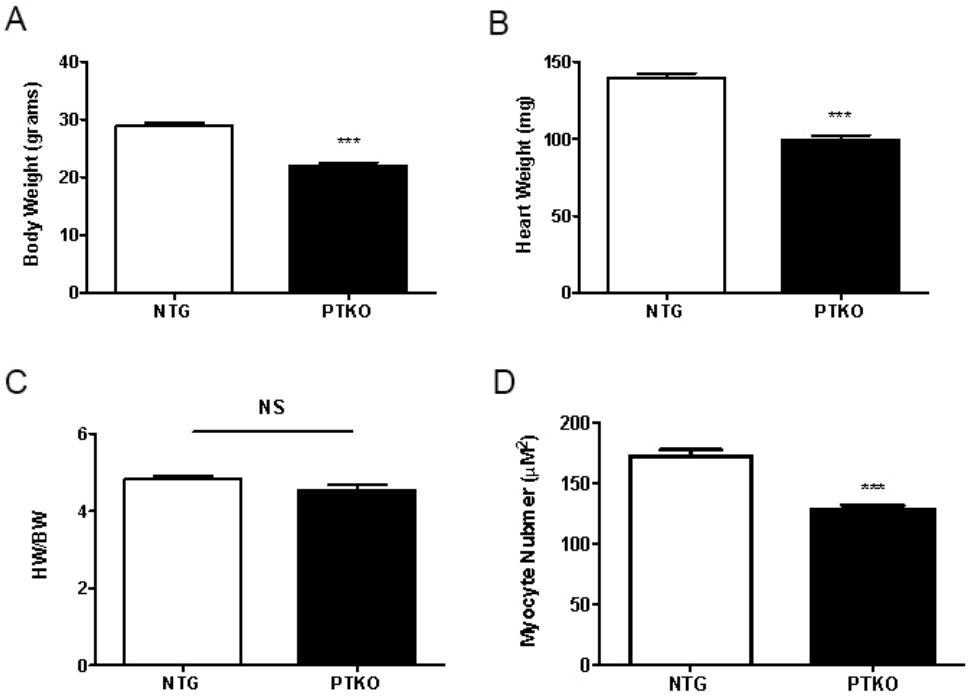


Supplementary Figure I: Expression of Pim kinases during aging and pathological challenge. A) Gene expression analysis of Pim 1,2, and 3 during physiological cardiac aging. B-D) Pim1, Pim2, and Pim3 gene expression respectively post myocardial infarction. E) Time course analysis of Pim kinase gene expression post transaortic constriction. F) PGC-1α gene expression post transaortic constriction. *, **, *** is significant compared to 3day (Figure 1A) or Sham. *p < 0.05; **p < 0.01; ***p < 0.001. #,##,### is significant compared to remote. #p < 0.05; ##p < 0.01; ###p < 0.001

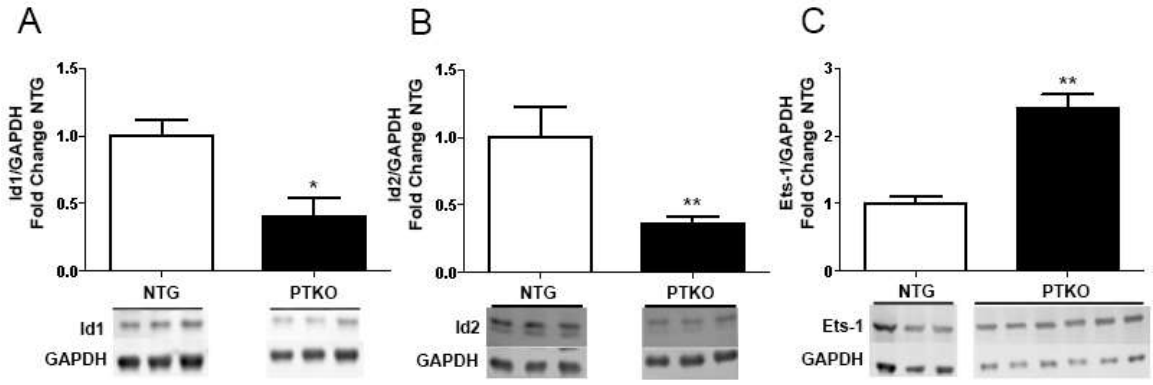


Supplementary Figure II: Hypertrophic pathways in PTKO mice. A-B) Immunoblot of mTORC1 targets phosphorylated and total ribosomal S6 and phosphorylated and total 4EBP1 with loss of Pim. C) Phosphorylated Akt levels in PTKO mice assessed by immunoblot. D) Gene expression of RCAN1.4 in PTKO mice. n=6 NTG and n=6 PTKO. *, **, *** is significant compared to NTG. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

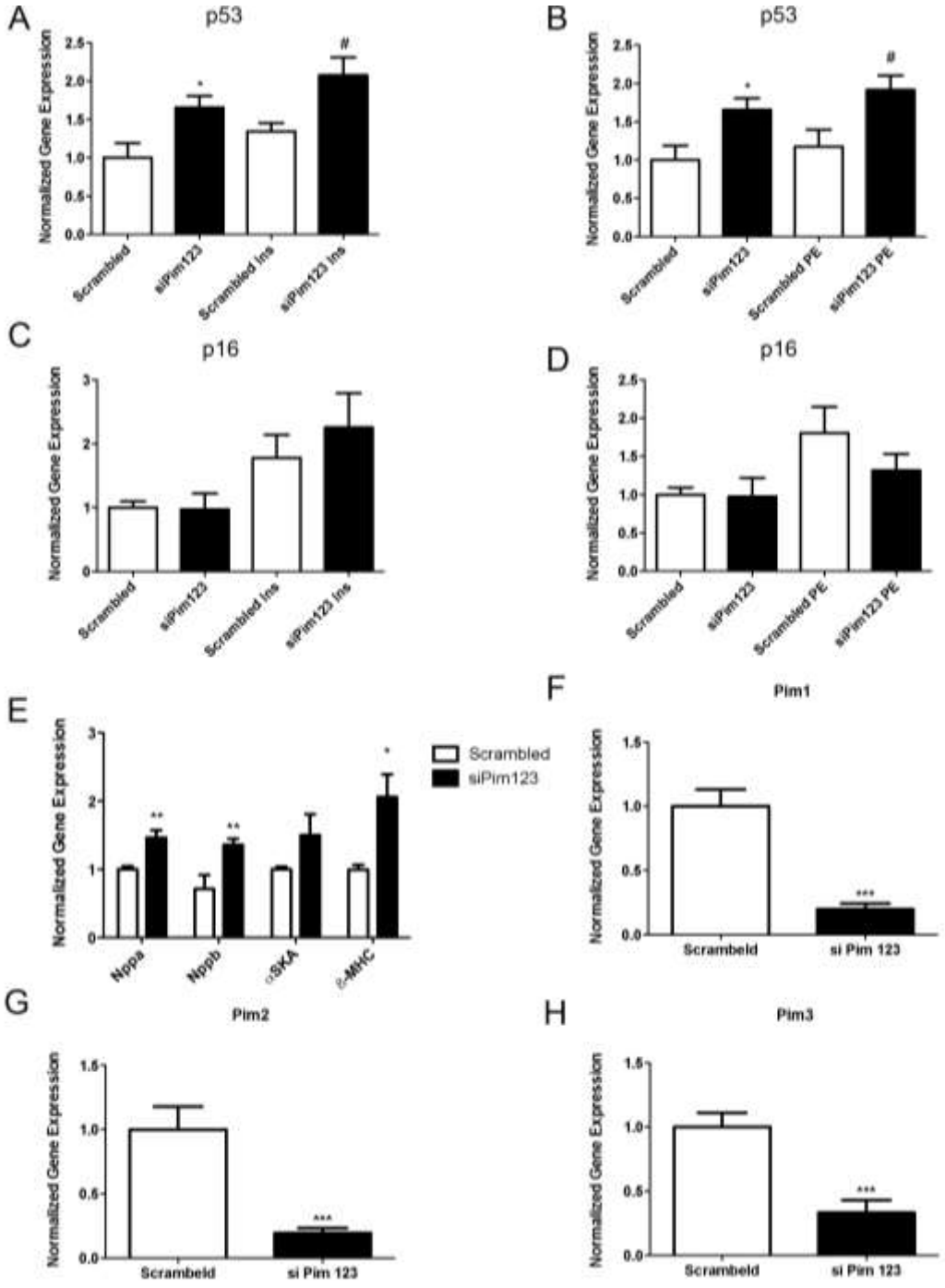
Supplementary Figure 3



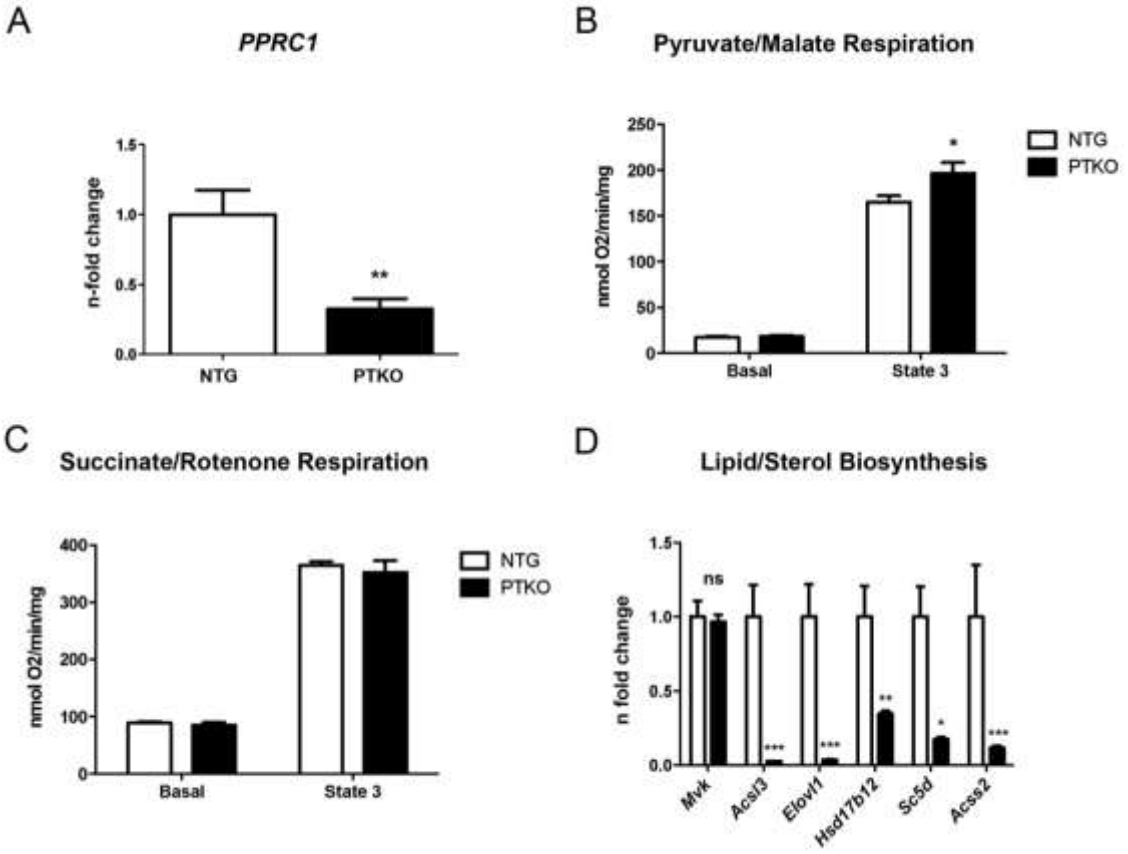
Supplementary Figure III: Biometric parameters in PTKO mice. A-C) Body weight, heart weight, and heart weight to body weight ratio in NTG and PTKO mice at six months respectively. n=8 NTG and n=9 PTKO. D) Myocyte number in PTKO hearts assessed by confocal microscopy at 1 month. n=4 NTG and n=4 PTKO. *** is significant compared to NTG mice. ***$p < 0.001$.



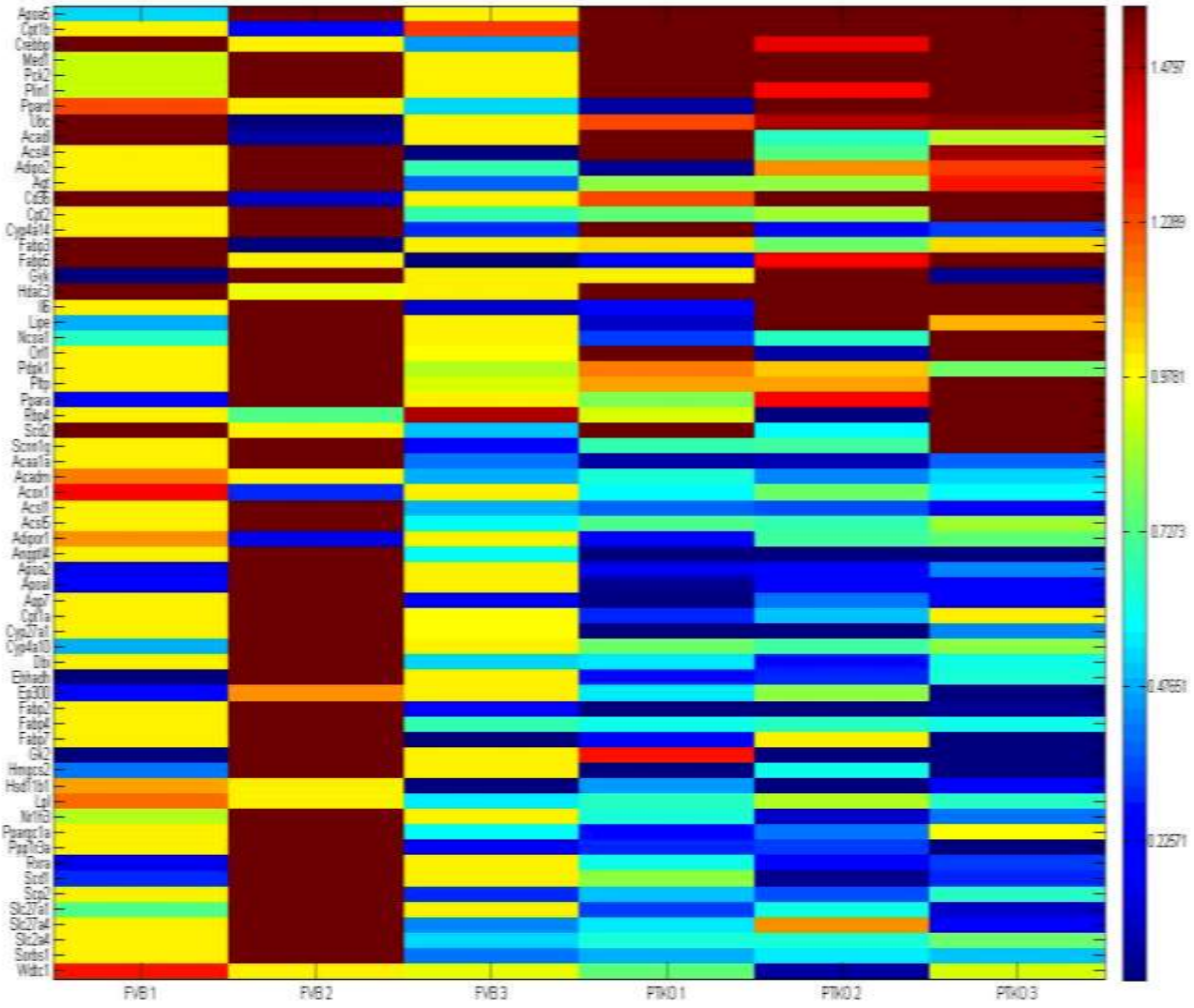
Supplementary Figure IV: Pim deletion activates p16 pathway. A & B) Immunoblot of p16 transcriptional repressors Id1 and Id2 in NTG (n=3) and PTKO (n=3) mice. C) Protein analysis of p16 transcriptional activator Ets-1 in NTG (n=3) and PTKO (n=4) cardiac lysates. * and ** is significant compared to NTG mice. *$p < 0.05$ and **$p < 0.01$.



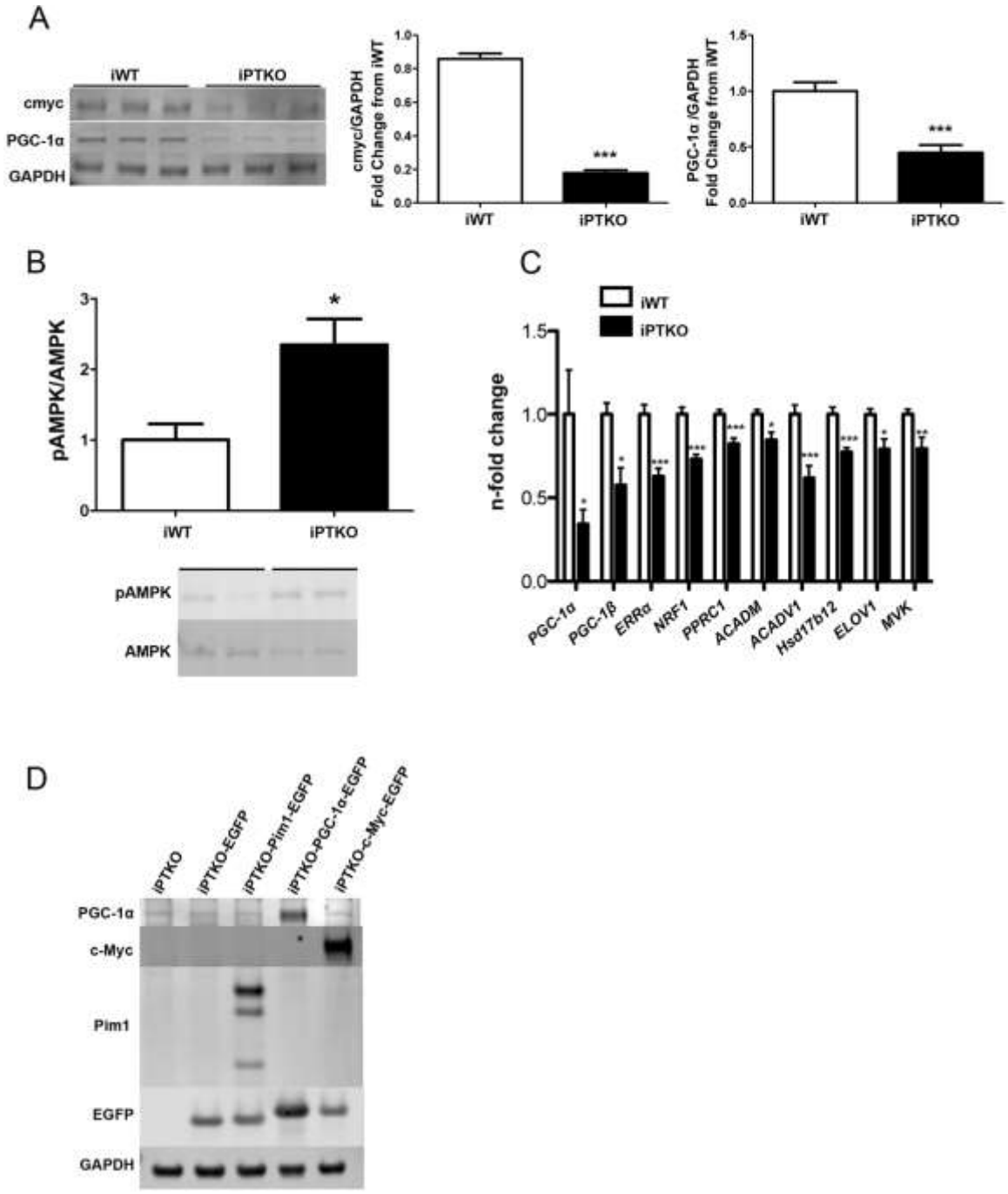
Supplementary Figure V: Increased senescence occurs in NRCMs with Pim kinase knockdown. A&B) Gene expression of p53 in NRCMs with siRNA to Pim123 followed by insulin or phenylephrine stimulation respectively (n=4). C&D) mRNA analysis of p16 following Pim123 knockdown and growth stimulation (n=4). E) Analysis of fetal gene program in NRCMs with Pim kinase knockdown (n=4). F-H) Validation of siRNA efficiency to Pim kinaes in NRCMs by quantitative PCR. *p < 0.05, **p < 0.01, and *** p<.001 when compared to scrambled alone. # p<.05 when compared to scrambled Ins or PE.



Supplementary Figure VI: Functional mitochondrial alterations and reduced PPAR signaling in PTKO mice. A and B) Mitochondrial respiration assay using glutamate and succinate as substrates in NTG (n=4) and PTKO (n=4) one month isolated mitochondria. C) mRNA levels of PPRC1 in NTG (n=6) and PTKO mice (n=6) at one month D) mRNA analysis of genes downstream of PPAR signaling involved in lipid sterol biosynthesis in NTG (n=6) and PTKO (n=6) one month hearts. *, **, *** is significant compared to NTG. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



Supplementary Figure VII: Microarray analysis of genes involved in PPAR signaling. Microarray analysis of cardiac mRNA from NTG and PTKO mice at 1 month. 62 PPAR-related signaling genes were detected in cardiac tissue, 32 showed a downregulation, 21 genes remained unchanged, and 8 genes were induced. Three out of the four genes included in the targeted screen (Acadm, PGC-1α, Cpt1b, and Cpt2) demonstrated similar results to our targeted approach. Acadm and PGC-1α showed decreased gene expression, Cpt1b exhibited increased expression. Genes included in the microarray panel are involved in fatty acid oxidation, fatty acid transport, cholesterol synthesis and lipid/sterol biosynthesis.



Supplementary Figure VIII: iPTKO MEFs demonstrate similar metabolic dysfunction as PTKO mice. A) Immunoblot of c-Myc and PGC-1α in iWT MEFs and iPTKO MEFs (n=3) B) Phosphorylation levels of AMPK and quantification of pAMPK : AMPK in iPTKO MEFs compared to iWT MEFs (n=3). C) q-PCR of genes involved in the PPAR signaling circuit (n=3). D) Protein analysis of adenoviral overexpression of PGC-1α, c-Myc, Pim1, and EGFP. *, **, *** is significant compared to iWT MEFs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table I: List of Antibodies

Application	Antibody	Dilution	Amplification	Company
Immunoblot	PGC-1α	1:500	no	Calbiochem ST-1204
Immunoblot	PGC-1β	1:500	no	R and D systems AF5656
Immunoblot	c-Myc	1:500	no	CST 9402
Immunoblot	p16	1:250	no	Santa Cruz sc-1661
Immunoblot	MDM2	1:500	no	Life Tech 337100
Immunoblot	P53	1:500	no	CST 9282
Immunoblot	Id1	1:250	no	Santa Cruz sc488
Immunoblot	Id2	1:250	no	Santa Cruz sc489
Immunoblot	Ets-1	1:250	no	Santa Cruz sc350
Immunoblot	pAMPK	1:500	no	CST 2535
Immunoblot	AMPK	1:500	no	CST 2532
IHC	p16	1:50	no	Santa Cruz sc-1661
IHC	Desmin	1:200	no	Abcam 15200
IHC	Wheat Germ Agglutinin	1:200	no	Life Tech W32464

Table II: List of Mouse Primers 5' to 3'

PGC-1α Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha	For primer Rev primer	TCGGGAGCTGGATGGCTTGGGA ACCAACCAGAGCAGCACACTCTA
PGC-1β Peroxisome proliferator-activated receptor-gamma coactivator 1 beta	For primer Rev primer	CTTTGCGGCACGGCAGGACT TGGCCTGGGCTGAGCTTGGT
Errα Estrogen-related receptor alpha	For primer Rev primer	CAAGAGCATCCCAGGCTT GCACTTCCATCCACACACTC
NRF1 Nuclear respiratory factor 1	For primer Rev primer	GAACTGCCAACCACAGTCAC TTTGTTCCACCTCTCCATCA
TFAM Mitochondrial transcription factor A	For primer Rev primer	AGGCTTGGAAAAATCTGTCTC TGCTCTTCCCAAGACTTCATT
Acate3 acyl-Coenzyme A thioesterase 3, mitochondrial	For primer Rev primer	CAGAAGCCTGTTGAAGTTGGT GACTTGAAATGTCGCTGTCC
Acadv1 Aceyl coenzyme A long chain	For primer Rev primer	ATCTCTGCCCAGCGACTTT TTCTGGCTTGTCCAGAACTG
Acadm FAO-acetyl CoenzymeA dehydrogenase	For primer Rev primer	GGAAATGATCAACAAAAAAAGAAGTATTT ATGGCCGCCACATCAGA
Cpt1b Carnatine palmitoyl transferase	For primer Rev primer	TCTAGGCAATGCCGTTCAC GAGCACATGGGCACCATAC
Cpt2 Carnatine palmitoyl transferase 2	For primer	AGTATCTGCAGCACAGCATCGTA GGCTTCTGTGCACTGAGGTATCT

	Rev primer	
Hk2 Hexokinase 2	For primer Rev primer	TGCTACAGGTCCGAGCCA ATGCTGTCGTCACACGTGC
Pfk Phosphofructokinase	For primer Rev primer	CGTTGAGGTAGGAATACTTCTGCA ACCTCTTCCGAAAGGAGTGGA
Pdk4 Pyruvate dehydrogenase kinase 4	For primer Rev primer	CCGCTGTCCATGAAGCA GCAGAAAAGCAAAGGACGTT
Idh2 Isocitrate dehydrogenase 2	For primer Rev primer	CCCTATTGCCAGCATCTTTG TGTCCAGGAAGTCTGTGGTG
PDHA1 Pyruvate dehydrogenase E1 alpha 1	For primer Rev primer	GGGACGTCTGTTGAGAGAGC TGTGTCCATGGTAGCGGTAA
PFKFB2 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	For primer Rev primer	CGGGAATGGATCTACACTGG GGAGAGCAAAGTGAGGGATG
Cytc Cytochrome C	For primer Rev primer	ACCAAATCTCCACGGTCTGTT GGATTCTCCAAATACTCCATCAG
Ndufa NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (Ndufa9)	For primer Rev primer	ATCCCTTACCCTTTGCCACT CCGTAGCACCTCAATGGACT
Ndufv NADH dehydrogenase (ubiquinone) flavoprotein 1 (Ndufv1)	For primer Rev primer	TGTGAGACCGTGCTAATGGA CATCTCCCTTCACAAATCGG
PPRC1 Peroxisome proliferator-activated receptor gamma	For primer	AATACCTGGCCGAATGACTC AGTTGTCACCTTGGACACGA

coactivator-related protein 1	Rev primer	
Mvk mevalonate kinase	For primer Rev primer	GGGACGATGTCTTCCTTGAA GAACTTGGTCAGCCTGCTTC
Acsl3 Long-chain-fatty-acid—CoA ligase 3	For primer Rev primer	CCAGCAGCTGCGTCAGGGTC TAAGACCCGCGGGCTCCGAC
Elov1 Fatty acid elongase 1	For primer Rev primer	GCTCCAGGAGGAATGGGCTCC CACAGGGCCAAGGGCAGACA
Hsd17b12 hydroxysteroid (17-beta) dehydrogenase 12	For primer Rev primer	GGGCCTTCCAGGTGTGGTGC GTGCCACCTGTAACAACTGCCCA
Sc5d sterol-C5-desaturase	For primer Rev primer	CCAAATGGCTGGATTCATCT GTCCACAGGGTGAAAAGCAT
Acss2 acyl-CoA synthetase short-chain family member 2.	For primer Rev primer	CTGTGGAGGAGCCACGGGAGTT TGGAGGAATGGGCCAGGGCAT
Nppa (ANP) Atrial natriuretic peptide	For primer Rev primer	TGGGTCTTGTTAGGGCTCAAACCT TGAAACTCAAGGGACACCCATCGT
Nppb (BNP) Brain natriuretic peptide	For primer Rev primer	AATGGCCCAGAGACAGCTCTTGAA CTTGTGCCCAAAGCAGCTTGAGAT
αSKA alpha skeletal actin	For primer Rev primer	CGCCAGCCTCTGAAACTAGA AGCCGTTGTCACACACAAGA
Myh7, β-MHC Beta Myosin Heavy Chain	For primer	GAGCCTTGGATTCTCAAACG GTGGCTCCGAGAAAGGAAG

	Rev primer	
Collagen 1α1	For primer	ACGCCATCAAGGTCTACTGC
	Rev primer	ACTCGAACGGGAATCCATCG
Collagen 3α1	For primer	CCCTGGACCTCAGGGTATCA
	Rev primer	GGGTTTCCATCCCTTCCAGG
MCIP	For primer	TCCAGCTTGGGCTTGACTGAG
	Rev primer	ACTGGAAGGTGGTGTCCTTGT
Pim1	For primer	ATCCGCGTCGCCGACAACTT
	Rev primer	TCGGGTGCCATTGGGCAGTT
Pim2	For primer	GGCTGGTCCACCGTGTCAGACT
	Rev primer	GGCGTATCACACCCGGATGGC
Pim3	For primer	GTCCAAGTTCGGCTCCCTGGC
	Rev primer	TTGTCAGCCTTGGCTGGCTGT
Primers for quantification of Mitochondrial DNA		
CoxI (mitochondrial)	For primer	CTGAGCGGGAATAGTGGGTA
	Rev primer	TGGGGCTCCGATTATTAGTG
Cytochrome B (mitochondrial)	For primer	ATTCCTTCATGTCGGACGAG
	Rev primer	ACTGAGAAGCCCCCTCAAAT
H19 (nuclear)	For primer	GTCCACGAGACCAATGACTG
	Rev primer	GTACCCACCTGTCGTCC

B1 globin (nuclear)	For primer Rev primer	GCACCTGACTGATGCTGAGAA TTCATCGGCGTTCACCTTTCC

1. Kubli DA, Zhang X, Lee Y, Hanna RA, Quinsay MN, Nguyen CK, Jimenez R, Petrosyan S, Murphy AN, Gustafsson AB. Parkin protein deficiency exacerbates cardiac injury and reduces survival following myocardial infarction. *The Journal of biological chemistry*. 2013;288:915-926
2. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative pcr method. *Nucleic acids research*. 2009;37:e21
3. Konstandin MH, Volkers M, Collins B, Quijada P, Quintana M, De La Torre A, Ormachea L, Din S, Gude N, Toko H, Sussman MA. Fibronectin contributes to pathological cardiac hypertrophy but not physiological growth. *Basic research in cardiology*. 2013;108:375