SUPPLEMENTAL MATERIAL

For Bai *et al*

Seipin knockout mice develop heart failure with preserved ejection fraction

Mice

We used several mouse strains in the current study. (1) The generation of whole body seipin knockout (SKO) mice was achieved by deletion of exons 5-7 of *Bscl2* gene in ES cells harboring the targeting vector cassette with recombination to replace the wild-type allele. Mice were crossed with oocyte-specific ZP3-Cre transgenic mice to generate heterozygous seipin pups (seipin$^{+/-}$). The SKO mice were generated by intercrossing of heterozygous mice (1). The wild-type mice from littermates were used as control mice (Ctrl) for experiments. (2) To generate cardiomyocyte-specific seipin knockout mice (SHKO), homozygous Bscl2 (fl/fl) mice were crossed with heterozygous Bscl2 mice also containing Cre recombinase driven by the cardiac-specific murine alpha myosin heavy chain promoter (Myh6-Cre). The homozygous Bscl2 (fl/fl) mice without Cre recombinase were used as corresponding control mice. Myh6-Cre mice were obtained from Jackson Laboratory (Stock no:011038). Male mice were used for all studies. The animals were housed in a temperature and humidity-controlled environment with 12-hour light /12-hour dark cycle, and maintained on a standard chow diet with free access to water.

Experimental Protocol

A schematic of the protocol was shown in Figure 1A. The physiological, cardiac and exercise parameters of Ctrl and SKO mice were characterized serially by nuclear magnetic resonance (NMR), echocardiography, and treadmill running test at the age of 8 weeks (8±1 weeks), 15 weeks (15±1 weeks), 23 weeks (23±1 weeks), 36 weeks (36±1 weeks), and 60 weeks. Mouse blood pressure was monitored by implantable telemetry from young to adult stage (10 to 27 weeks). LV pressure-volume (PV) loop assessment was performed in adult (23 weeks) and aged (44 weeks) Ctrl and SKO mice via PV catheters. Mouse heart tissues were harvested at a different age for histological examination, analysis of titin phosphorylation, and examination of protein and mRNA levels. The number of animals in each experiment/group was shown in the figure legends.

Metabolic Characterization of Mice

Body composition of conscious and un-anesthetized mice was assessed by NMR using a Bruker Minispec Live Mice Analyzer (LF50, Bruker Optics Inc., MA, USA). Lean mass was examined for comparison between Ctrl and SKO mice at a different age. Blood glucose levels of adult mice during basal feeding were monitored by tail nicking using an Accu-Check Advantage II Glucometer (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured by utilizing the highly sensitive mouse insulin immunoassay kit according to the instruction (Antibody and Immunoassay Services, Hong Kong, China).

Echocardiography

We performed echocardiography assessment using a Vevo 2100 Imaging System (Fujifilm Visual-Sonics, Canada) as described previously (2). Mice were anesthetized by inhalation of isoflurane (induction 3-4%, maintenance 1-2% mixed with 100% oxygen). Standard imaging planes, M-mode, Pulsed Wave Doppler, structure and functional calculations were conducted to animals according to the American Society of Echocardiography guidelines. The systolic function was evaluated by calculating EF and fractional shortening (FS). The Doppler echocardiography was used to examine diastolic function by measuring the isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT), and ejection time (ET) of mitral valve flow. The myocardial performance index (MPI) was calculated by using the formula: MPI=(IVCT+IVRT)/ET. Heart rate during echocardiography study was maintained in the range of 500 to 550 bpm for M-mode and 400 to 450 bpm for Doppler studies.

Pressure-volume Analysis

In vivo LV pressure-volume measurements were performed in isoflurane-anesthetized mice (induction 3-4%, maintenance 1.5-2.5% mixed with 100% oxygen). The catheter (PVR-1045, ADI instruments, Australia) was inserted through the right carotid artery without opening the chest cavity as described in the protocol (3). The baseline PV loops at a steady state or varying preload during the vena cava inferior occlusions were recorded. Data acquisition and analysis were performed using LabChart8 (ADI instruments, Australia). The LV end-diastolic pressure (EDP) was recorded. The end-

diastolic PV relationship (EDPVR) was analyzed with an exponential fit ($P=Ce^{\beta V}$), with the exponent β indicated as the stiffness (4).

Exercise Tolerance Test

Exercise tolerance test was performed using a 5-lane rodent treadmill system (LE8710, Harvard Apparatus, UK). The mice were habituated to this system for three days before actual experiments. After an acclimation period during which mice ran at low speed (10 m/min for 5 minutes), exercise testing was performed by having mice run at progressively increasing speeds (speed steps of 4 m/min). The accumulative running distance and running time were recorded before the mice left the treadmill and remained on a shock pad for at least five seconds (5).

Blood Pressure Measurement

Blood pressure was measured using an implantable telemetry system (Data Sciences International, MN, USA) as described (6). In brief, the anesthetized mice with Hypnorm/ Dormicum mixture (Hypnorm: 0.315 mg/mL of fentanyl and 10 mg/mL of fluanisone [VetaPharma Ltd, Leeds, UK]; Dormicum: 5 mg/mL of midazolam [Roche A/S, Hvidovre, Denmark]) were inserted with a telemetry catheter (PA-C10; Data Sciences International, MN, USA) through left common carotid artery. Mice were housed individually in a temperature and humidity-controlled facility and maintained under a 12-hour dark (lights off 7 pm) and 12-hour light (lights on 7 am) cycle. Blood pressure was recorded using the Dataquest A.R.T. system (Data Sciences International,

MN, USA). The Ctrl and SKO mice (8 weeks old) were implanted with the telemetry transmitter and allowed two weeks of recovery from the surgery. Both systolic and diastolic blood pressure (Bp) collected during the dark cycle (7 pm to 7 am) was compared between Ctrl and SKO mice from the age of 10 to 27 weeks.

Quantification of Cross-sectional Area of Mouse Hearts and Cardiomyocytes

The dissected mouse heart tissues were fixed overnight in neutral buffered formaldehyde, dehydrated and embedded in paraffin. Tissue blocks were then sectioned at five micrometers. The longitudinal sections were subjected to the hematoxylin-eosin staining (H&E) according to the manufacturer's specifications from Sigma (MO, USA). The whole heart cross-sectional area was quantified using Image J software (National Institutes of Health, USA) for comparison. In brief, the images were converted to greyscale followed by thresholding to remove background and to reveal the positively stained area. The images were then binary-converted to black and white. The positively stained area was then automatically measured by software. To quantify the cross-sectional area of cardiomyocytes, images with the magnification of 400× were captured using Ni-E motorized upright microscope (Nikon, Japan). At least ten H&E-stained cross-sections were analyzed from each heart sample to count 1,000 cardiomyocytes. The average cardiomyocyte area was calculated by dividing the total stained area by the total number of cells.

Picrosirius Red Staining

Picrosirius Red staining was used to evaluate the interstitial collagen content. The dissected heart tissues were fixed, dehydrated, and embedded in paraffin as described above. Tissue blocks were then sectioned at five micrometers. The staining was performed according to manufacturer's specifications from Sigma (MO, USA). The pixel area of positively stained collagen was quantified using Image J software for comparison.

Neutrophil Extracellular Traps (NETs) Staining

For *in situ* NETs staining, dissected mouse heart tissues were fixed overnight in neutral buffered formaldehyde, dehydrated and embedded in paraffin. Tissue blocks were then sectioned at five micrometers. The tissue sections were blocked with 3% bovine serum albumin in PBS after antigen retrieval. The NETs components were detected with primary antibodies recognizing myeloperoxidase (MPO) and citrullinated histone 3 (Cit-H3, citrulline R2+R8+R17) and then incubated with secondary antibodies including Alexa Fluor 568-conjugated chicken anti-goat and subsequent Alexa Fluor 488-conjugated goat anti-rabbit. Finally, tissue sections were stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) and determined by computerized digital planimetry, using a video microscope (Olympus BX41, Japan) with DP2-BSW imaging system (7).

Western Blotting

The procedures were carried out as described (2,8). In brief, equal amounts of proteins

were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with specific antibodies to determine the expression of the target proteins. Band intensities were quantified and analyzed using Image J software. Antibodies against tumor necrosis factor (TNF), Cit-H3, transforming growth factor beta (TGFB), α-smooth muscle actin (ACTA2), β-tubulin and neutrophil elastase were purchased from Abcam (Cambridge, UK). Antibodies recognizing β-actin, cyclic guanosine monophosphate-dependent protein kinase (PRKG), protein kinase C alpha (PRKCA), signal transducer and activator of transcription 3 (STAT3), phospho-STAT3 (Tyrosine 705), Janus kinase 2 (JAK2), phospho-JAK2 (Tyrosine 1007/1008), extracellular signal-regulated protein kinases 1 and 2 (Erk1/2), and phospho-Erk1/2 (Tyrosine 202/204) were from Cell Signaling Technology (MA, USA). The antibody against MPO was purchased from R&D Systems (MN, USA). The antibody recognizing cyclic adenosine monophosphate–dependent protein kinase catalytic, alpha (PRKACA) was obtained from Enzo Life Sciences (NY, USA).

RNA Extraction and Quantitative Real-time PCR (QPCR)

Total RNA was isolated from mouse heart tissues using Trizol Reagent (Thermo Fisher Scientific, Massachusetts, USA). The purity and concentration of total RNA were determined with a spectrophotometer at 260 nm and 280 nm. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. The cDNA was synthesized using the QuantiTect Reverse Transcription kit from Qiagen (Venlo, Germany). Quantitation of target genes was performed using SYBR Green PCR Master Mix (Qiagen) and a

StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, CA, USA). Primers were listed in Supplementary Table 1. Quantification was achieved using Ct values that were normalized with β-actin or GAPDH as a reference control.

Total titin analysis

The total titin analysis was performed according to the procedure as described (9). Snap-frozen mouse left ventricular tissues were weighed and pulverized in liquid nitrogen. The tissue powder was dissolved in 50% urea buffer (8 mol/L Urea，2 mol/L Thiourea, 0.05 mol/L Tris-HCl, 0.075 mol/L Dithiothreitol with 3% SDS and 0.03% Bromophenol blue, pH 6.8) and 50% glycerol with protease inhibitors at 60°C for 10 min. After centrifugation at 13,000 rpm for 15 min, the solubilized samples were electrophoresed on 1% agarose gels using a vertical SDS-agarose gel system. Gels were run at 15 mA per gel for three to four hours, then stained with silver staining reagent (Sigma). The scanned gel images were analyzed to get band intensity using Image J software. The ratio of total titin expression relative to myosin heavy chain was calculated for comparison.

Tandem Mass Spectrometry Coupled to Liquid Chromatography

Snap-frozen mouse left ventricular tissues were weighed and pulverized in liquid nitrogen. Proteins were extracted with lysis buffer containing 6 mol/L urea, 2 mol/L thiourea and 20 mmol/L HEPES. Total 4 mg of protein from each sample was subjected to reduction, alkylation and in-solution digestion with Lys-C (Wako, Japan) and

sequencing-grade trypsin (Promega, USA). After 4 hours of digestion at 37°C, the peptide solution was cleaned up using a C_{18} cartridge (3M™ Empore™, Sigma). IMAC beads were employed for selective enrichment of phosphorylated peptides. IMAC beads were prepared as described with minor modifications (10). Briefly, Ni-NTA agarose conjugates (Qiagen, Venlo, Germany) was rinsed thrice with MilliQ water and incubated with 100 mmol/L EDTA pH 8.0 for 30 minutes at room temperature to strip the nickel. Residual nickel was rinsed thrice with MilliQ water, followed by incubation with 100 mmol/L iron chloride solution for 30-45 minutes. The resin was then washed thrice with MilliQ water, followed by 80% acetonitrile / 0.1% trifluoroacetic acid. The tryptic peptides were then enriched using the iron chloride treated IMAC beads. Tryptic peptides were reconstituted in 50% acetonitrile / 0.1% trifluoroacetic acid, followed by 1:1 dilution with 99.9% acetonitrile / 0.1% trifluoroacetic acid. The peptides were incubated with 10 µl of IMAC beads for 30 minutes with end-over-end rotation, and the beads were subsequently loaded onto self-packed C_{18} stage tips pretreated with 200 µl methanol (11), washed with 50% acetonitrile / 0.1% formic acid and equilibrated with 1% formic acid. Following loading onto the stage tip, the IMAC beads were washed with 80% acetonitrile / 0.1% trifluoroacetic acid and 1% formic acid. The phosphopeptides were eluted from the IMAC beads onto C_{18} membranes using 500 mmol/L dibasic sodium phosphate (pH 7.0), followed by washing using 1% formic acid. The phosphopeptides were stored on the stage tip until they were ready to be analyzed by LC-MS. The phosphopeptides were eluted from the C_{18} membranes with 50% acetonitrile / 0.1% formic acid, dried using a speed vac and reconstituted in 0.1% formic

acid for LC-MS analysis. Reconstituted peptides were analyzed using an EASY-nLC 1000 liquid chromatograph (Thermo Fisher Scientific, Massachusetts, USA) attached to a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer. (Thermo Fisher Scientific). Peptides were enriched using a C_{18} precolumn and separated on a 50 cm analytical column (EASY-Spray Columns, Thermo Fisher Scientific) at 50°C using a 265 min gradient ranging from 0 to 40% acetonitrile /0.1 % formic acid, followed by a 10 min gradient ranging from 40 to 80% acetonitrile / 0.1 % formic acid and maintained for 10 min at 80% acetonitrile / 0.1% formic acid. Survey full scan MS spectra (m/z 310–2000) were collected with a resolution of r = 70,000, an AGC target of 3e6 and a maximum injection time of 10 ms. Twenty of the most intense peptide ions in each survey scan with an intensity threshold of 10,000, underfill ratio of 1%, and a charge state ≥2 were sequentially isolated with a window of 2 Th to a target value of 50,000 with a maximum injection time of 50 ms. These were fragmented in the high energy collision cell by dissociation, using normalized collision energy of 25%. The MS/MS was acquired with a starting mass of m/z 100 and a resolution of 17,500 and dynamic exclusion of duration of 15 s. Data were processed using MaxQuant (Version 1.5.0.30) and searched against Uniprot2016-12 mouse database containing 245 commonly observed contaminants. Database search was performed with tryptic specificity allowing a maximum of two missed cleavages as well as an initial mass tolerance of 4.5 ppm for precursor ions and 20 ppm for fragment ions. Cysteine carbamidomethylation was searched as a fixed modification, and N-acetylation, oxidized methionine, phosphorylated serine/threonine/tyrosine were searched as

variable modifications. Maximum false discovery rates were set to 0.01 for both protein and peptide. Proteins were considered identified when supported by at least one unique peptide with a minimum length of seven amino acids. Each detected phosphorylation site has a minimum Andromeda Score of 40.

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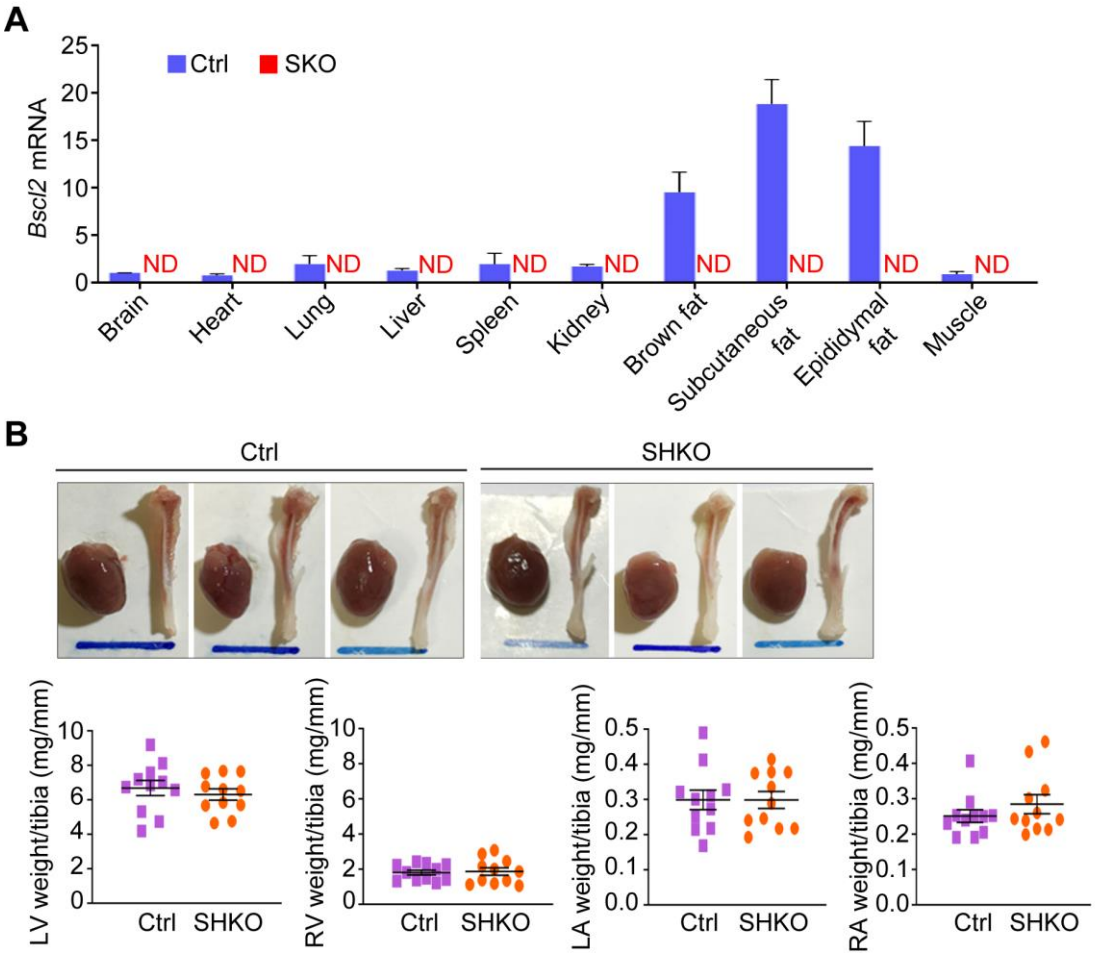
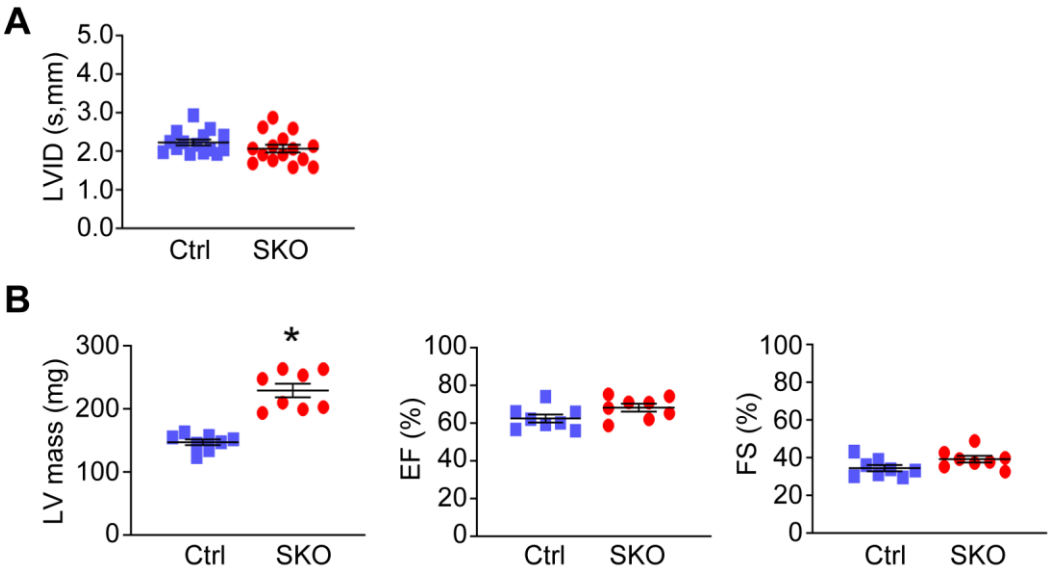
ONLINE FIGURES:



Online Figure 1: Normal cardiac characteristics in cardiomyocyte-specific seipin knockout mice

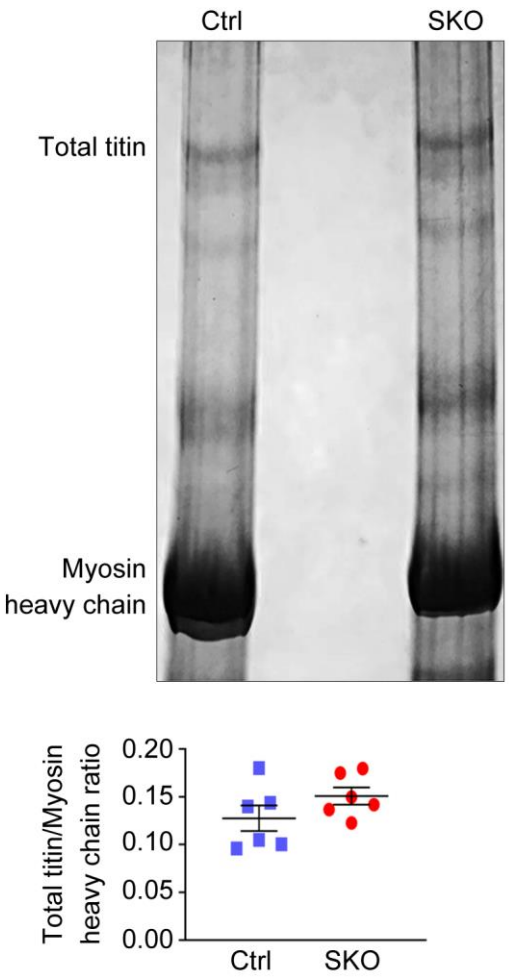
(A) The mRNA expression profile of *Bscl2/Seipin* gene was analyzed in tissues of adult Ctrl and SKO mice (18 to 24 weeks, n=6). Fold change was calculated for comparison.

(B) The whole heart tissues were harvested from aged cardiomyocyte-specific seipin knockout mice (SHKO) and control mice without Cre recombinase (55 to 60 weeks). The ratios between left ventricular (LV), left atrial (LA), right ventricular (RV), right atrial (RA) weight to tibia length were calculated for comparison (scale bar=10 mm, n=11). ND: Non-detectable.



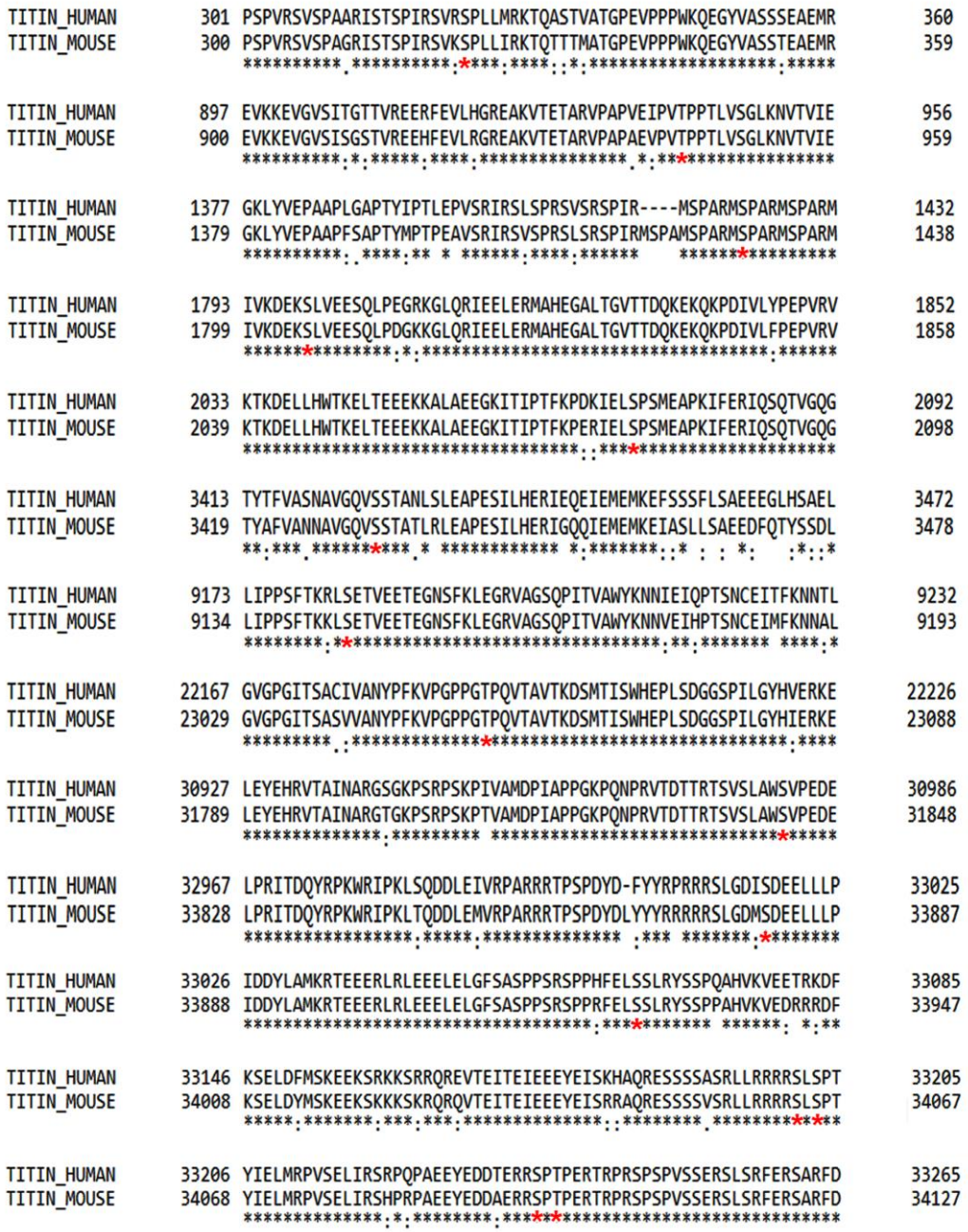
Online Figure 2: Increased left ventricle mass, but without reduction of ejection fraction or fractional shortening in aged SKO mice

(A) Left ventricle internal diameter (LVID) in systole (s) of adult Ctrl and SKO mice (23 to 36 weeks) was examined via Vevo 2100 echocardiography (n=15). **(B)** Left ventricle mass (LV mass), ejection fraction (EF), and fractional shortening (FS) of aged mice (~60 weeks) were examined by echocardiography. Ctrl: Control; SKO: Seipin knockout; ***p*<0.05, compared with age-matched Ctrl mice (n=8).



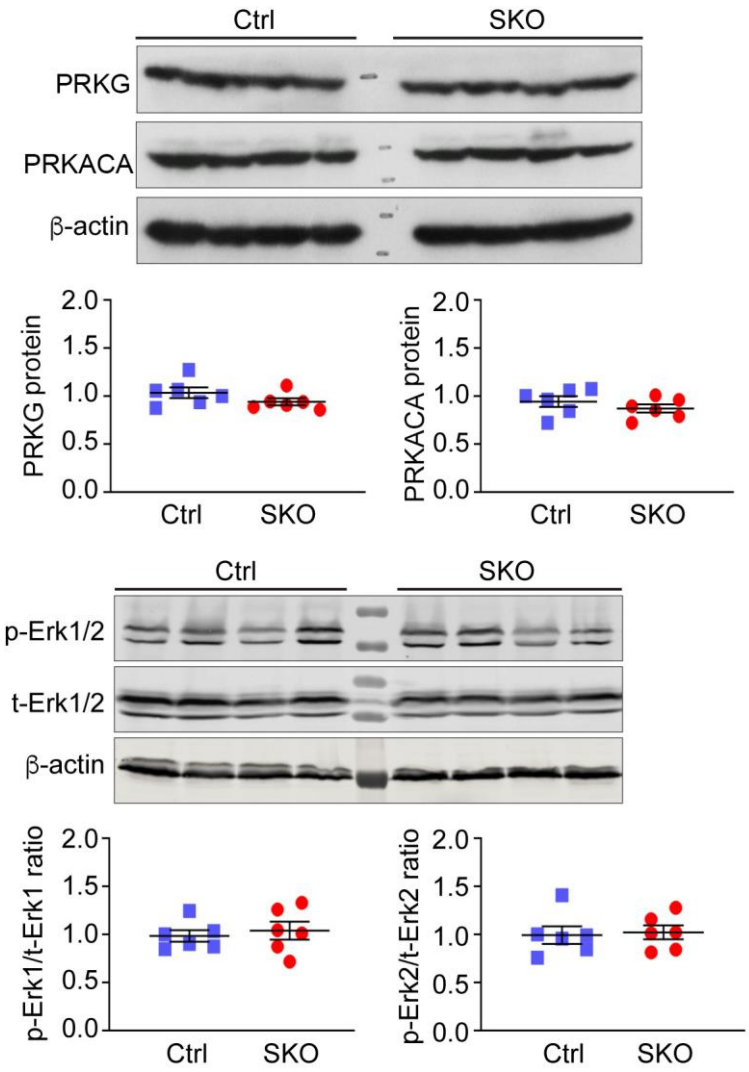
Online Figure 3: Comparable levels of total cardiac titin between Ctrl and SKO mice

Mouse left ventricular tissues were harvested from adult Ctrl and SKO mice (23 to 36 weeks). Expression of total titin was analyzed by using agarose gel electrophoresis. Total titin was normalized to myosin heavy chain for comparison (n=6).



Online Figure 4: Sequence alignment of human and mouse titin proteins

Sequence alignment of human and mouse titin (UniProtKB Q8WZ42 and A2ASS6, respectively). Red stars: phosphosites that were altered in SKO LV tissues.



Online Figure 5: Unaltered protein kinase levels in SKO mouse heart tissues

Protein expression of cyclic guanosine monophosphate-dependent protein kinase (PRKG), cyclic adenosine monophosphate–dependent protein kinase catalytic, alpha (PRKACA), total extracellular signal-regulated protein kinases 1 and 2 (Erk1/2), and phospho-Erk1/2 was examined in mouse heart tissues (23 to 36 weeks, n=6) by Western blotting. Fold change was calculated for comparison.

Supplementary Table 1. Sequences of primers used for QPCR analysis.

Gene	Species	Accession ID	Primer sequences
β-actin (*Actb*)	*Mus musculus*	GI:11461	Forward 5'-AGTGTGACGTTGACATCCGT-3'
			Reverse 5'-CCACCGATCCACACACAGAGTA-3'
α-skeletal actin (*Acta1*)	*Mus musculus*	GI: 11459	Forward 5'-TGAGACCACCTACAACAGCA-3'
			Forward 5'-CCAGAGCTGTGATCTCCTTC-3'
Seipin (*Bscl2*)	*Mus musculus*	GI: 14705	Forward 5'-ATCTCCACTTCTTCACGC-3'
			Forward 5'-CTGCTTCTGTTCAGCGAA-3'
Collagen I (*Col1a1*)	*Mus musculus*	GI:12842	Forward 5'-AGCCTGAGTCAGCAGATTGAGAA-3'
			Reverse 5'-TGGTTAGGGTCGATCCAGTACTCT-3'
GAPDH (*Gapdh*)	*Mus musculus*	GI:14433	Forward 5'-AACTTTGGCATTGTGGAAGGG-3'
			Reverse 5'-GGATGCAGGGATGATGTTCT-3'
IL-6 (*Il6*)	*Mus musculus*	GI:16193	Forward 5'-CAAAGCCAGAGTCCTTCAGAG-3'
			Forward 5'-GCCACTCCTTCTGTGACTCC-3'
ICAM-1 (*Icam1*)	*Mus musculus*	GI:15894	Forward 5'-GTGATGCTCAGGTATCCATCCA-3'
			Reverse 5'-CACAGTTCTCAAAGCACAGCG-3'
ANP (*Nppa*)	*Mus musculus*	GI:230899	Forward 5'-ATTGACAGGATTGGAGCCCAGAGT-3'
			Forward 5'-TGACACACCACAAGGGCTTAGGAT-3'

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