FKBP8 protects the heart from hemodynamic stress by preventing the accumulation of misfolded proteins and endoplasmic reticulum-associated apoptosis in mice

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Supplementary Methods

Yeast two-hybrid assay

A yeast two-hybrid assay was performed as previously reported [1]. FKBP8 or its mutant containing a W83A I86A W379A L382A substitution and LC3B were cloned into pGADT7 and pGBKT7, respectively. The cloned constructs were co-introduced into an AH109 yeast strain using lithium acetate/polyethylene glycol with herring testis carrier DNA. The transformants were on agar plates containing a synthetic dropout medium (Clontech, Palo Alto, CA) lacking Leu and Trp for maintenance of the plasmids, and those additionally lacking His and Ade to suppress background, and grown at 30°C for 4 days.

Cell culture and transfection with small interfering RNA (siRNA) and plasmid DNA

H9c2 rat embryonic cardiac myoblasts and HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 μg/ml of streptomycin and 100 IU/ml of penicillin at 37°C in the presence of 5% CO_2. For siRNA transfection in H9c2 cells, cells were transfected with scrambled negative control siRNA (4390843, Thermo Fisher Scientific) or FKBP8-specific siRNA (4390771, s66102, Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to manufacturer's instructions. For plasmid DNA transfection, HEK293 cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's protocols.

Western blot analysis and mitochondrial fractioning

Frozen left ventricular tissues or cultured cells were initially homogenized in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L Na-orthovanadate, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L β-glycerophosphate, 1% Triton X-100) with protease inhibitors (protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO). Mitochondrial fractions were isolated from mouse hearts by differential centrifugation methods [2]. The extracted hearts were incubated with the buffer containing 225 mmol/L mannitol, 5 mmol/L sucrose, 0.2% bovine serum albumin and 0.1% collagenase type II for 15 min at 4°C and homogenized by glass-Teflon homogenizer. The lysate was initially centrifuged at 960 g and the supernatant containing the mitochondrial contents was centrifuged at 8540 g. After washing the pellet twice using the oxygraph solution (250 mmol/L sucrose, 5 mmol/L KH_2PO_4, 2 mmol/L HEPES, pH 7.4, 10 mmol/L EGTA), the final pellet was obtained as the mitochondrial fraction.

The protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The samples were electrophoresed and transferred onto nitrocellulose membranes (Amersham Protran Premium 0.2 NC, GE Healthcare, Waukesha, WI). After the membrane was blocked using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) or 5% skimmed milk for 1 h, membranes were incubated overnight at 4°C with the following specific antibodies; FKBP8 (AF3580, R&D Systems, Minneapolis, MN), GAPDH (G8795, Sigma-Aldrich), SDHA (11998, Cell Signaling Technology, Danvers, MA), COX IV (Ab33985, Abcam, Cambridge, UK), VDAC (4866, Cell Signaling Technology), LC3B (2775, Cell Signaling Technology), LC3A (ab62720, Abcam), phosphorylated S6 (2215, Cell Signaling Technology), S6 (2217, Cell Signaling Technology), 4E-BP1 (ab2606, Abcam), caspase-3 (8G10) (9665, Cell Signaling Technology), cleaved caspase-3 (9661, Cell Signaling Technology), caspase-8 (D35G2) (4790, Cell Signaling Technology), caspase-9 (9504, Cell Signaling Technology), caspase-12 (C7611, Sigma-Aldrich), Bax (2772, Cell Signaling Technology), Bcl-2 (2876, Cell Signaling Technology), KDEL (ADI-SPA-827, Enzo Life Sciences, Farmingdale, NY), PDI (ADI-SPA-891-D, Enzo Life Sciences), p-PERK (Thr 981) (sc-32577, Santa Cruz Biotechnology, Santa Cruz, CA), PERK (sc-13073, Santa Cruz Biotechnology), ATF4 (11815, CST), K_V11.1 (HERG) (APC-016, Alomone Labs, Jerusalem, Israel), and CFTR (ab2784, abcam) and, and then incubated with the appropriate IRDye 680 or IRDye 800 secondary antibodies (LI-COR Biosciences). Fluorescent immunoreactive bands were detected by an Odyssey CLX imaging system (LI-COR Biosciences).

Quantitative analysis was performed by Image Studio software (LI-COR Biosciences) and the values were expressed as a relative ratio over the control group [3].

Confocal microscopy for assessment of mitophagy

HEK293 cells stably expressing mitochondrial-targeted mKeima were established as previously demonstrated [1]. Cells were seeded on a 35 mm imaging dish (μ-Dish 35 mm, high, ibiTreat, ibidi, Martinsried, Germany) and transfected with scrambled negative control siRNA (4390843, Thermo Fisher Scientific) or FKBP8-specific siRNA (4390824, s24387, Thermo Fisher Scientific) using ScreenFect A (Wako, Osaka, Japan). Forty-eight hours after transfection, cells were incubated with 15 μmol/L mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or dimethylsulphoxide (DMSO) for 4 h and stained with LysoTracker Green (Thermo Fisher Scientific). The images of live cells were acquired by a Nikon Ti-Eclipse inverted microscope (Nikon, Tokyo, Japan) equipped with a Yokagawa CSU-X1-M2 spinning disk unit (Yokagawa, Musashino, Japan) and an Andor Neo sCMOS camera (Andor Technology, Belfast, United Kingdom) using a 60x oil immersion objective lens. For imaging of the mKeima signal, 488 nm and 561 nm laser diodes were used for excitation in combination with a 607/36 nm emission filter. For visualization of the LysoTracker Green, 488 nm excitation was used in combination with a 525/50 nm bandpass emission filter. Ratiometric images (561/488 nm) of mKeima were created using NIS-Elements software (Nikon). The rainbow-color ratio images were converted into a single-color intensity channel for the purpose of quantitative analysis. The level of mitophagy was assessed by quantification of high-ratio mKeima signals colocalized with LysoTracker-positive punctate structures.

H9c2 cells were seeded on a 35 mm imaging dish (μ-Dish 35 mm, high, ibiTreat) and transfected with scrambled negative control siRNA or FKBP8-specific siRNA. Forty eight h after transfection, pEGFP-LC3 was transfected by using Lipofectamine 3000 (Thermo Fisher Scientific). H9c2 cells were incubated with MitoTracker Red CMXRos (M7512, Thermo Fisher Scientific) for 30 min and then incubated with CCCP (10 μmol/L) or DMSO for 4 h. Cells were fixed by 4% paraformaldehyde 96 h after siRNA transfection and the images were captured by a Nikon Ti-Eclipse inverted microscope using a 60x oil immersion objective lens. The level of mitophagy was evaluated by quantification of mitochondria positive for pEGFP-LC3 [4]. pEGFP-LC3 was obtained from Prof. Noboru Mizushima [5].

Generation of cardiac-specific FKBP8-deficient mice

We designed the *Fkbp8* targeting vector to generate the *Fkbp8*^{flox} allele, in which exon 3 – 6 of the *Fkbp8* gene is flanked by two *loxP* sites. We referred to the genomic sequence database of C57BL/6J mice. The targeting vector was electroporated into embryonic stem (ES) cells (KY1.1) and transfected ES clones were selected for *neomycin* resistance according to standard methods [6]. We screened for the ES clones with homologous recombination by PCR and confirmed further by Southern blotting analysis. Karyotyping was carried out to obtain ES clones exhibiting the desired homologous recombination and normal karyotype. The pCAGGS-Flpo-IRES-puro plasmid was electroporated into the selected ES clones. The transfected ES clones were selected for puromycin resistance. The neomycin cassette excised ES clones were screened by PCR and injected into C57BL/6J mouse blastocysts to generate chimeric mice. The chimeric mice were crossed with C57BL/6J mice to obtain *Fkbp8*$^{flox/+}$ mice. We crossed the *Fkbp8*$^{flox/flox}$ mice with the mice expressing α-myosin heavy chain promoter driven *Cre* recombinase transgenic mice (*αMHC-Cre*) with C57BL/6J background. All procedures were carried out in accordance with the King's College London Ethical Review Process Committee and UK Home Office (Project License No. PPL70/7260 and 70/8889).

Transverse aortic constriction (TAC), echocardiography and blood pressure monitoring

The 8 – 11 weeks old mice were subjected to TAC or sham surgeries as previously described [7,8]. Briefly, the mice were anesthetized by injection intraperitoneally with medetomidine (1 mg/kg) and ketamine (75 mg/kg), followed with buprenorphine (0.12 mg/kg) intramuscularly and flunixin (2 mg/kg) subcutaneously for analgesia. After deep anesthesia was confirmed, ligation of the aortic arch was performed using a 6-0 suture between the brachiocephalic artery and left common carotid artery with a 27-gauge blunt needle. After ligation, the needle was removed. Sham surgeries were carried out without the ligation procedure. Echocardiography was conducted with a Vevo 2100 system using a 22 – 55 MHz linear transducer (Visual Sonics, Toronto, Canada) on conscious mice [2]. M-mode images of the parasternal short-axis view at papillary muscle level were captured and all parameters were averaged over at least 4 cardiac cycles for analysis. Left ventricular (LV) fractional shortening and LV mass were calculated as 100 x [end-diastolic LV internal dimension (LVIDd) – end-systolic LV internal dimension (LVIDs)]/LVIDd and 1.05 x [LVIDd + diastolic interventricular septum wall thickness (IVSd) + diastolic LV posterior wall thickness (LVPWd)]^3 – (LVIDd)^3], respectively. Non-invasive measurement of tail blood pressure was performed on conscious mice using a BP Monitor (Model MK-2000, Muromachi Kikai, Tokyo, Japan) as previously described [3].

Histological analysis

LV samples were embedded in the O.C.T. compound (Cryomatrix, Thermo Fisher Scientific), and frozen in liquid nitrogen immediately. The samples were sectioned into 6 μm thickness using a cryostat (Microm, HM560, Thermo Fisher Scientific) and stained with hematoxylin-eosin, Masson's trichrome (Masson's Trichrome Stain Kit, Polysciences, Warrington, PA), wheat germ agglutinin (Alexa Fluor 488 conjugated, Thermo Fisher Scientific), or immunohistochemical staining with S100A4 (fibroblast-specific protein-1) antibody (ab41532, Abcam) followed by avidin peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA), DAB peroxidase substrate kit (Vector Laboratories) and counterstaining with hematoxylin. Fibrosis fraction and cross-sectional areas of cardiomyocytes were measured using the NIH ImageJ software (National Institutes of Health, Bethesda, MD) [2,9] .

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the left ventricle using the Trizol reagent (Thermo Fisher Scientific) and cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) for RT-PCR according to the manufacturer's instructions. The quantitative PCR was performed to determine mRNA expression using the power SYBR green PCR mastermix (Thermo Fisher Scientific) with a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The specific primers are shown in the table below. Standard curve methods were applied by serially diluting the templates. All data were normalized to *Actb* mRNA and expressed as a fold increase of the control group [3].

	Forward	Reverse
Nppa	TCGTCTTGGCCTTTTGGCT	TCCAGGTGGTCTAGCAGGTTCT
Nppb	AAGTCCTAGCCAGTCTCCAGA	GAGCTGTCTCTGGGCCATTTC
Myh7	ATGTGCCGGACCTTGGAAG	CCTCGGGTTAGCTGAGAGATCA
Col1a2	ACGCGGACTCTGTTGCTGCT	GCGGGACCCCTTTGTCCACG
Col3a1	CCCGGGTGCTCCTGGACAGA	CACCCTGAGGACCAGGCGGA
Actb	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC

Transmission electron microscopy
The heart was perfused with ice cold 0.1 mmol/L PIPES buffer containing 2.5% polyvinylpyrrolidone and 0.1% sodium sitrite and then fixed with 0.1 mmol/L PIPES buffer containing 2% glutaraldehyde, 2% paraformaldehyde, 0.1% sodium itrite and 2.5% polyvinylpyrrolidone. Transfected H9c2 cells were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 mmol/L cacodylate buffer at 4°C overnight and then washed with 0.1 mmol/L cacodylate buffer. Samples were processed according to standard procedures [2] and viewed by a FEI Technai 12 (FEI, Hillsboro, OR) running at 120 kV or a JEOL JEM-1400Plus (JEOL, Akishima, Japan) at 120 kV.

Mitochondrial enzyme activities
The mitochondrial fraction was freshly prepared from mouse hearts as described above. Mitochondrial protein (7.5 μg) was used for the measurement steps. Mitochondrial electron transport chain complex activities of NADH cytochrome-c oxidoreductase (complex I + III) and succinate cytochrome-c oxidoreductase (complex II + III) were evaluated using spectrophotometric methods [2]. The data were expressed as a relative ratio to the control group.

Mitochondrial DNA quantification
DNA from mouse hearts was extracted and purified by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction with the inclusion of RNAse A digest. Quantitative PCR was performed using the power SYBR green PCR mastermix with a StepOnePlus Real-Time PCR System using mitochondrial DNA specific primer, cytochrome oxidase subunit 1 (*Co1*) gene and nuclear DNA specific primer, NADH:Ubiquinone oxidoreductase core subunit V1 (*Ndufv1*). The sequences of primers were as follows: forward 5'-TGCTAGCCGCAGGCATTAC-3' and reverse 5'-GGGTGCCCAAAGAATCAGAAC-3' for *Co1*, and forward 5'-CTTCCCCACTGGCCTCAAG-3' and reverse 5'-CCAAAACCCAGTGATCCAGC-3' for *Ndufv1*. All reactions were run in duplicate. The level of mitochondrial DNA content was calculated using the delta CT method and the data were expressed as a fold increase of the control group [1,10].

Terminal deoxynucleotidyl transferase mediated dUTP nick end-labelling (TUNEL) staining
O.C.T.-embedded heart sections were incubated with proteinase K and then stained with TUNEL kit (Takara Bio, Otsu, Japan) and anti-actin (α-sarcomeric) antibody (A2172, Sigma-Aldrich) followed by the secondary antibody Texas Red Anti-Mouse IgM (Vector Laboratories). Samples were mounted with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). For H9c2 cells, the cells were fixed in 4% paraformaldehyde, stained with the TUNEL kit and mounted with ProLong Gold Antifade Reagent with DAPI. All images were captured by a IX81 fluorescence inverted microscope (Olympus, Tokyo, Japan) using x20 objective lens. The number of TUNEL-positive nuclei and total nuclei was counted, and expressed as the number of TUNEL-positive cardiomyocytes per 10^5 nuclei for the heart tissue and as the percentage of TUNEL-positive nuclei to total nuclei for H9c2 cells.

Cell viability assay
H9c2 cells were seeded at 0.8 x10^4/well in 96-well plates and transfected with siRNA. The numbers of viable cells were analyzed using a Cell-Titer Blue assay (Promega, Fitchburg, WI) [11]. The caspase-12 specific inhibitor, Z-ATAD-FMK (BioVision, Milpitas, CA), was incubated prior to hydrogen peroxide (Sigma-Aldrich) stimulation. The data were expressed as a relative ratio to the control.

Isolation of mouse adult cardiomyocytes and rat neonatal cardiomyocytes and immunofluorescence

Mouse adult cardiomyocytes were isolated using a Langendorff system as previously described [8]. Isolated cardiomyocytes were pelleted, fixed by 4% paraformaldehyde at 37°C for 15 min and then the fixed cells were layered over the cover glasses coated with Corning Cell-Tak Cell and Tissue Adhesive (Corning, Corning, NY). The adhered cells were permeabilized with 1% Triton X-100 for 30 min, blocked with 10% normal donkey serum (ab7475, Abcam) for 1 h at room temperature, and then incubated with specific primary anti-FKBP8 (AF3580, R&D Systems), KDEL (ADI-SPA-827, Enzo Life Sciences) and Tomm20 (ab56783, Abcam) antibodies overnight at 4°C, followed by appropriate secondary antibodies such as Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (A-11057, Thermo Fisher Scientific) and Chicken anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21469, Thermo Fisher Scientific), and then mounted with ProLong Gold Antifade Reagent with DAPI. Rat neonatal cardiomyocytes were isolated from hearts of 1 to 2 day old Sprague-Dawley rats as previously reported [8]. Cells were grown onto coverslips or a 35 mm imaging dish (μ–Dish 35 mm, high, ibiTreat) and fixed with 4% paraformaldehyde at 37°C for 15 min, permeabilized with 0.05% or 0.2% Triton X-100 for 3 min or 15 min, and then blocked with 2% bovine serum albumin for 1 h at room temperature. Transfection was performed using tdTomato-ER-3 by TransFectin transfection reagent (BioRad, Hercules, CA) as instructed by the manufacturer. TdTomato-ER-3 was a gift from Michael Davidson (Addgene plasmid # 58097). MitoTracker Red CMXRos or MitoTracker Deep Red FM (M22426, Thermo Fisher Scientific) were added into the cultured medium prior to fixation. Confocal imaging was performed on a Nikon Ti-Eclipse inverted microscope equipped with a Yokagawa CSU-X1-M2 spinning disk unit using a 60x or 100x oil immersion objective lens. Acquired images were processed using NIS-Elements software and displayed images represent one z plane [12].

Construction of DNA plasmid

Mouse *Fkbp8* (NM_001111066.1) and heat shock protein 90 alpha family class A member 1 (*Hsp90aa1*) (NM_010480.5) cDNA was obtained by RT-PCR using total RNA from C57BL/6J mouse heart. *Fkbp8* was amplified by PCR with the forward primer 5'-AGCAGAATTCAGCATGGCGTCTTGGGCTGA-3' and reverse primer 5'-AGCAGAATTCGGGGGACAGTCAGTTCCTGGCA-3'. The obtained fragment was directly cloned into pTA2 (Takara Bio) and the products digested by EcoRI were subcloned into pGADT7 vector (Clontech). To obtain HA-tagged FKBP8, the plasmid DNA was amplified using the forward primer 5'-AGCAACTAGTGCCGCCATGGAGTACCCATACGACGTA-3' and the same reverse primer as above, and then inserted into pCR2.1-TOPO (Thermo Fisher Scientific). The fragment was subcloned into pcDNA3.1 at BamHI and XhoI sites. *Hsp90aa1* was amplified by PCR with the forward primer 5'-AAAGCGGCCGCAATGCCTGAGGAAACCCAGACCCA-3' and reverse primer 5'-CCGGTCGACGACTTAGTCTACTTCTTCCATGCGTG-3'. The fragment was inserted into pFLAG-CMV-2 at NotI and SalI sites. All plasmid constructs were verified by restriction digestion and DNA sequencing.

Immunoprecipitation

Samples were prepared from HEK293 cells transfected with HA-FKBP8 and Flag-HSP90 for 48 h or mouse heart tissue. For HEK293 cells, samples were lysed with lysis buffer (75 mmol/L NaCl, 50 mmol/L Tris-HCl, 0.5% Nonidet P-40, pH 8.0) with a protease inhibitor cocktail. Protein concentration was determined by BCA Protein Assay and then 300 μg of protein was subjected to immunoprecipitation using protein G-coupled magnetic beads (Thermo Fisher Scientific) and anti-HA antibody (3724, Cell Signaling Technology) or anti-Flag antibody (F1804, Sigma-Aldrich) for 10 min at room temperature in the presence of 1 mmol/L $CaCl_2$ or EGTA. Rabbit or mouse immunoglobulin G (IgG) was used as control, respectively. The precipitate complexes were washed 3 times with the lysis buffer containing 1 mmol/L $CaCl_2$ or EGTA. The bound proteins were analyzed by immunoblotting. For the

heart tissue, the mouse heart was harvested and homogenized with the lysis buffer (37.5 mmol/L NaCl, 50 mmol/L Tris-HCl, 0.5% Nonidet P-40, pH 8.0) and a protease inhibitor cocktail. After protein concentration was measured by BCA Protein Assay, 500 μg lysates were immunoprecipitated with anti-FKBP8 antibody (11173-1-AP, ProteinTech, Chicago, IL) or rabbit IgG using protein G-coupled magnetic beads for 2 h at 4°C. After washing the immunoprecipates 5 times with lysis buffer, the samples were subjected to immunoblot analysis.

Adenovirus construction and infection

Target-specific short hairpin RNA (shRNA) against rat *Fkbp8* mRNA (sense: 5'-GCTGAAGGTCAAGTGTCTGAA-3') was designed by using BLOCK-iT RNAi Designer (Thermo Fisher Scientific). Recombinant adenoviral vector expressing shRNA was constructed with BLOCK-iT U6 RNAi Entry Vector Kit and BLOCK-iT Adenoviral RNAi Expression System (Thermo Fisher Scientific) according to the manufacturer's instructions. Adenovirus was amplified in HEK293A cells and purified by using Adenovirus Mini Purification Virakit (Virapure, San Diego, CA) and virus titer was determined by Adeno-X Rapid Titer kit (Clontech, Palo Alto, CA**).** Rat neonatal cardiomyocytes were infected with adenoviral vector expressing shRNA targeted to FKBP8 (Ad-shFKBP8) at a multiplicity of infection of 100. LacZ (Ad-shLacZ) was used as control.

Detection for protein aggregates and Tat-Beclin 1 treatment

Protein aggregates were detected with Proteostat Aggresome Detection Kit (Enzo Life Sciences) according to manufacturer's instruction [13]. H9c2 cells were seeded onto coverslips and transfected with siRNA, and then cells were fixed with 4% paraformaldehyde, permeabilized by Triton X-100, labeled with Proteostat aggresome dye, and mounted by ProLong Gold Antifade Reagent with DAPI. For co-staining with Proteostat dye, cells were incubated with anti-ubiquitin (BML-PW8810-0100, Enzo Life Sciences) or anti-p62 (ab56416, Abcam) antibody overnight following blocking with 2% bovine serum albumin after permeabilization, and then stained with Chicken anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-21200, Thermo Fisher Scientific) or Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A31571, Thermo Fisher Scientific). Images were captured by Nikon Ti-Eclipse inverted confocal microscope using a 60x oil immersion objective lens. Rat neonatal cardiomyocytes were seeded on a 35 mm imaging dish (μ-Dish 35 mm, high, ibiTreat) and infected with adenoviral vector expressing shRNA, and then fixed and stained with the same procedure as H9c2 cells. For the image acquisition, a x100 oil objective lens was used. Tat-Beclin 1 L11 Autophagy Inducing Peptide (Novus Biologicals, Littleton, CO) was used for induction of autophagy [14]. Transfected H9c2 cells were washed by PBS and treated for 3 h with Tat-Beclin 1 dissolved in Opti-MEM (Thermo Fisher Scientific) acidified with 0.15% 6 N HCl according to the protocol.

20S proteasome activity

Proteasome activity was evaluated using a 20S Proteasome Activity Assay Kit (APT280, Merck-Millipore, Darmstadt, Germany) according to the manufacturer's protocol. The cells were lysed in 50 mmol/L HEPES (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100 and 2 mmol/L ATP. The lysates were cleared by centrifugation at 13,200 rpm for 15 min at 4°C. The protein concentration was measured by BCA Protein Assay and 18 μg of protein was used for preparing assay mixtures in a 96-well plate. The fluorogenic substrate, LLVY-7-amino-4-methylcoumarin (AMC), was added and proteasome activities were determined by the intensity of free AMC after cleavage from the substrate. Fluorescence was quantified using a fluorescence microplate reader with excitation at 380 nm and emission at 460 nm. The values were expressed as a relative ratio.

Statistical analysis

All data are expressed as the mean ± SEM. Student's *t*-test was used for the comparison of paired data. One way analysis of variance followed by the Bonferroni's post hoc test was applied for multiple comparisons. All data were analyzed with IBM SPSS Statistics 22.0 (IBM, Armonk, NY). A probability value < 0.05 was considered statistically significant.

Supplementary Table 1. Echocardiographic and physiological parameters in *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ mice at baseline.

	Fkbp8$^{+/+}$	*Fkbp8*$^{-/-}$
IVSd, mm	0.92 ± 0.01	0.94 ± 0.02
LVPWd, mm	0.91 ± 0.03	0.93 ± 0.02
LVIDd, mm	3.00 ± 0.08	2.98 ± 0.04
LVIDs, mm	1.40 ± 0.04	1.43 ± 0.05
LVFS, %	53.16 ± 1.12	52.08 ± 1.16
LV mass, mg	90.30 ± 3.33	91.87 ± 1.88
Heart rate, beats/min	663 ± 7	684 ± 10
SBP, mmHg	114 ± 6	109 ± 3
BW, g	25.8 ± 0.6	26.2 ± 0.5
HW, mg	133.3 ± 6.1	140.6 ± 6.8
LVW, mg	101.7 ± 4.4	108.4 ± 5.4
RVW, mg	15.7 ± 1.7	14.9 ± 0.7
Lung weight, mg	136.3 ± 2.7	137.8 ± 1.6
Liver weight, mg	1188 ± 48	1177 ± 67
TL, mm	17.6 ± 0.1	17.3 ± 0.1
HW/BW, mg/g	5.12 ± 0.27	5.45 ± 0.32
HW/TL, mg/mm	7.57 ± 0.32	8.12 ± 0.35
LVW/BW, mg/g	3.91 ± 0.22	4.20 ± 0.25
LVW/TL, mg/mm	5.77 ± 0.23	6.26 ± 0.29
Lung weight/BW, mg/g	5.28 ± 0.08	5.28 ± 0.12
Lung weight/TL, mg/mm	7.73 ± 0.13	7.97 ± 0.15
Liver weight/BW, mg/g	46.10 ± 1.54	44.93 ± 1.79
Liver weight/TL, mg/mm	67.47 ± 2.50	67.99 ± 3.48

IVSd, diastolic interventricular septum wall thickness; LVPWd, diastolic left ventricular posterior wall thickness; LVIDd, end-diastolic left ventricular internal dimension; LVIDs, end-systolic left ventricular internal dimension; LVFS, left ventricular fractional shortening; LV mass, left ventricular mass; SBP, systolic blood pressure; BW, body weight; HW, heart weight; LVW, left ventricular weight; RVW, right ventricular weight; TL, tibia length. *Fkbp8*$^{+/+}$ mice (n = 4), *Fkbp8*$^{-/-}$ mice (n = 4). Data are expressed as the mean ± SEM.

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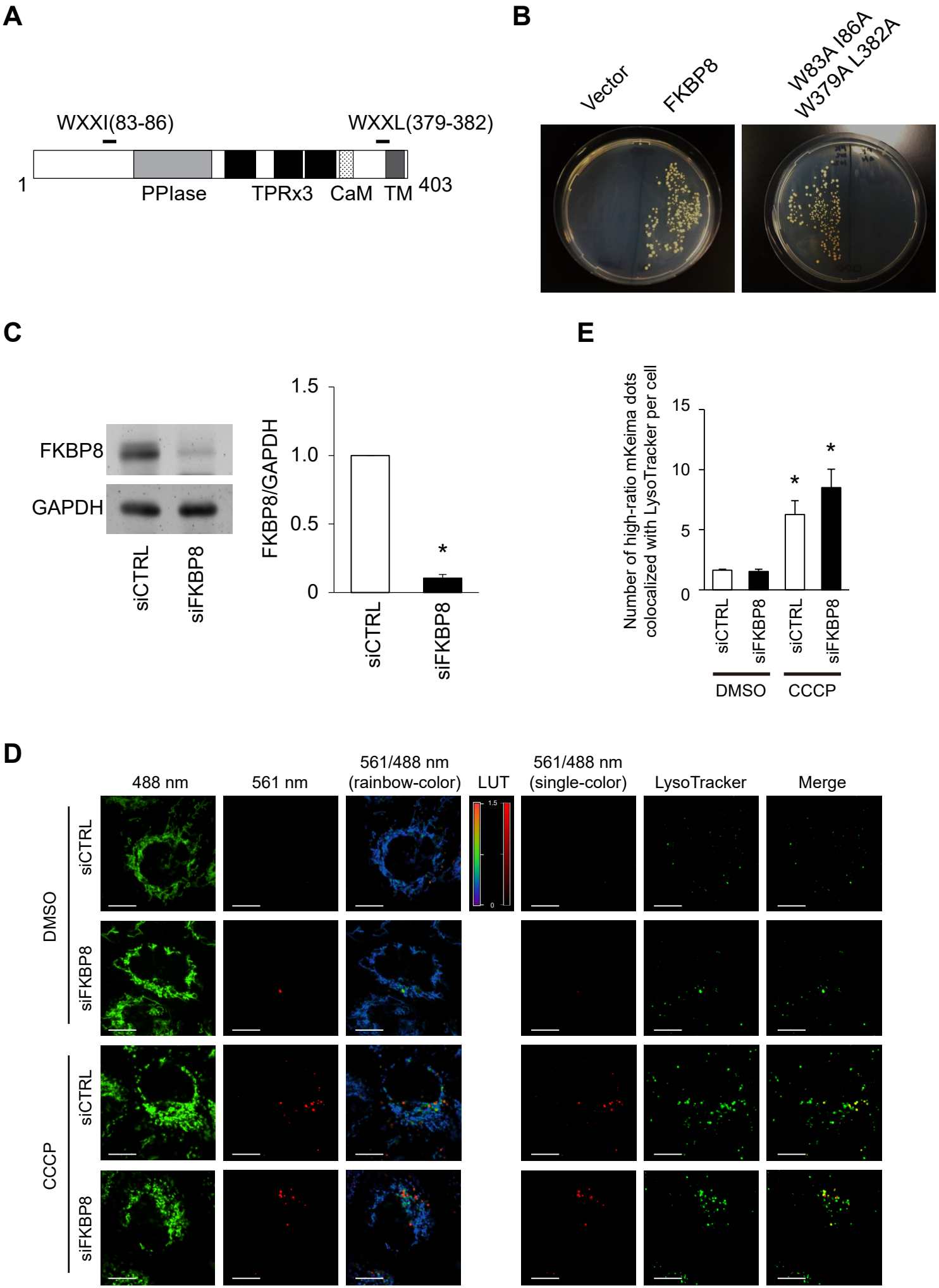
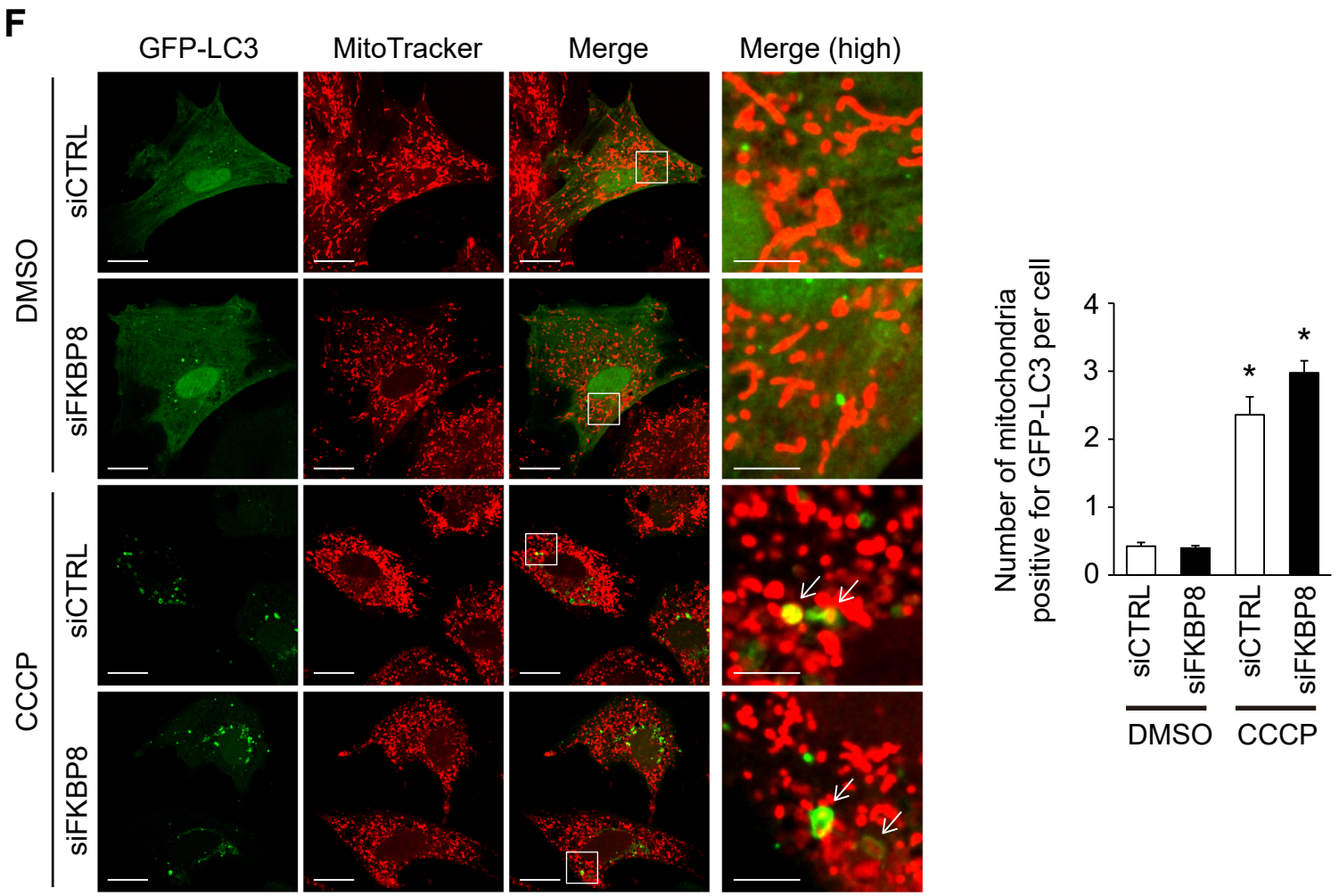
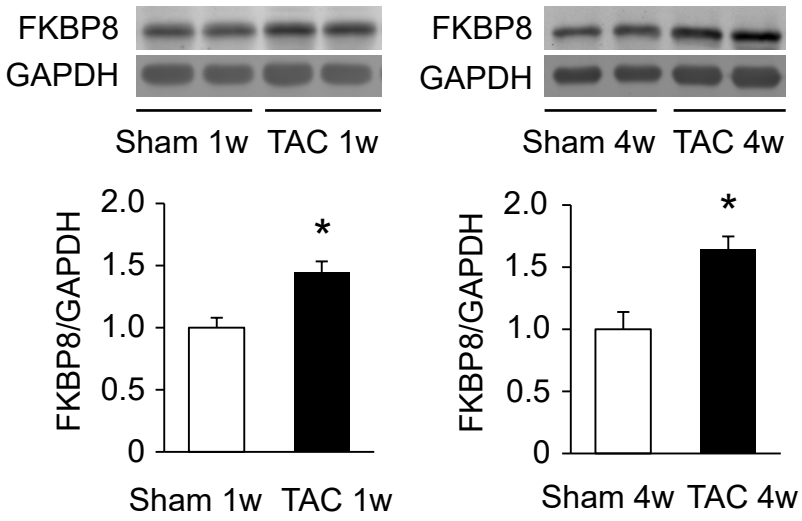
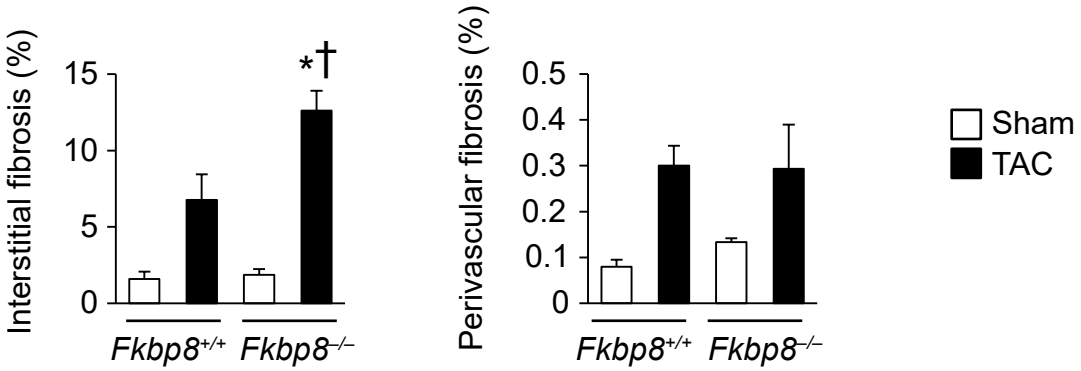

A
WXXI(83-86)
WXXL(379-382)
1
PPIase
TPRx3
CaM
TM
403
B
Vector
FKBP8
W83A I86A
W379A L382A
C
FKBP8
GAPDH
siCTRL
siFKBP8
FKBP8/GAPDH
1.5
1.0
0.5
0
*
siCTRL
siFKBP8
E
Number of high-ratio mKeima dots colocalized with LysoTracker per cell
15
10
5
0
*
*
siCTRL
siFKBP8
siCTRL
siFKBP8
DMSO
CCCP
D
488 nm
561 nm
561/488 nm (rainbow-color)
LUT
561/488 nm (single-color)
LysoTracker
Merge
DMSO
siCTRL
siFKBP8
CCCP
siCTRL
siFKBP8



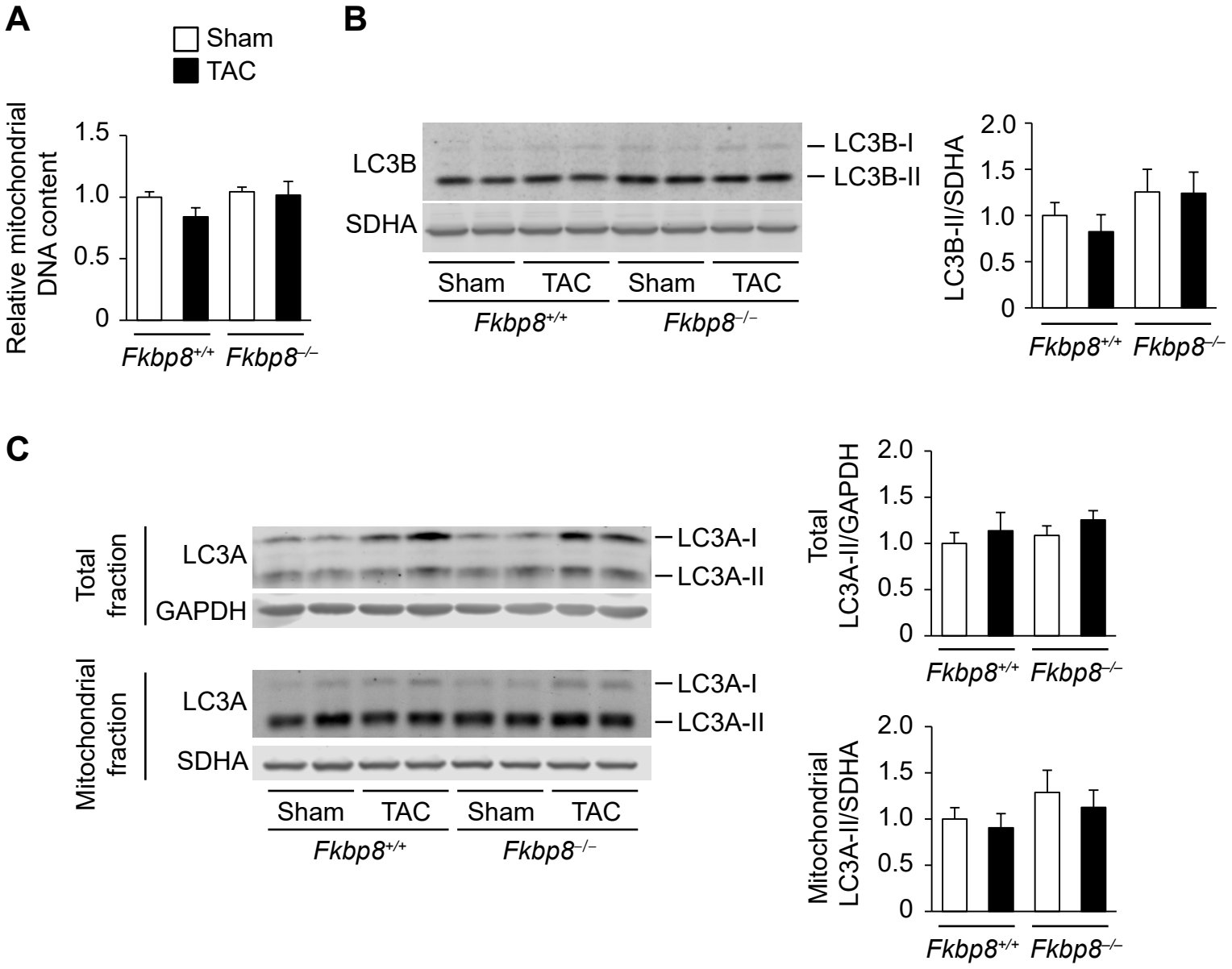
Supplementary Fig. 1. Characterization of FKBP8 and its role in mitophagy.
A. Schematic diagram of mouse FKBP8 structure. The putative peptidyl prolyl cis/trans-isomerase (PPIase) domain, three-unit tetratricopeptide repeat (TPRx3), calmodulin binding (CaM) domain, transmembrane (TM) domain, and two WXXL/I motifs are indicated. Numbers indicate amino acid positions. B, Yeast two-hybrid assay to test the interaction of LC3B and FKBP8. Wild-type and its mutant FKBP8 are indicated. The transformants were cultured on agar plates lacking Leu, Typ, His and Ade. C, HEK293 cells stably expressing mKeima were transfected with negative control siRNA (siCTRL) or FKBP8 siRNA (siFKBP8). Forty-eight h after transfection, cell lysates were subjected to Western blot analysis using anti-FKBP8 antibody. Representative blots are shown in left panels and densitometric analysis is shown in right graph. The value of FKBP8-to-GAPDH ratio in siCTRL was set equal to 1. Results are presented as the mean ± SEM (n =3). *P < 0.05 versus siCTRL. D, To induce mitophagy, the transfected cells were incubated with 15 μmol/L CCCP or DMSO for 4 h and stained with LysoTracker Green. The images were sequentially acquired using 488 nm and 561 nm laser diodes in combination with a 607/36 nm emission filter for mKeima (as shown in green and red, respectively) and 488 nm laser diode with a 525/50 nm emisson filter for LysoTracker Green (green). The ratio images of mKeima (561 nm/488 nm) were generated to visualize mitochondria in an acidic environment (rainbow look up table (LUT) scale). In the 4th column from left, the ratio images are displayed using a red intensity LUT scale to visualize the merged images of high-ratio mKeima and LysoTracker. Scale bar, 10 μm. E, The number of high-ratio mKeima dots colocalized with LysoTracker per cell was quantified from these merged images as displayed in D. More than 50 cells were counted in each group (n = 3). *P < 0.05 versus DMSO group. F, H9c2 cells were transfected with siCTRL or siFKBP8 and 48 h later the cells were additionally transfected with GFP-LC3 plasmid. Cells were stained with MitoTracker Red for 30 min and incubated with 10 μmol/L CCCP or DMSO for 4 h, and then fixed 96 h after siRNA transfection. Boxed areas are highlighted in the right panels. White arrows indicate the mitochondria characterized with GFP-LC3-labeled autophagic structures. Scale bars are 20 μm and 5 μm. Quantification of mitochondria positive for GFP-LC3 is shown in the graph. More than 30 GFP-expressing cells were counted in each group (n = 3). *P < 0.05 versus DMSO group.



Supplementary Fig. 2. Changes in cardiac FKBP8 expression levels during pressure overload.
Left ventricular homogenates obtained from C57BL/6J mice 1 (1w) and 4 weeks (4w) after TAC were analyzed by immunoblotting with anti-FKBP8 antibody. Densitometric analysis is shown in the graphs. The average value of FKBP8-to-GAPDH ratio in sham-operated mice was set equal to 1. Results are presented as the mean ± SEM from 3 to 5 mice in each group. **P* < 0.05 versus the corresponding sham-operated mice.

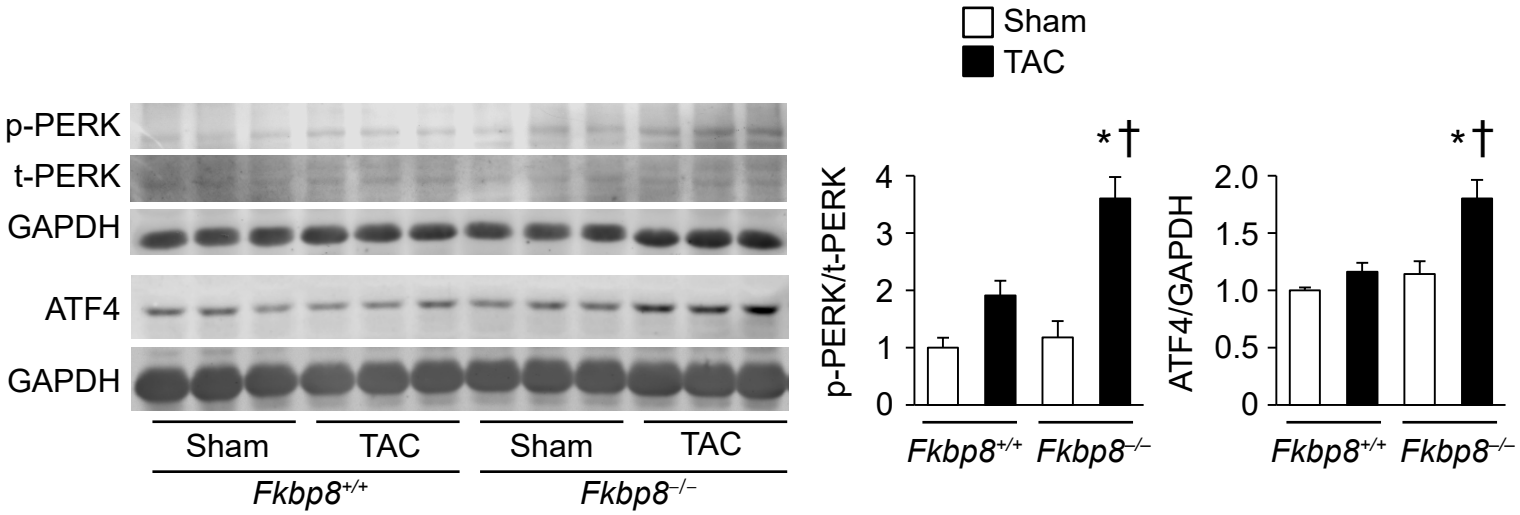


Supplementary Fig. 3. Evaluation of interstitial and perivascular fibrosis in sham- and TAC-operated *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ hearts.
Interstitial and perivascular fibrosis fractions were measured from Masson's trichrome-stained heart sections. Quantitative analysis is shown in the graphs. The values represent the mean ± SEM (n = 3). Open and closed bars represent sham- and TAC-operated groups, respectively. **P* < 0.05 versus the corresponding sham-operated group. †*P* < 0.05 versus TAC-operated *Fkbp8*$^{+/+}$ mice.

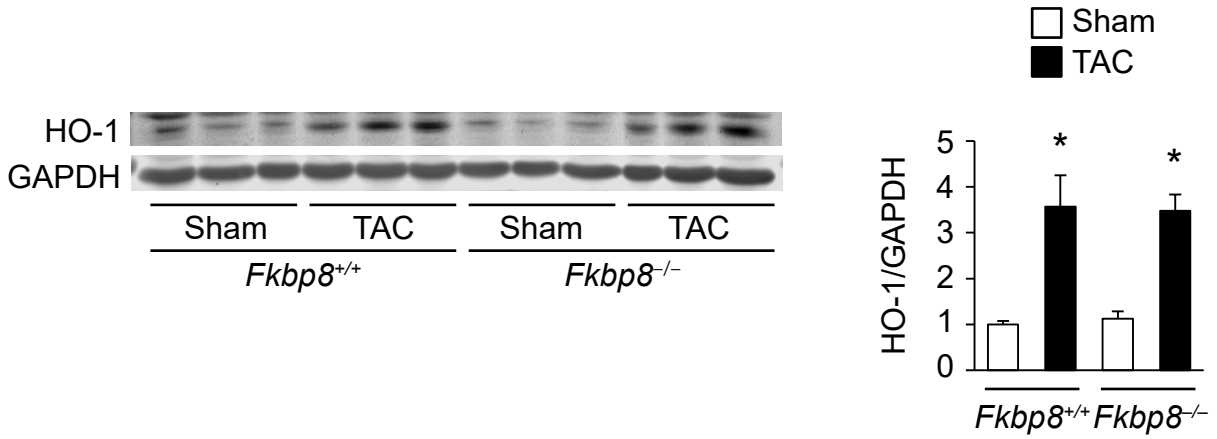


Supplementary Fig. 4. Assessment of mitophagy in sham- and TAC-operated *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ hearts.

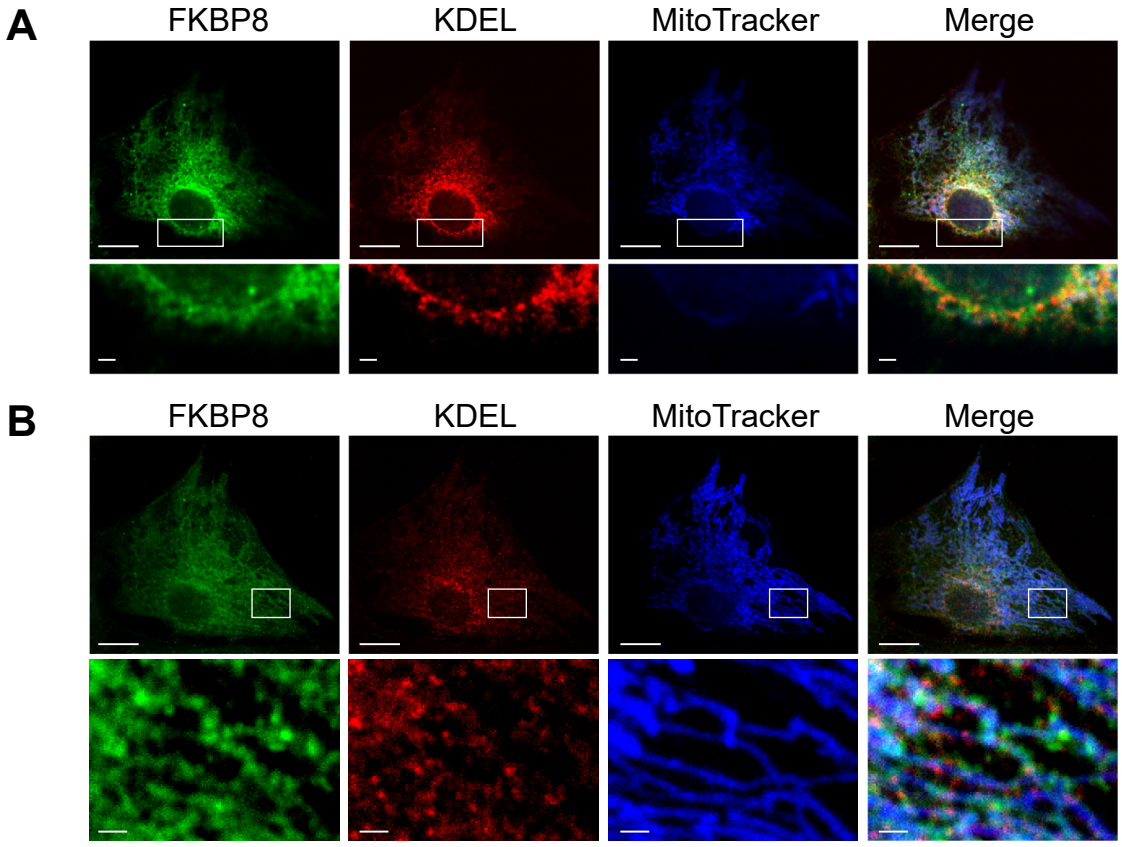
A, Relative mitochondrial DNA content determined by quantitative PCR in sham- and TAC-operated hearts. Values are expressed as the mean ± SEM (n = 6). B, Immunoblot analysis for LC3B in mitochondrial fraction from sham- and TAC-operated hearts. The level of LC3B-II was normalized by SDHA. Data are presented as the mean ± SEM (n = 6). C, Immunoblot analysis for LC3A in total and mitochondrial fractions from sham- and TAC-operated hearts. The levels of LC3A-II were normalized by GAPDH and SDHA in total and mitochondrial fraction, respectively. Data are presented as the mean ± SEM (n = 6). The average value for sham-operated *Fkbp8*$^{+/+}$ mice was set to 1. Open and closed bars represent sham- and TAC-operated groups, respectively.



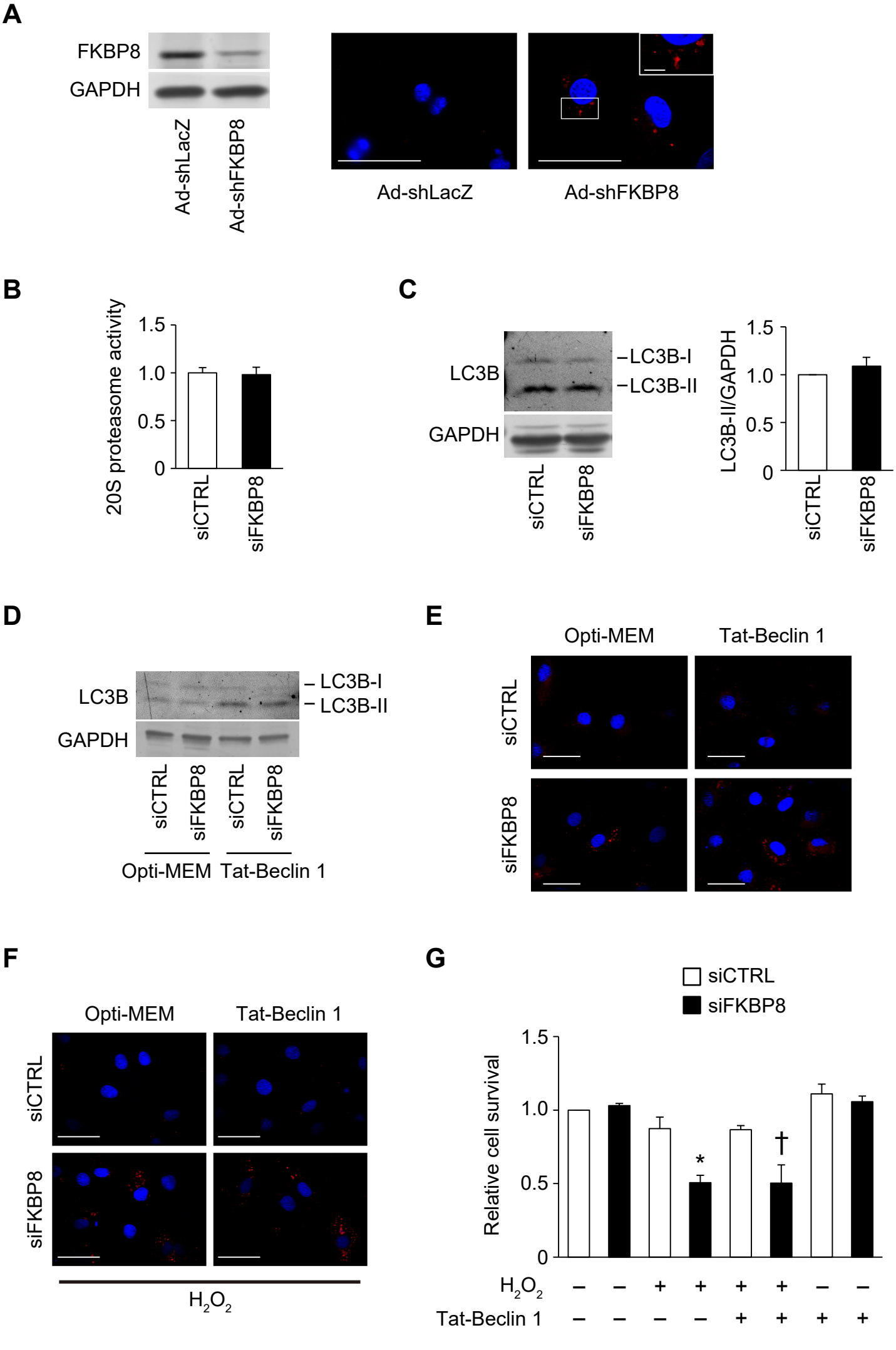
Supplementary Fig. 5. Western blot analysis for PERK and ATF4 in sham- and TAC-operated *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ hearts.
Representative Western blots are shown in the left panels. p-PERK and t-PERK indicate phosphorylated and total PERK, respectively. p-PERK-to-t-PERK ratios (n = 4 - 6) and ATF4-to-GAPDH ratios (n = 7) are shown in the graphs. The average value for sham-operated *Fkbp8*$^{+/+}$ mice was set to 1. Values are expressed as the mean ± SEM. Open and closed bars represent sham- and TAC-operated groups, respectively. *P < 0.05 versus the corresponding sham-operated group. $^{\dagger}P$ < 0.05 versus TAC-operated *Fkbp8*$^{+/+}$ mice.



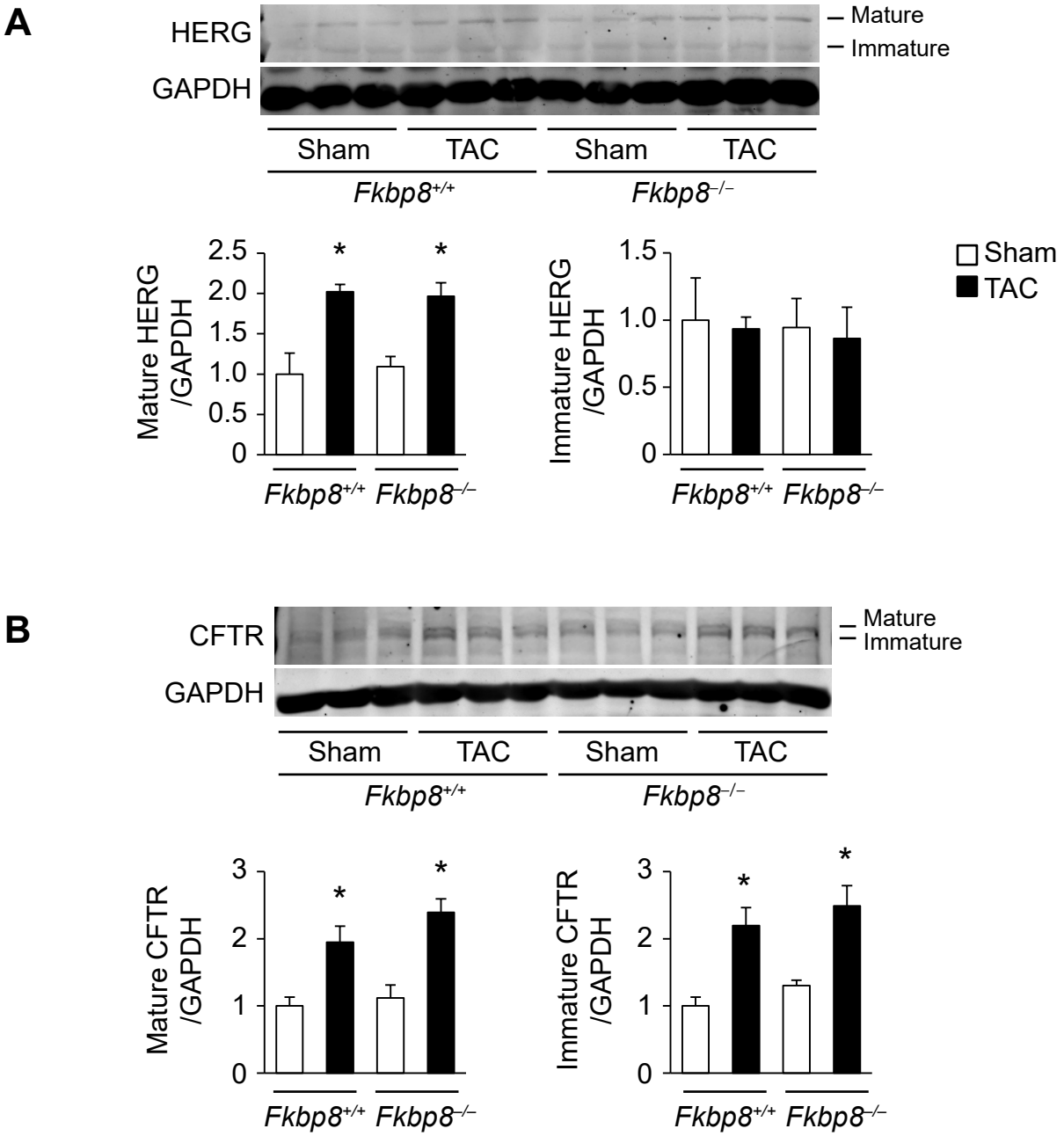
Supplementary Fig. 6. Western blot analysis for HO-1 in sham- and TAC-operated *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ hearts.
Representative Western blots and densitometric analysis are shown. The expression level of HO-1 was normalized by GAPDH. The average value for sham-operated *Fkbp8*$^{+/+}$ mice was set to 1. Values are expressed as the mean ± SEM (n = 6). Open and closed bars represent sham- and TAC-operated groups, respectively. *P < 0.05 versus the corresponding sham-operated group.



Supplementary Fig. 7. Subcellular localization of FKBP8 in rat neonatal cardiomyocytes.
A, B. Cultured rat neonatal cardiomyocytes were stained with MitoTracker to label mitochondria (shown in blue) and then fixed, stained with anti-KDEL antibody (red) to visualize ER and anti-FKBP8 antibody (green). The images were captured by confocal microscopy with Z stack. The representative images of different Z section in one cell are shown. Images in boxed areas at higher magnification are shown in the lower panels. Scale bars are 10 μm and 1 μm.


A
FKBP8
GAPDH
Ad-shLacZ
Ad-shFKBP8
Ad-shLacZ
Ad-shFKBP8
B
20S proteasome activity
1.5
1.0
0.5
0
siCTRL
siFKBP8
C
LC3B
GAPDH
LC3B-I
LC3B-II
siCTRL
siFKBP8
LC3B-II/GAPDH
1.5
1.0
0.5
0
siCTRL
siFKBP8
D
LC3B
GAPDH
LC3B-I
LC3B-II
siCTRL
siFKBP8
siCTRL
siFKBP8
Opti-MEM
Tat-Beclin 1
E
Opti-MEM
Tat-Beclin 1
siCTRL
siFKBP8
F
Opti-MEM
Tat-Beclin 1
siCTRL
siFKBP8
H2O2
G
siCTRL
siFKBP8
Relative cell survival
1.5
1.0
0.5
0
*
†
H2O2 – – + + + + – –
Tat-Beclin 1 – – – – + + + +

Supplementary Fig. 8. Detection of misfolded protein aggregates in rat neonatal cardiomyocytes and involvement of proteasome and autophagy in FKBP8-mediated protein aggregation.
A, Detection of misfolded protein aggregates in FKBP8 knockdown rat neonatal cardiomyocytes. Cultured rat neonatal cardiomyocytes were infected with adenoviral vectors expressing short hairpin RNA targeted to LacZ (Ad-shLacZ) or FKBP8 (Ad-shFKBP8) at a multiplicity of infection of 100. Five days after infection, cell lysates were subjected to Western blot analysis using anti-FKBP8 antibody (left panels). Cells were stained with Proteostat fluorescent dye (red) and DAPI (blue). Representative confocal images are shown in the right panels. Inset is highlighted from the boxed area in main panel. Scale bars represent 50 μm in the main panels and 25 μm in the inset. B, Proteasome activity. Proteasome activity in the lysates from H9c2 cells transfected with siCTRL or siFKBP8 was measured using a 20S Proteasome Activity Assay Kit. The average value in siCTRL-transfected cells was set equal to 1. Data are presented as the mean ± SEM (n = 5). C, Western blot analysis for autophagic activity. The densitometric analysis is shown in the graph. The value of LC3B-II-to-GAPDH ratio in siCTRL-transfected H9c2 cells was set equal to 1. Data are presented as the mean ± SEM (n = 3). D, Western blot analysis using anti-LC3B antibody after Tat-Beclin 1 or vehicle (Opti-MEM) treatment. Transfected H9c2 cells were incubated with Tat-Beclin 1 peptides (5 μmol/L) for 3 h. E, Transfected H9c2 cells were treated with Tat-Beclin 1 peptides (5 μmol/L) for 3 h. siRNA-transfected Tat-Beclin 1-treated cells were fixed and stained with Proteostat dye (red) and DAPI (blue). Scale bar, 50 μm. F, No effect of Tat-Beclin 1 on protein aggregation in H_2O_2-treated FKBP8 knockdown H9c2 myocytes. Transfected H9c2 cells were treated with Tat-Beclin 1 peptides (5 μmol/L) for 3 h and incubated with H_2O_2 (50 μmol/L) for 1.5 h. The cells were fixed and stained with Proteostat dye (red) and DAPI (blue). Scale bar, 50 μm. G. No effect of Tat-Beclin 1 on cellular viability in H_2O_2-treated FKBP8 knockdown H9c2 cells. Transfected H9c2 cells were incubated with Tat-Beclin 1 (5 μmol/L) for 3 h before H_2O_2 administration (50 μmol/L). Cellular viability was determined by Cell-Titer Blue assay 6 h after H_2O_2 stimulation. The value in the cells transfected with siCTRL and incubated without H_2O_2 nor Tat-Beclin 1 was set to 1. Values represent the mean ± SEM from 3 independent experiments with triplicates. $^*P < 0.05$ versus all other groups except the siFKBP8-transfected group followed by H_2O_2 with Tat-Beclin 1 treatment. $^{\dagger}P < 0.05$ versus all other groups except the siFKBP8-tranfected group followed by H_2O_2 without Tat-Beclin 1 treatment. Open and closed bars represent siCTRL- and siFKBP8-transfected groups, respectively.



Supplementary Fig. 9. Western blot analysis for HERG and CFTR in sham- and TAC-operated *Fkbp8*$^{+/+}$ and *Fkbp8*$^{-/-}$ hearts.

A, Representative Western blots of HERG and densitometric analysis are shown. The levels of mature and immature HERG were normalized by GAPDH. Data are presented as the mean ± SEM (n = 3). B, Representative Western blots of CFTR and densitometric analysis are shown. The levels of mature and immature CFTR were normalized by GAPDH. Data are presented as the mean ± SEM (n = 5 - 6). The average value for sham-operated *Fkbp8*$^{+/+}$ mice was set to 1. Open and closed bars represent sham- and TAC-operated groups, respectively. *P < 0.05 versus the corresponding sham-operated group.