SUPPLEMENTAL MATERIAL

Spliced X-box Binding Protein 1 Stimulates Adaptive Growth through Activation of mTOR

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Supplemental Methods

Animals

Mice were maintained on a 12 hr dark/light cycle from 6 AM to 6 PM and housed in a cage of no more than 5 with unlimited access to water and chow diet (Teklad, 2916). To achieve cardiac-specific deletion of XBP1, floxed mice were bred to the αMHC-Cre transgenic mice to generate $XBP1^{F/F}$;αMHC-Cre animals (cKO). To obtain cardiomyocyte-restricted inducible overexpression of XBP1s, the TRE-XBP1s transgenic mice were crossed with the αMHC-tTA animals. Transgene XBP1s expression was suppressed by inclusion of doxycycline in the drinking water (0.1 mg/mL). Induction of XBP1s was achieved by removal of doxycycline. Single transgenic mice of TRE-XBP1s or αMHC-tTA were used as controls. Liver or fat-specific overexpression of XBP1s was conducted as we did previously.[1, 2] XBP1s induction was triggered by feeding doxycycline-containing chow diet for 4 weeks (Bio-Serv, S3888). All mice have been bred to the pure C57BL/6 genetic background for more than 9 generations. The Institutional Animal Care and the Use Committee of University of Texas Southwestern Medical Center has approved all animal protocols.

Thoracic aortic constriction (TAC)

Thoracic aortic banding was performed according to standard procedures with animals of ~ 8 weeks old.[3] Briefly, anesthesia was achieved with ketamine (100 mg/kg, I.P.) and xylazine (5 mg/kg, I.P.). Sterile instruments, supplies, and suture materials were used. Intubation was achieved orally and a small incision in the anterior neck was made to reveal the trachea to facilitate cannulation of the upper airway. Simultaneously, respiratory rate, body temperature, and heart rate were monitored. The aortic arch was accessed via left lateral thoracotomy and the suture material (5-0 silk) was used to ligate the aorta (between the innominate and left carotid arteries) and an overlying 27G needle, which was then removed, immediately leaving a discrete region of stenosis. At the end, chest was closed and animals were observed during recovery from anesthesia.

Echocardiography

Echocardiograms were recorded using a Vevo 2100 system (VisualSonics) with a MS400C scanhead on gently constrained, conscious animals. M-mode images at the level of papillary muscles were captured and analyzed to determine various parameters. Heart rates were also recorded.

Neonatal rat ventricular myocytes (NRVMs) culture

Neonatal Sprague-Dawley rats of 1-2 days old were used to harvest ventricles, which were subjected to cardiomyocyte isolation using a neonatal rat/mouse cardiomyocyte isolation kit (Cellutron, NC-6031). After pre-plating for 2 hrs to remove neonatal fibroblasts, myocytes were plated at a density of 1,250 cells/mm^2 in plating medium (DMEM/M199 = 3:1, high glucose) with 5% FBS, 10% horse serum, and bromodeoxyuridine (BrdU, 100 μM).

After 24 hrs, NRVMs were incubated with 1% FBS medium (DMEM/M199 = 3:1, high glucose, 1% FBS) with BrdU (100 μM). After another 24 hrs, cells were washed twice with PBS, and medium was changed to serum-free DMEM (high glucose)/M199 = 3:1.

Cells were then subjected to various treatments, including siRNA (Sigma), adenoviral infection, and pharmacological stimulation. Adenovirus expressing XBP1s-IRES-GFP or FKBP11-IRES-GFP was used to infect NRVMs, and GFP-positive cells suggest infection positivity. Adenovirus expressing GFP alone was used as a negative control.

Adult mouse cardiomyocyte isolation

Adult mouse cardiomyocytes were isolated from $XBP1^{F/F}$ and cKO mouse hearts according to previous procedures.[4] Briefly, the hearts were perfused in the retrograde manner with the Krebs-Ringer buffer (35 mM NaCl; 4.75 mM KCl, 1.19 mM KH_2PO_4, 16 mM Na_2HPO_4, 134 mM sucrose, 25 mM $NaCO_3$, 10 mM glucose, and 10 mM HEPES, pH 7.4). Collagenase II (Worthington, 0.8 mg/mL) was used to digest the heart. Atria were removed and ventricles were dissociated by pipetting. After filtration by a cell strainer, cardiomyocytes were separated by sedimentation twice and used for Western blotting.

^{3}H-leucine incorporation assay

L-[3, 4, 5-^{3}H]-leucine (PerkinElmer, NET460A001MC) at a final concentration of 2 μCi/mL was included in culture medium when phenylephrine (50 μM) or Angiotensin II (1 μM) treatments commenced.[5] After 24 hrs, cells were washed twice with ice-cold PBS. Trichloroacetic acid (LabChem, 10% W/V, LC262302, 2 mL) was added to each well of 6-well plates for 30 min with agitation at 4°C, followed by 2 washes with 95% ice-cold ethanol. NaOH (1 mL, 0.5 N) was then added to each well. The plates were sealed by parafilm and samples were incubated at 37°C for 6 hrs or overnight while shaking. Equal volume of HCl (1 mL, 0.5 N) was added to each well to neutralize the pH, and the entire content was transferred to a scintillation vial, followed by mixing with 18 mL scintillation solution (MP Biomedicals, EcoLite, 882475) for radioactivity measurements using a bench-top liquid scintillation counter (Beckman, LS5000TA).

Immunoblotting

Approximately 20 mg animal tissues of each sample were used for protein isolation in 500 μL RIPA buffer with a 2 mL Dounce glass tissue grinder homogenizer. Protease and phosphatase inhibitors were included (Thermo, 88669). Whole lysates were then cleared by centrifugation at 14, 000 RPM for 10 min and the supernatants were collected for a BCA assay (Thermo, 23225). Equal total amount of proteins (30 μg) for each sample was loaded onto Criterion gels (Bio-Rad) and subjected for immunoblotting.

For tissue culture, NRVMs were first washed once with ice-cold PBS. Ice-cold RIPA buffer (80 μL) supplemented with protease and phosphatase inhibitors was then added to each well. Cells were stored at -80°C until future use. When processing, 5 X SDS-PAGE sample buffer (20 μL) was added to each well. Cells were then scraped on ice and transferred to tubes. Samples were boiled for 10 min at 95°C, briefly centrifuged, and filtered by glass wool. Lysates were then subjected for SDS-PAGE using 26-well Criterion gels (Bio-Rad), followed by transferring to the nitrocellulose membrane. After blotting, the membrane was scanned by an Odyssey scanner (LI-COR). The following antibodies were used: GAPDH (Fitzgerald, 10R-G109A), Rcan1 (Sigma, D6694), βMHC (Abcam, ab50967), XBP1 (Santa Cruz Biotechnology, sc-7160), GRP78 (BD

Biosciences, 610979), mTOR (Cell Signaling, 4517), p-mTOR (Cell Signaling, 2971), S6K1 (Cell Signaling, 2708), p-S6K1 (Cell Signaling, 9206), S6 (Cell Signaling, 2317), p-S6 S235S236 (Cell Signaling, 4858), p-S6 S240S244 (Cell Signaling, 5364), 4EBP1 (Cell Signaling, 9644), p-4EBP1 (Cell Signaling, 2855), ANF (Abcam, ab180649), IRE1α (Cell Signaling, 3294), p-IRE1α (Novus Biologicals, nb100-2323), FKBP11 (Santa Cruz Biotechnology, sc-83818), Histone H3 (Cell Signaling, 4499), goat anti-mouse secondary antibodies Alexa Fluor 680 (Thermo, A21057), and goat anti-rabbit secondary antibodies 800 CW (LI-COR, 925-32211).

Immunofluorescence staining

To culture NRVMs for staining, glass cover slips were sterilized by U.V. exposure for 20 min and put into 12-well plates. Gelatin (1 mL) was used for overnight coating. NRVMs were then seeded. After treatment, cells were washed twice with ice-cold PBS, and fixed by 4% paraformaldehyde for 30 min at 4°C. The cells were then washed once with ice-cold PBS before permeabilization with 0.1% Triton X-100 in PBS on ice for 10 min. After washing, NRVMs were blocked with 1.5% normal goat serum in PBS with 1% BSA at room temperature for 30 min, followed by incubation with primary antibodies in blocking buffer in a humid chamber at 4°C overnight. NRVMs were then washed 6 times and incubated with secondary antibodies at room temperature for 1 hr. After washing, the cells were sealed with the ProLong Gold anti-fade mountant with DAPI (Thermo, P36931) on tissue slides. The following antibodies were used: α-actinin (Sigma, A7811, 1:100), LAMP2 (Abcam, ab13524, 1:200), mTOR (Cell Signaling, 2983, 1:200), and goat anti-mouse IgG Alexa Fluor 568 (Thermo, A11004, 1:300).

For wheat germ agglutinin (WGA) staining, heart sections (5 μm) were first deparaffinized by incubation at 60°C for 30 min, followed by rehydration. Antigen retrieval (Biogenex, HK086-9K) was conducted before blocking for 1 hr with 5% goat serum/1% BSA in PBS. Sections were then incubated with Alexa Fluor 594-WGA (Thermo, W11262, 10 μg/mL, 1:100) in blocking buffer for 1 hr at room temperature. After washing with PBS for three times, samples were mounted with the ProLong Gold anti-fade/DAPI mountant (Thermo, P36931).

FKBP11 promoter cloning and the luciferase assay

The FKBP11 promoters from different species were aligned and surveyed for putative consensus unfolded protein response elements. Sequence between the 2nd and 3rd exons of 380 bp was cloned into a pGL3-TATA vector (Promega) between *Kpn* I and *Xho* I sites. This construct was co-transfected into HEK293T cells together with different amount of a XBP1s-expressing plasmid. Luciferase assay (Promega, E1500) was then conducted.[4] β-galactosidase activity was used to normalize transfection efficiency (Thermo, 75707).

Adeno-associated virus serotype 9 production

The mouse FKBP11 cDNA was amplified from a mouse heart cDNA library and cloned into the pAAV:cTnT::Luciferase construct by replacing the luciferase open reading frame. This plasmid contains a chicken troponin T promoter to enhance gene expression in a cardiac-specific manner.[6, 7] AAV9 virus packaging, production, and

concentration were conducted in the Viral Core of Boston Children's Hospital. Viruses were delivered into mice after pressure overload with AAV9-GFP as a negative control.

Chromatin immunoprecipitation assay (ChIP)

ChIP was performed with the EZ-ChIP kit (Millipore, 17-371). Briefly, NRVMs were first infected by adenovirus expressing XBP1s. Cells were cross-linked by 37% formaldehyde, lysed with the SDS Lysis Buffer, and subjected to sonication to shear DNA. Cross-linked protein/DNA was precipitated using anti-flag tag antibody (Sigma, F1804) with normal mouse IgG antibody as a control. PCR was then conducted to compare XBP1s-FKBP11 promoter binding between groups.

RNA isolation, reverse transcription and quantitative PCR

Total RNA from cardiac tissues and NRVMs was isolated using an Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, 732-6870) and a Quick-RNA Microprep kit (Zymo Research, R1051), respectively. A total of 250 ng RNA was used for reverse transcription (Bio-Rad, iScript, 1708891) and cDNA was then diluted 10-fold by ddH_2O. For each sample, 2 μL cDNA was used for quantitative PCR to determine relative mRNA levels to 18s rRNA using a LightCycler machine (Roche) and the SYBR green reagent (Bimake, B21203). Real time PCR for human XBP1s was conducted according to a previous report.[8] All primer sequences are listed in Table I in the online-only Data Supplement.

Human patients

Human cardiac tissues were harvested from patients with dilated cardiomyopathy. The informed content was obtained under an IRB protocol that was approved by the Tongji Hospital Human Research Ethics Committee. Control samples were obtained from healthy donors. This study conformed to the principles outlines in the Declaration of Helsinki. Cardiac samples were subjected to RNA isolation, quantitative PCR analysis, and immunoblotting. Patient information is included in Table II in the online-only Data Supplement.

Statistical analysis

Data are expressed as means ± SEM. The Student's *t* test (2-tailed) was used for comparison between two groups. The log rank test was conducted to calculate the statistical significance for survival curve. For comparison of more than two groups, two-way ANOVA was conducted, followed by Tukey's test. $P < 0.05$ was considered as statistically significant. Statistical analysis was done with Prism Graphpad 7.0.

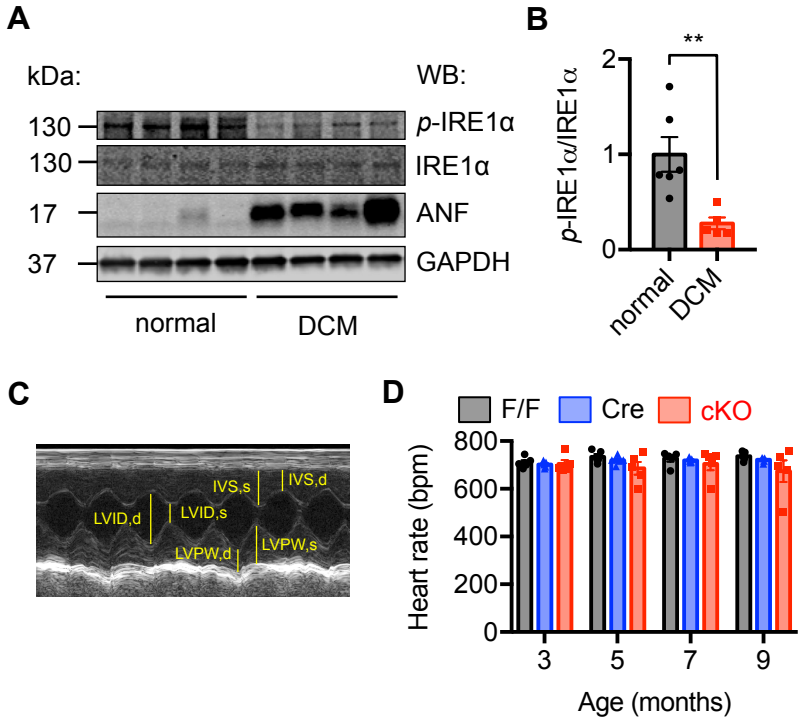
Supplemental Table 1. Primers used in this study.

Gene	Species	Primer	Test
XBP1s	mouse/rat	GGTCTGCTGAGTCCGCAGCAGG	qPCR
		GAAAGGGAGGCTGGTAAGGAAC	
XBP1s	human	TGCTGAGTCCGCAGCAGGTG	qPCR
		GCTGGCAGGCTCTGGGGAAG	
18s	mouse	AGGGTTCGATTCCGGAGAGG	qPCR
		CAACTTTAATATACGCTATTGG	
18s	rat/human	AAACGGCTACCACATCCAAG	qPCR
		CCTCCAATGGATCCTCGTTA	
Anf	rat	CTTCTTCCTCTTCCTGGCCT	qPCR
		TTCATCGGTCTGCTCGCTCA	
Bnp	rat	TCCTTAATCTGTCGCCGCTG	qPCR
		AGGCGCTGTCTTGAGACCTA	
Rcan 1.1	mouse/rat	GACCCGCGCGTGTTC	qPCR
		TGTCATATGTTCTGAAGAGGGAATC	
Rcan 1.4	mouse/rat	CCCGTGAAAAAGCAGAATGC	qPCR
		TCCTTGTCATATGTTCTGAAGAGGG	
Anf	mouse	CTTCTTCCTCGTCTTGGCCT	qPCR
		CTGCTTCCTCAGTCTGCTCA	
Bnp	mouse	CATGGATCTCCTGAAGGTGC	qPCR
		CCTTCAAGAGCTGTCTCTGG	
Edem1	mouse	CTGCAATGAAGGAGAAGGAG	qPCR
		TAGAAGGCGTGTAGGCAGAT	
GalE	mouse	CCATAACGCCATTCCGTGGAG	qPCR
		TCCAGAGGCTTCTGCACTGA	
FKBP11	mouse	GGAATCTGCTGCCATTGGAG	qPCR
		GCCAGGTGAGAAGGAATGAC	
FKBP11	rat	AGGCTGAGGCTGAGGTAGAA	qPCR
		CATGTCCAGAAGGCTCTGCT	
FKBP11	rat	CGCTCCACATACACTACACG	ChIP
flanking UPRE		GAAGCTGGCGAGAGGACAATC	
FKBP11	rat	CCTGACTCCTTGACCTTGAT	ChIP
distal		CAGGCCACGCTAGTATAAGA	
Xbp1 F/F	mouse	ACTTGCACCAACACTTGCCATTTC	Genotyping
		CAAGGTGGTTCACTGCCTGTAATG	

Supplemental Table 2. Clinical characteristics of patients with dilated cardiomyopathy.

Category	Patient study #	Age (years)	Sex	Body weight	Height (m)	BMI	Heart rate (BPM)	LVEDd (cm)	EF%
donor	1	18	M	61	1.7	21.1	83	40	60
donor	2	25	M	65	1.65	23.9	78	41	61
donor	3	38	M	70	1.71	23.9	74	43	59
donor	4	27	M	59	1.57	23.9	67	44	63
donor	5	33	F	69	1.64	25.7	65	42	60
donor	6	35	M	80	1.81	24.4	72	41	64
patient	7	28	M	72	1.75	23.5	80	84	16
patient	8	30	M	70	1.8	21.6	84	73	25
patient	9	42	M	53	1.64	19.7	108	75	17
patient	10	46	M	69	1.7	23.9	86	71	17
patient	11	60	F	67.5	1.58	27	100	63	34
patient	12	58	F	40	1.55	16.6	78	80	21
patient	13	54	F	73	1.6	28.5	95	89	16

Supplemental Figure 1.



Cardiac-specific conditional knockout of XBP1s leads to early mortality in mice.

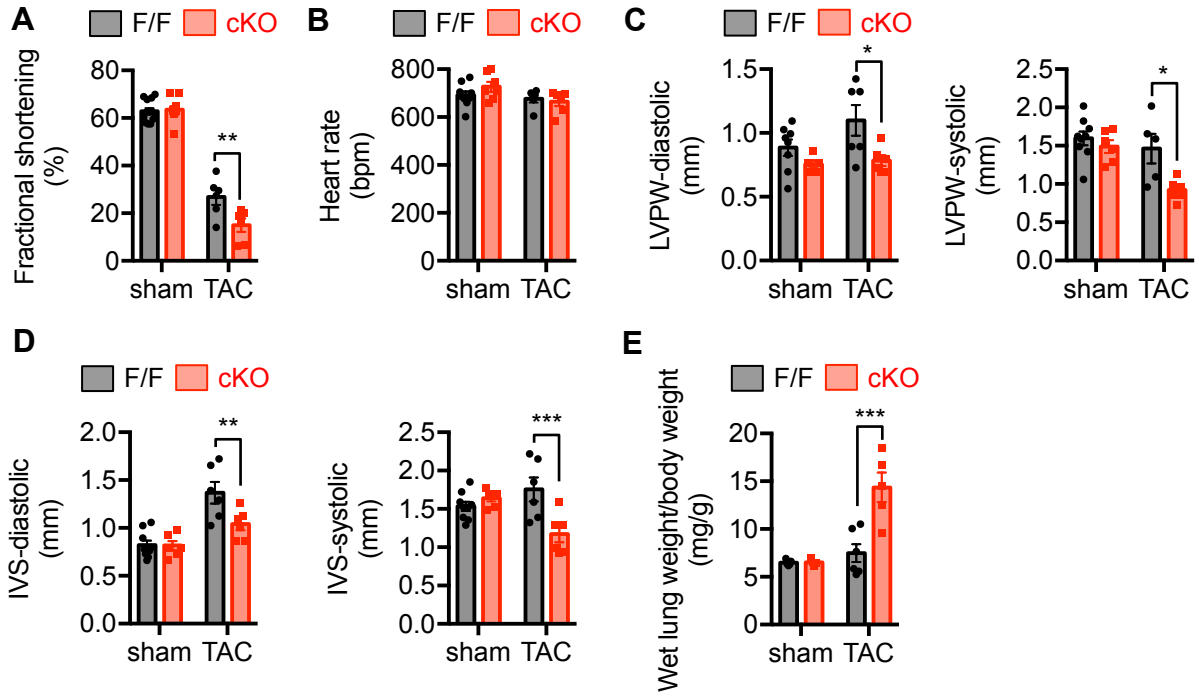
A. IRE1α phosphorylation (Ser724) was reduced in cardiac tissues from heart failure patients. Note that the failing hearts showed elevated expression of ANF. GAPDH was used as a loading control. DCM, dilated cardiomyopathy.

B. Quantification of **A** showed a significant decrease in the *p*-IRE1α/total IRE1α level under heart failure condition. N = 6 for normal hearts; n = 5 for failing hearts. Student's *t* test was performed.

C. Representative short-axis M mode image of echocardiography. Vertical yellow lines depict the calculation of various functional parameters.

D. XBP1s cKO did not lead to changes in heart rate, compared to $XBP1^{F/F}$ and αMHC-Cre control mice. N = 5 for each group. **, $P<0.01$.

Supplemental Figure 2.



XBP1s deficiency exacerbates cardiac dysfunction by pressure overload.

A. XBP1s cKO mice showed a significant decrease in fractional shortening (%) after thoracic aortic constriction (TAC), in comparison to F/F controls. N = 6-13.

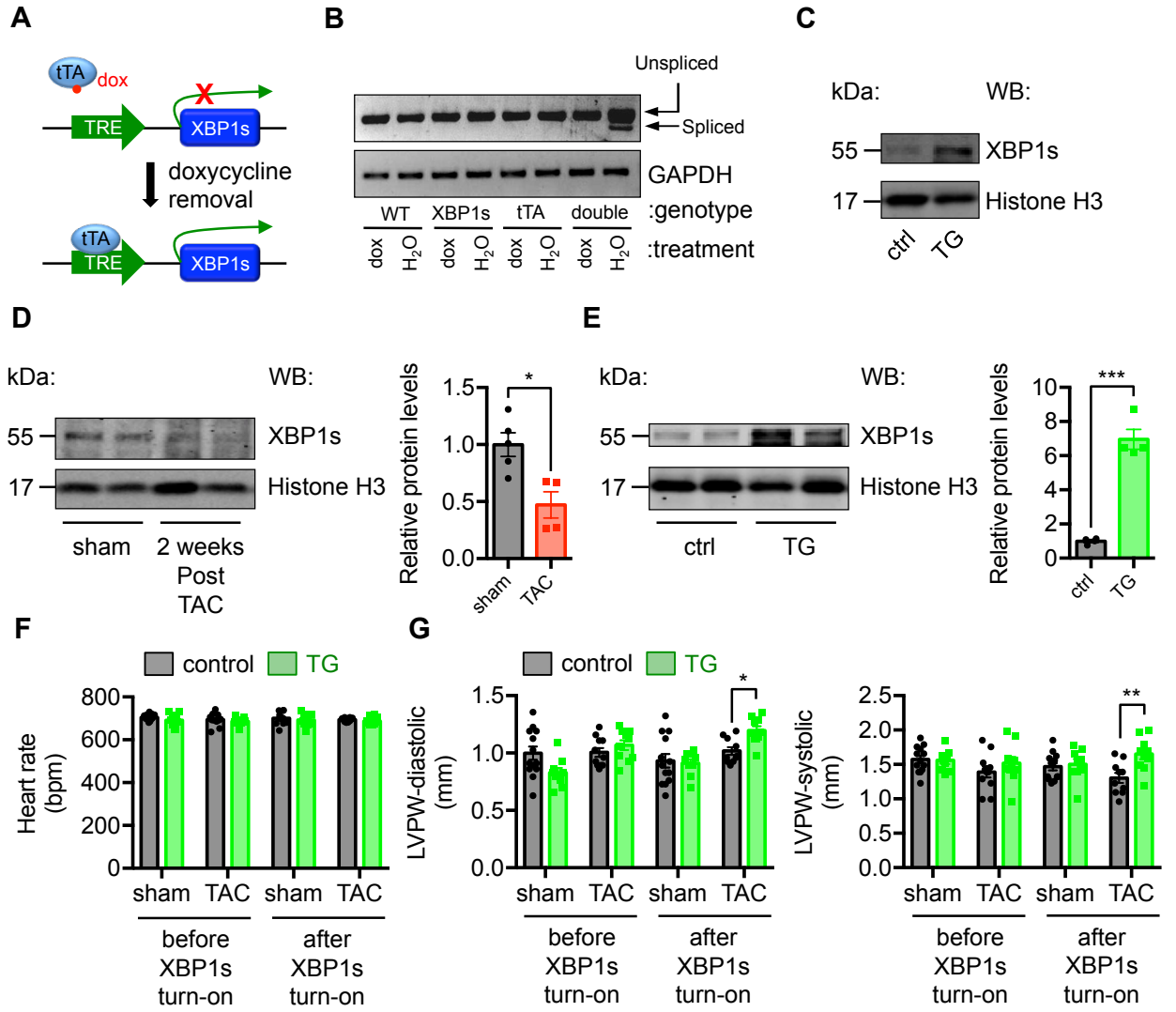
B. Heart rate was not affected. N = 6-9.

C. Left ventricular posterior wall (LVPW) thickness was decreased at both systole and diastole, indicating a defect in cardiac growth in the cKO mice after pressure overload. N = 5-9.

D. Interventricular septum (IVS) thickness was reduced in the cKO mice after TAC. N = 6-9.

E. A significant increase in the ratio of wet lung weight/body weight indicates cardiac dysfunction and heart failure in the cKO mice post TAC. N = 3-6. Two-way ANOVA analysis was performed, followed by Tukey's test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplemental Figure 3.



Cardiac-specific overexpression of XBP1s improves cardiac function in response to pressure overload.

A. Scheme of the inducible transgenic mouse model. In the double transgenic mouse of cardiac-specific tTA (αMHC-tTA) and tetracycline response element-driven XBP1s (TRE-XBP1s), inclusion of doxycycline in drinking water prevents XBP1s expression. Upon removal of doxycycline, tTA is activated, which in turn stimulates XBP1s expression only in cardiac myocytes.

B. Induction of XBP1s was only detected in the double transgenic heart in the absence of doxycycline. Animals were maintained in doxycycline-containing water during breeding, pregnancy, and weaning. XBP1s induction was achieved by switching doxycycline water to regular drinking water for 1 week. The hearts were harvested for RT-PCR analysis. GAPDH was used as an internal control.

C. XBP1s was induced in the double transgenic heart by removal of doxycycline at the protein level. Nuclear fractions were isolated from control and transgenic mice for immunoblotting. Histone H3 was used as a loading control.

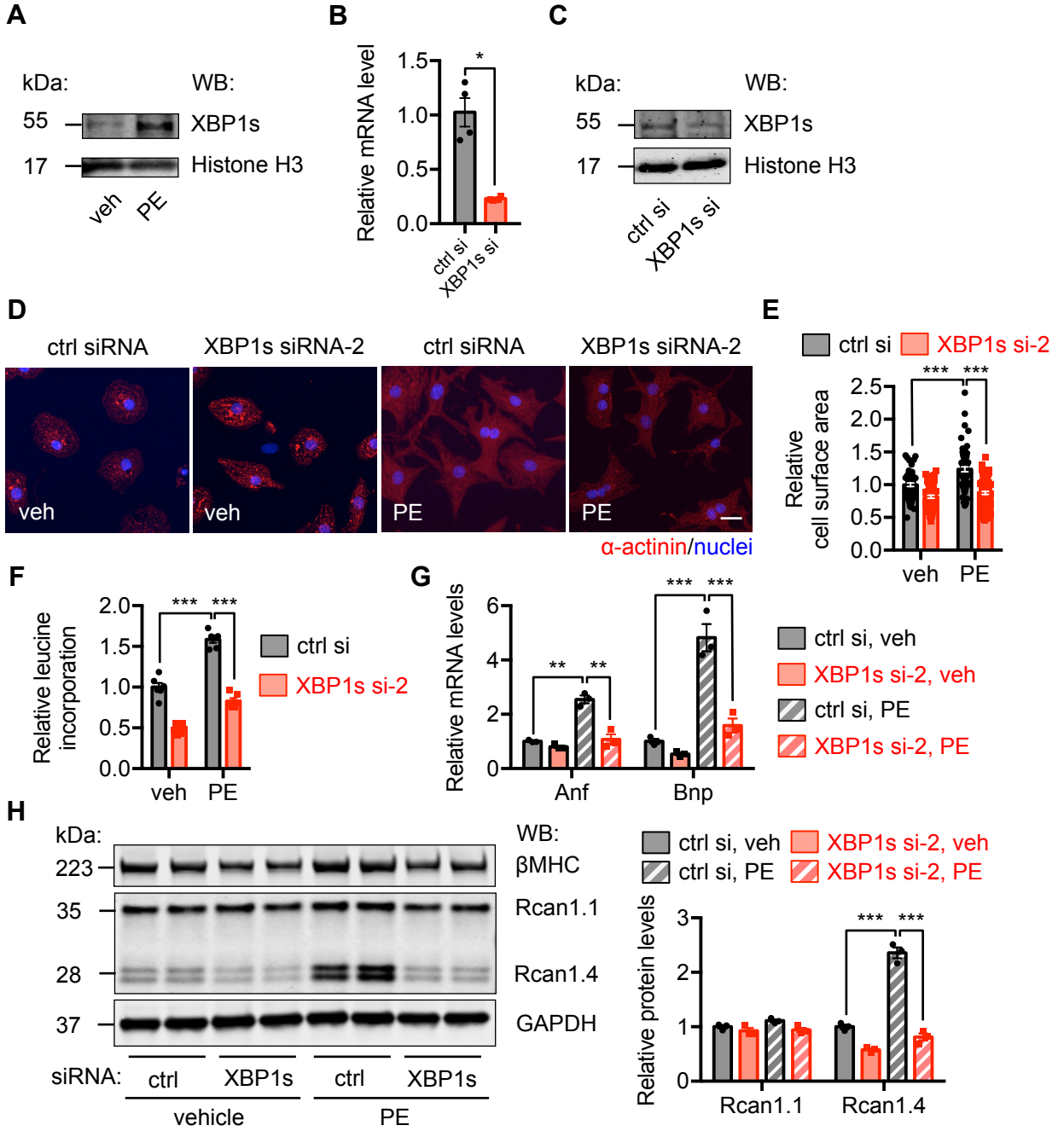
D. Endogenous XBP1s in the heart was decreased by pressure overload after 2 weeks of TAC surgery. Control mice were subjected to TAC and cardiac nuclear extracts were isolated after 2 weeks for Western blotting. Histone H3 was used as a loading control for nuclear fractions. N = 4-5.

E. XBP1s protein level was significantly increased after transgene turn-on in the transgenic (TG) TAC mice, compared to control animals. N = 4 for each group. Student's *t* test was conducted.

F. Heart rate was not altered in either control or transgenic mice. N = 9-13.

G. LVPW was significantly elevated in the TG mice at both systole and diastole, suggesting XBP1s overexpression in the heart stimulates cardiac growth in response to pressure overload. N = 9-13. Two-way ANOVA analysis was performed, followed by Tukey's test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplemental Figure 4.



XBP1s is required for cardiomyocyte growth in response to hypertrophic stimulus.

A. XBP1s protein expression was increased in cardiomyocytes after hypertrophic stimulation. NRVMs were treated with PE (50 μM) for 24 hrs. Nuclear fractions were isolated and used for immunoblotting. Histone H3 was used as a loading control.

B. siRNA knockdown of XBP1s in NRVMs as revealed by quantitative RT-PCR at the mRNA level. N = 4 for each group.

C. XBP1s silencing in NRVMs reduced its expression at the protein level, as shown by immunoblotting. Histone H3 was used as a loading control for the nuclear fractions**.**

D. NRVMs were transfected by an independent siRNA against XBP1s (XBP1s siRNA-2). PE treatment was then conducted for 24 hrs and the cells were used for immunofluorescence staining (α-actinin, red). Scale bar: 20 μm.

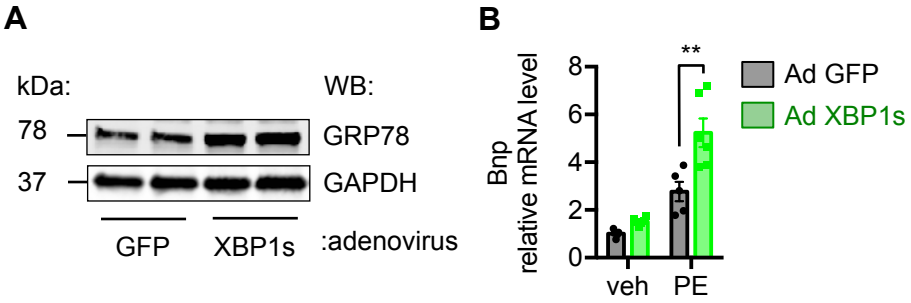
E. Cardiomyocyte surface area quantification of **D** showed that PE induced an increase in cell size, which was significantly diminished by XBP1s silencing. N = 58 for veh/ctrl si; 64 for veh/XBP1s siRNA-2; 64 for PE/ctrl si; 86 for PE/XBP1s siRNA-2.

F. Knockdown of XBP1s with the independent siRNA-2 led to a decrease in protein synthesis, as determined by radioactive leucine incorporation. N = 6 for each group.

G. The induction of hypertrophic gene markers, Anf and Bnp, was decreased by XBP1s silencing. N = 3 per group.

H. Immunoblotting showed that the induction of Rcan1.4 by PE was reduced by XBP1s knockdown using XBP1s siRNA-2. In contrast, Rcan1.1 protein was not altered. GAPDH was used as a loading control. Quantification (relative to GAPDH) is shown at the right. N = 3 for each group. Two-way ANOVA analysis was performed, followed by Tukey's test. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

Supplemental Figure 5.

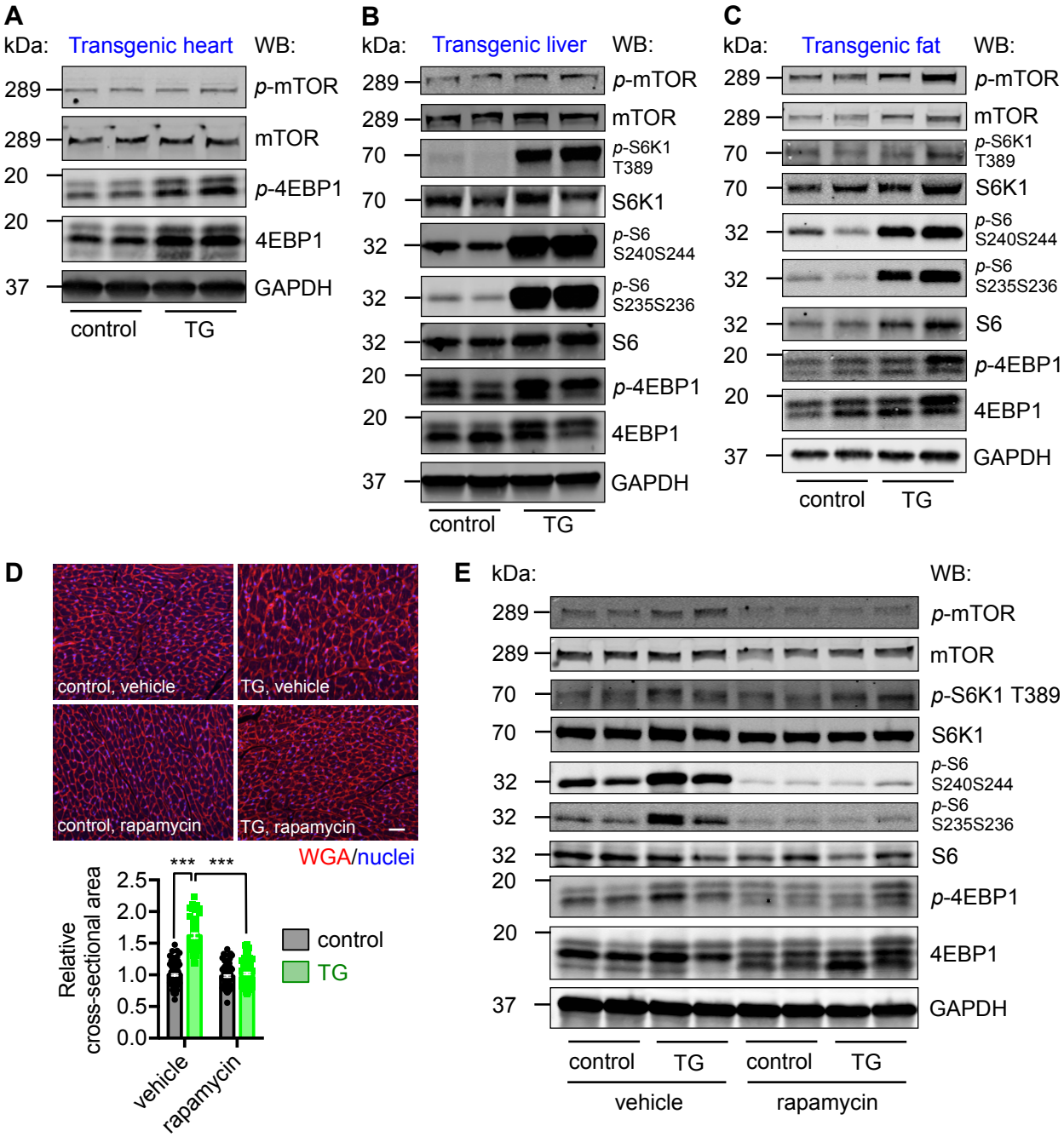


Overexpression of XBP1s in NRVMs by adenovirus infection.

A. Overexpression of XBP1s in NRVMs was achieved by adenovirus infection, and validated by induction of GRP78, a XBP1s downstream target. GAPDH was used as a loading control.

B. Induction of the fetal gene program was significantly augmented by XBP1s expression. Quantitative RT-PCR was conducted to determined the relative mRNA level of Bnp. N = 5 for Ad GRP; 6 for Ad XBP1s. Two-way ANOVA analysis was performed, followed by Tukey's test. **, *P*<0.01.

Supplemental Figure 6.



Activation of the mTOR pathway by XBP1s *in vivo* in various transgenic mouse models.

A. Cardiomyocyte-specific overexpression of XBP1s increased mTOR signaling in the heart. XBP1s expression was turned on for 2 weeks. Cardiac tissues were subjected to Western blotting. GAPDH was used as a loading control.

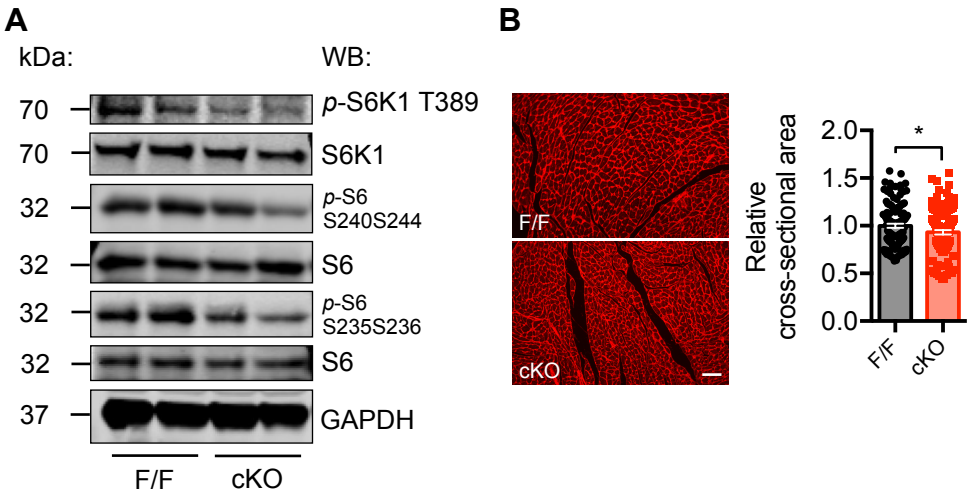
B. XBP1s induction in the liver stimulated mTOR signaling. XBP1s was induced only in hepatocytes in liver-specific XBP1s transgenic mice for 1 week. Liver samples were used for immunoblotting.

C. XBP1s induction in the fat tissue activated mTOR pathway. XBP1s expression was triggered in adipocytes in fat-specific transgenic mice for 4 weeks. Epididymal adipose tissues were harvested for immunoblotting.

D. Rapamycin suppressed XBP1s-induced cardiomyocyte hypertrophic growth. XBP1s overexpression was achieved by removal of doxycycline. Rapamycin administration (2 mg/kg body weight/day) was initiated for 1 week. WGA staining showed significant reduction in cell size by rapamycin treatment. Scale bar: 50 μm. N = 102 for veh/control; 51 for veh/TG; 100 for rapamycin/control; 119 for rapamycin/TG.

E. Rapamycin treatment inhibited XBP1s-induced mTOR activation. Cardiac tissues from control and transgenic mice, with or without rapamycin treatment, were harvested for immunoblotting. GAPDH was used as a loading control. Two-way ANOVA analysis was performed, followed by Tukey's test. ***, $P<0.001$.

Supplemental Figure 7.

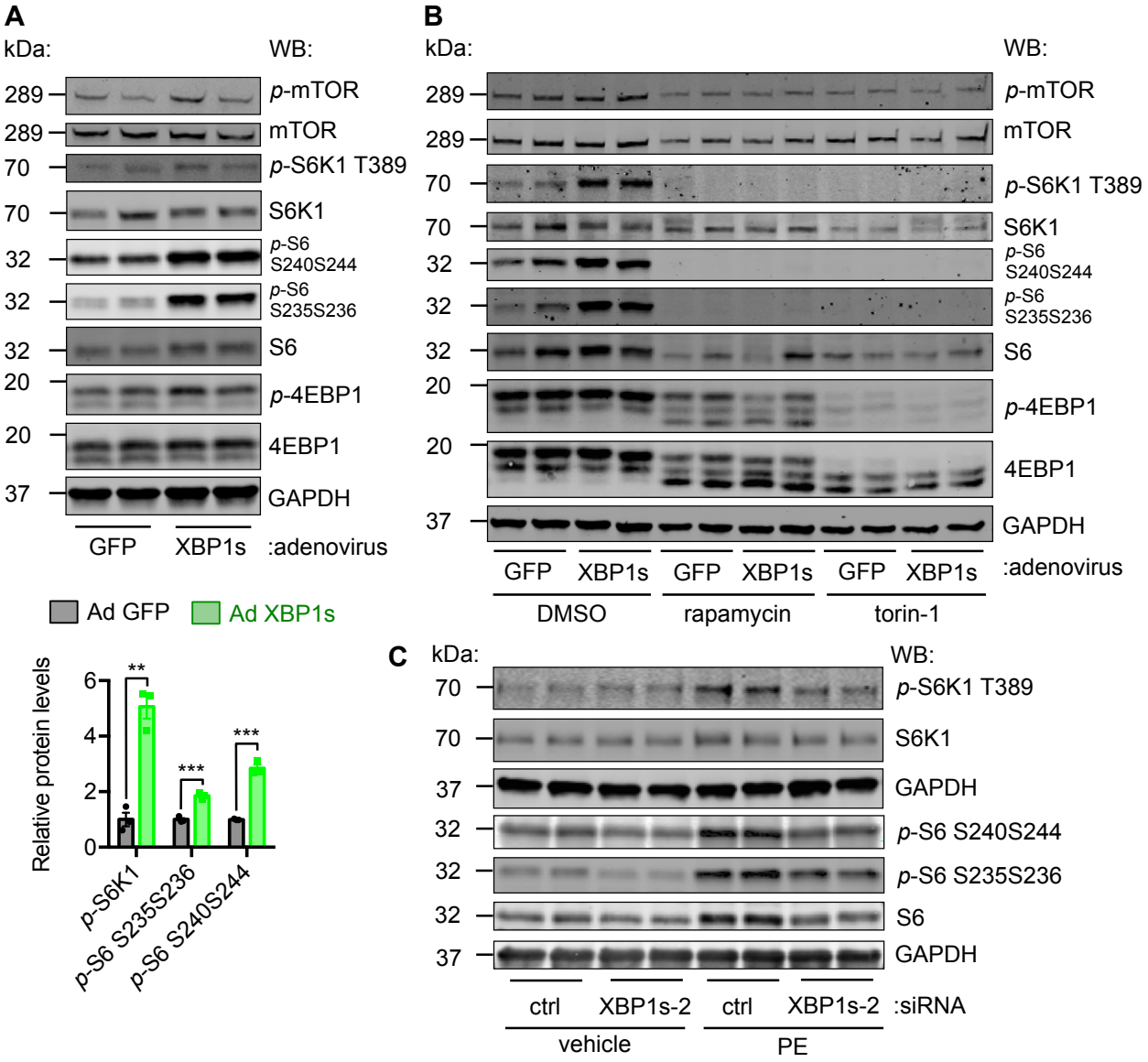


Cardiomyocyte size and mTOR signaling in XBP1s cKO mice.

A. XBP1s cKO mice (5 months) showed a decrease in mTOR signaling, compared to the F/F controls. Adult cardiomyocytes were isolated from age-matched mice and used for Western blotting. GAPDH was used as a loading control.

B. The hearts at baseline were isolated and subjected to WGA staining (left). Quantification at the right showed that deficiency of XBP1s caused a significant decrease in cardiomyocyte size in comparison to F/F controls. Scale bar: 50 μm. N = 120 for F/F; 113 for cKO. Student's *t* test was conducted. *, $P<0.05$.

Supplemental Figure 8.



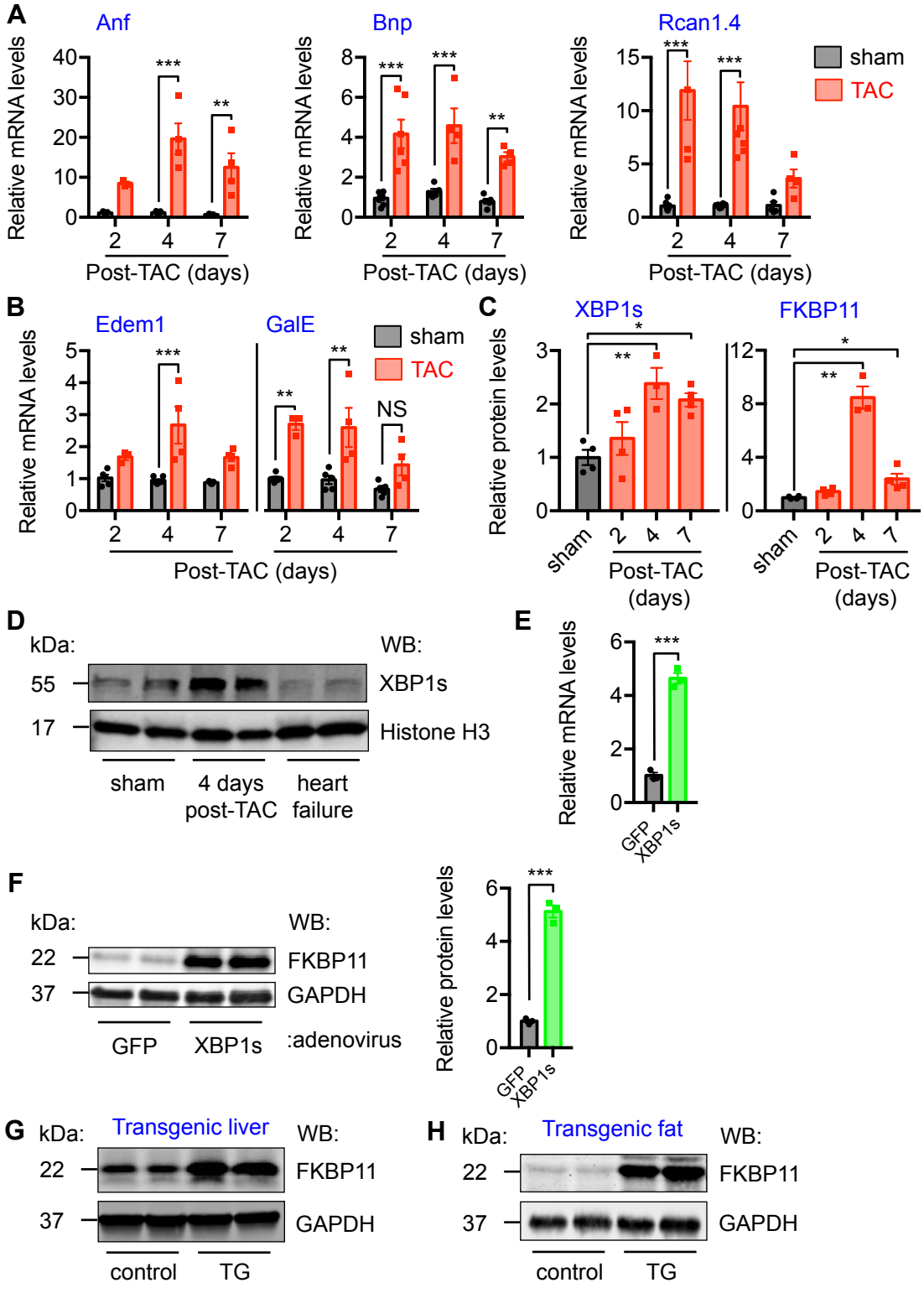
Activation of mTOR by XBP1s overexpression in NRVMs.

A. XBP1s overexpression *in vitro* in NRVMs led to activation of the mTOR signaling. NRVMs were infected by adenovirus expressing GFP or XBP1s. Cardiomyocyte lysates were used for Western blotting. Phosphorylated proteins were normalized to respective total proteins. N = 3 per group.

B. Treatments by mTOR inhibitors suppressed XBP1s-mediated activation of mTOR. NRVMs were infected by adenovirus expressing GFP or XBP1s. Rapamycin or torin-1 was used to treat the cells for 6 hrs.

C. XBP1s silencing by an independent siRNA (XBP1s siRNA-2) led to a decrease in mTOR activation by PE treatment. Student's *t* test was performed. **, $P<0.01$; ***, $P<0.001$.

Supplemental Figure 9.



Pressure overload leads to upregulation of hypertrophic markers and XBP1s targets in the heart

A. Markers of cardiac hypertrophy were elevated in the heart after TAC. N = 5-9.

B. XBP1s target genes were upregulated in TAC hearts. N = 3-6.

C. Quantification showed significant increases of XBP1s and FKBP11 at the protein level in the heart by TAC. XBP1s was normalized to Histone H3 for the nuclear fraction loading and FKBP11 was normalized to GAPDH. N = 3-4.

D. XBP1s protein expression was elevated after 4 days of TAC, which was significantly reduced in the hearts under failing conditions. Histone H3 was used as a loading control for nuclear fractions.

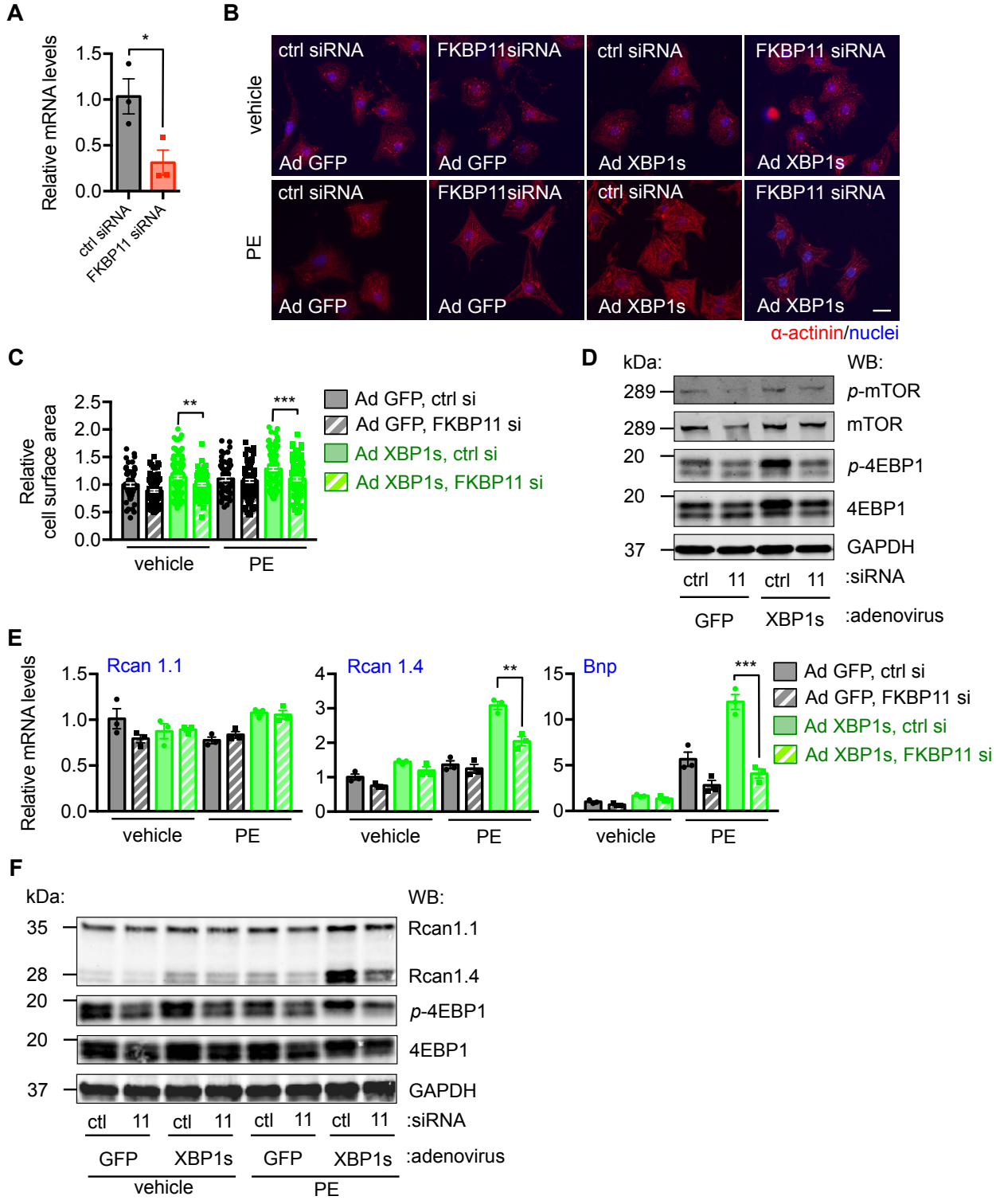
E. XBP1s expression *in vitro* in NRVMs by adenovirus upregulated FKBP11 gene expression at the mRNA level. N = 3 for each group. Student's *t* test was conducted.

F. XBP1s expression *in vitro* in NRVMs led to an increase in the protein level of FKBP11. N = 3 per group. Student's *t* test was conducted.

G. Protein level of FKBP11 was upregulated in the liver-specific XBP1s transgenic mice. XBP1s induction was triggered by feeding with doxycycline-containing diet for 1 week. GAPDH was used as a loading control.

H. Inducible overexpression of XBP1s in adipocytes caused an increase in FKBP11 expression at the protein level. Adipocyte-specific overexpression of XBP1s was achieved by feeding with doxycycline diet for 4 weeks. Epididymal adipose tissues were used for Western blotting. GAPDH was used as a loading control. Two-way ANOVA analysis was performed, followed by Tukey's test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplemental Figure 10.



FKBP11 is required for XBP1s-mediated mTOR activation in NRVMs.

A. FKBP11 expression was reduced by siRNA-mediated knockdown. Quantitative RT-PCR was conducted to assess the knockdown efficiency. N = 3 per group.

B. FKBP11 silencing decreased cardiomyocyte growth. NRVMs were first infected by adenovirus expressing either GFP or XBP1s. siRNA against FKBP11 was used to silence FKBP11. PE treatment was conducted for 24 hrs. The cells were then subjected to immunofluorescence staining. Scale bar: 20 μm.

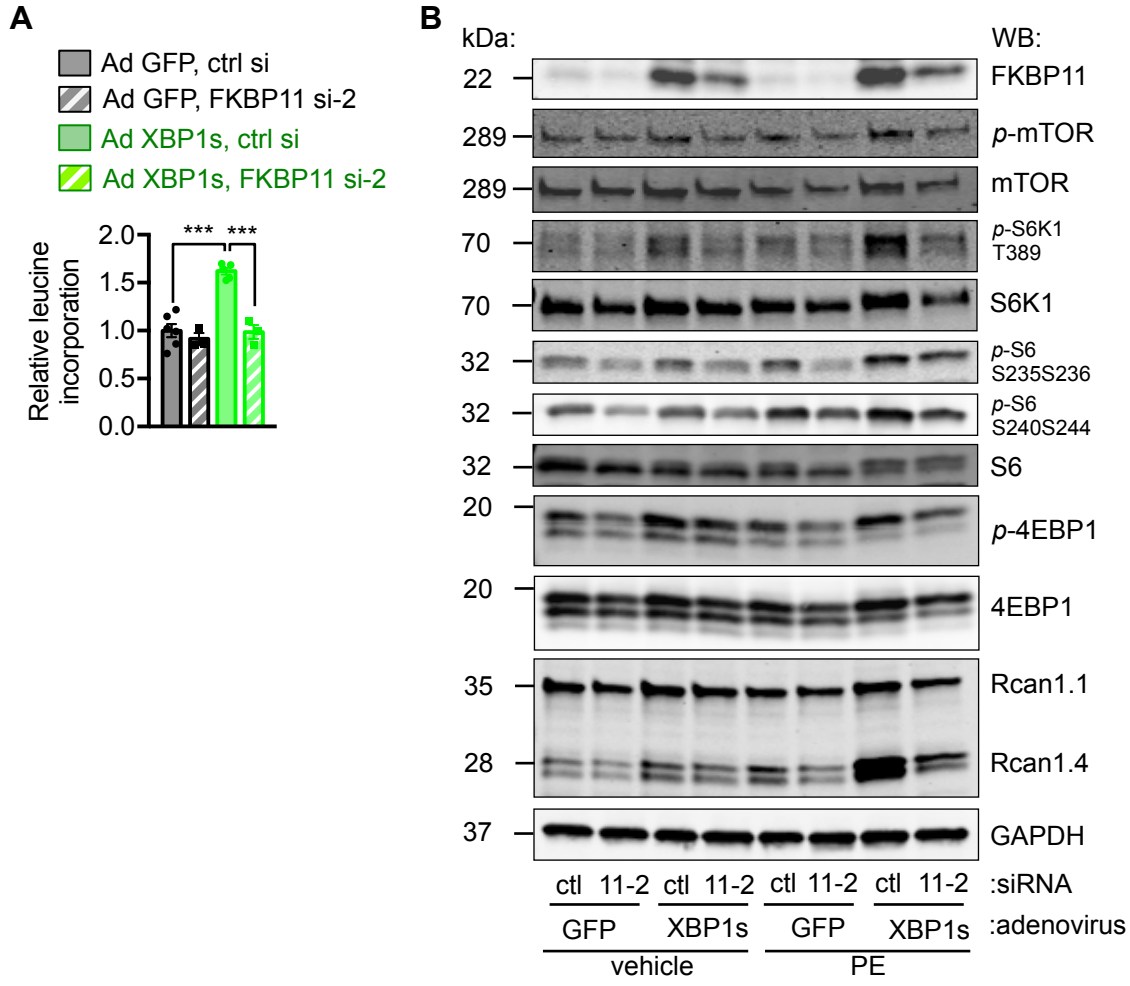
C. Quantification of cardiomyocyte size in **B** showed a significant decrease by FKBP11 knockdown in XBP1s expressing cells. N = 63-128.

D. FKBP11 knockdown led to a decrease in XBP1s-induced mTOR activation. NRVMs were first infected by adenovirus to overexpress GFP or XBP1s. FKBP11 siRNA was then used to transfect the cells, and NRVM lysates were extracted for immunoblotting. GAPDH was used as a loading control.

E. FKBP11 knockdown diminished the induction of the fetal gene program by PE and XBP1s at the mRNA level (Rcan1.4 and Bnp). Rcan1.1, as a control, was not affected. N = 3 per group.

F. Reduction of FKBP11 led to a decrease in PE/XBP1s-induced mTOR activation. NRVMs were first infected by adenovirus expressing GFP or XBP1s. FKBP11 specific siRNA was used to transfect NRVMs. PE treatment was conducted for 24 hrs and immunoblotting was performed to examine the mTOR signaling and Rcan1 expression. Two-way ANOVA analysis was performed, followed by Tukey's test. *, $P<0.05$; **; $P<0.01$; ***, $P<0.001$.

Supplemental Figure 11.

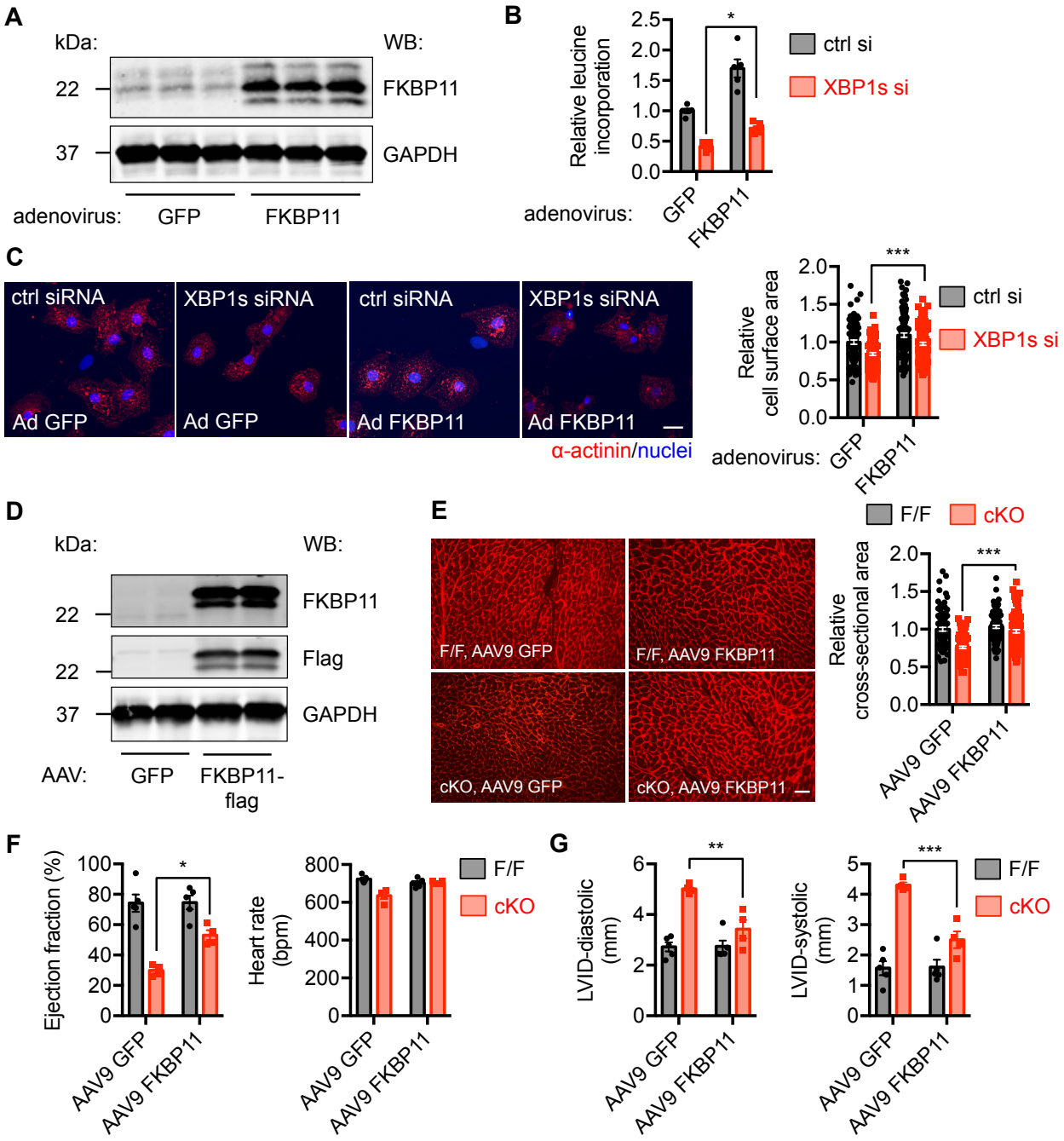


FKBP11 silencing leads to a decrease in mTOR signaling in NRVMs.

A. FKBP11 silencing with an independent siRNA (FKBP11 si-2) led to significant reduction of XBP1s-mediated increase of protein synthesis, as assessed by radioactively leucine incorporation. N = 3-6.

B. FKBP11 knockdown by an independent siRNA against FKBP11 (FKBP11 si-2) in NRVMs reduced XBP1s-induced mTOR activation. Two-way ANOVA analysis was performed, followed by Tukey's test. ***, $P<0.001$.

Supplemental Figure 12.



Overexpression of FKBP11 rescues XBP1s silencing-induced reduction of cell growth and development of cardiomyopathy.

A. FKBP11 expression was upregulated by adenovirus infection in NRVMs at the protein level. Adenovirus expressing GFP was used as a control.

B. Overexpression of FKBP11 rescued the decrease of protein synthesis from XBP1s silencing. NRVMs was transfected by siRNA against XBP1s. Adenovirus

expressing GFP control or FKBP11 was then used to infect the cells. Radioactive leucine incorporation was quantified after 24 hrs. N = 5-6.

C. FKBP11 overexpression in NRVMs rescued the reduction of cardiomyocyte size by XBP1s silencing. Adenovirus expressing GFP or FKBP11 was used to infect NRVMs. Immunofluorescence staining was then conducted and cell size was determined. Scale bar: 20 μm. N = 86 for ctrl si, Ad GFP; 102 for ctrl si, Ad FKBP11; 102 for XBP1s si, Ad GFP; 106 for XBP1s si, Ad FKBP11.

D. FKBP11 overexpression *in vivo* by AAV9 virus infection in mouse hearts. Note that the transgene FKBP11 was flag-tagged, which can be detected by flag antibody or FKBP11 antibody.

E. Overexpression of FKBP11 rescued cardiomyocyte growth in the XBP1s cKO mice after pressure overload. Control F/F and cKO mice were subjected to TAC. AAV9 virus expressing GFP or FKBP11 was then injected. Cardiac tissues were stained by WGA 2 weeks after TAC and cardiomyocyte size was quantified. Scale bar: 50 μm. N = 95 for F/F, AAV9 GFP; 103 for cKO, AAV9 GFP; 90 for F/F, AAV9 FKBP11; 107 for cKO, AAV9 FKBP11.

F. FKBP11 overexpression in mice led to an increase in ejection fraction (%) in the cKO mice compared to the GFP control. No significant changes in heart rate were detected. N = 5 for F/F; n = 4 for cKO.

G. LVID was reduced at both diastole and systole by FKBP11 overexpression in the cKO mice after pressure overload. N = 5 for F/F; n = 4 for cKO. Two-way ANOVA analysis was performed, followed by Tukey's test. *, $P<0.05$; **; $P<0.01$; ***, $P<0.001$.

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