SUPPLEMENTARY MATERIALS

GRP78 Promotes Cardiomyocyte Growth through Activation of GATA4

Guangyu Zhang[1,2], Xiaoding Wang[2], Xukun Bi[2,3], Chao Li[2], Yingfeng Deng[4], Ali A. Al-Hashimi[5], Xiang Luo[2], Thomas G. Gillette[2], Richard C. Austin[5], Yanggan Wang[1,6,*] and Zhao V. Wang[2,*]

[1]Department of Cardiology, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China.

[2]Division of Cardiology, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

[3]Department of Cardiology, Biomedical Research (Therapy) Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China.

[4]Touchstone Diabetes Center, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

[5]Department of Medicine, Division of Nephrology, McMaster University, St. Joseph's Healthcare Hamilton and Hamilton Center for Kidney Research, Hamilton, ON, Canada.

[6]Medical Research Institute of Wuhan University, Wuhan University, Wuhan, Hubei, China.

Correspondence:

*Yanggan Wang, M.D., Ph.D.

Department of Cardiology, Zhongnan Hospital of Wuhan University & Medical Research Institute of Wuhan University, 169 Donghu Road, Wuhan, Hubei, China, 430071.

Tel: 86-27-67813477;

Email: wb000813@whu.edu.cn

*Zhao V. Wang, Ph.D.

Division of Cardiology, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas, USA, 75390-8573.

Tel: 1-214-648-6686;

Fax: 1-214-648-1450;

Email: zhao.wang@utsouthwestern.edu

Supplementary materials and methods

GRP78 transgenic animals

To generate cardiomyocyte-specific GRP78 mouse model, we first created a transgenic mouse with GRP78 under the control of the universal CAG promoter.[1] A transcriptional/translational stop cassette was engineered between the promoter and GRP78. The stop region was spanned by two loxP sites. Transgenic positive lines were crossed with the cardiomyocyte-specific Cre (αMHC-Cre) mouse model. Cre-mediated excision of the stop cassette led to expression of GRP78 only in cardiomyocyte in the double transgenic hearts. Genotyping primers are provided in Table S1.

Thoracic aortic constriction

Banding of thoracic aorta was performed according to standard procedures.[2] Animals of 8-10 weeks old were used when the body weight was between 22 and 25 grams. Mice were anesthetized by one injection of ketamine (100 mg/kg body weight, intraperitoneally) and xylazine (5 mg/kg body weight, intraperitoneally). All instruments and suture materials were sterilized before use. Intubation was conducted orally. The aortic arch was exposed and the suture (5-0 silk) was used to ligate the aorta to desired constriction using a blunted needle as guild. The needle was then removed, the chest was closed, and animals were returned to original cages before analysis.

Echocardiography

Conscious, gently restrained mice were used to monitor *in vivo* cardiac function (Vevo 2100 system, MS400C probe, VisualSonics).[3] M-mode images were captured and analyzed at the level of papillary muscle. Various parameters were determined. Doppler imaging was conducted to assess pressure gradient across the aortic constriction site in a noninvasive manner.

Neonatal rat ventricular myocytes (NRVMs) culture

Ventricles from new born 1-2 day old Sprague-Dawley rats (Charles River) were harvested for cardiac myocyte isolation using a neonatal rat/mouse cardiomyocyte isolation kit (NC-6031, Cellutron). Fibroblasts were maximally separated by pre-plating for 2 hours. Cardiomyocytes were plated in 6-well or 12-well plates at the density of 1,250 cells/mm^2 in the plating medium (DMEM, 5% FBS, 10% horse serum, and 100 μM bromodeoxyuridine). Immunofluorescence staining for α-actinin is conducted for each preparation, and we routinely obtain approximate 90% cardiomyocytes.

To overexpress GRP78, adenovirus for either control GFP or GRP78 was used to infect NRVMs (MOI: 5-10). After 6 hours, culture medium was replenished and phenylephrine (50 μM) treatment was conducted for 24 hours. To silence GRP78 or GATA4, specific siRNA (Sigma) was dissolved in Opti-MEM (Thermo) and stored at -20°C. NRVMs were transfected by siRNA using Lipofectamine RNAiMAX (Thermo) for 24 hours. PE treatment was then initiated for another 24 hours and cells were harvested for further analysis.

Histology

Mouse hearts were harvested and immediately fixed in 4% paraformaldehyde for 48

hours. Paraffin-embedded tissue blocks were used for sectioning (5 μm). Hematoxylin and eosin staining and Masson's Trichrome staining were performed by the Molecular Pathology Core at UT Southwestern. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) was performed as before.[4]

For wheat germ agglutinin (WGA) staining, transverse heart sections were blocked by 5% normal goat serum after deparaffinization and rehydration. After antigen retrieval (Biogenex, HK086-9K), Alexa Fluor 594-conjugated WGA was used (W11262, 10 μg/ml, Thermo) for 1 hour at room temperature. After mounting with ProLong Gold antifade (P36931, Thermo), sections were imaged on a Leica DM2000 compound epi-fluorescence microscope (Leica). Cardiac myocyte size was then quantified by choosing cells with circularity coefficient of more than 0.7.

For immunostaining *in vitro*, NRVMs were cultured in 12-well plates with cover slips. After treatments, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 10 min before incubation with primary antibodies for α-actinin (A7811, Sigma). After 6 washes with 1 X PBS, NRVMs were incubated with goat anti-mouse IgG Alexa Fluor 568 (A11004, Thermo) for 1 hour. The cells were then sealed with ProLong Gold antifade (Thermo) and visualized with a fluorescent microscope (Leica).

Immunoblotting analysis

NRVMs were washed by cold 1 X PBS and lysed in RIPA (radioimmunoprecipitation assay) buffer supplemented with protease and phosphatase inhibitors (A32959, Thermo). After a cycle of freezing/thawing, cells were scraped from plates and the lysates were spun at 4°C (14,000 RPM, 10 min). Supernatant was removed for protein concentration quantification using the BCA assay (23225, Thermo).

Cardiac tissues of 10-20 mg were homogenized using dounce homogenizer in 500 μl RIPA supplemented with protease and phosphate inhibitors, followed by clearance at 4°C and protein concentration determination. Equal amount of total protein was loaded onto a Criterion 26-well gel (Bio-Rad). After transferring to the nitrocellulose membrane, western blotting was done by incubating with different primary antibodies overnight, followed by incubating with secondary antibodies for 1 hour. Immunoblots were visualized with an Odyssey scanner (Li-Cor) and quantified with the ImageStudio software (Li-Cor). The following antibodies were used: GRP78 (610979, BD Biosciences), GAPDH (10R-G109A, Fitzgerald), RCAN1 (D6694, Sigma), mTOR (4517, Cell Signaling), p-mTOR (2971, Cell Signaling), S6K1 (2708, Cell Signaling), p-S6K1 (9206, Cell Signaling), S6 (2317, Cell Signaling), p-S6 S235S236 (4858, Cell Signaling), p-S6 S240S244 (5364, Cell Signaling), 4EBP1 (9644, Cell Signaling), p-4EBP1 (2855, Cell Signaling), Anf (ab180649, Abcam), Bnp (ab19645, Abcam), p-p38 (9216, Cell Signaling), p-38 (9212, Cell Signaling), ERK (9107, Cell Signaling), p-ERK (9101, Cell Signaling), MEF2a (9736, Cell Signaling), GATA4 (SC-1237, Santa Cruz Biotechnology), NFATc1 (MA3-024, Thermo), βMHC (ab50967, Abcam), p-PERK (3179, Cell Signaling), p-IRE1 (nb100-2323, Novus Biologicals), IRE1 (3294, Cell Signaling), XBP1s (sc-7160, Santa Cruz Biotechnology), ATF6 (BAM-73-505-EX, Cosmo), ATF4 (sc-200, Santa Cruz Biotechnology), CHOP (MA1-250, Thermo), p-AKT (S473, 4060, Cell Signaling), p-AKT (T308, 13038, Cell Signaling), AKT (9272,

Cell Signaling), p-NFATc3 (sc-32982, Santa Cruz Biotechnology), NFATc3 (sc-8321, Santa Cruz Biotechnology), goat anti-rabbit secondary antibody 800 CW (925-32211, Li-Cor), goat anti-mouse secondary antibody Alexa Fluor 680 (A21057, Thermo), and donkey anti-goat secondary antibody Alexa Fluor 488 (A11055, Thermo).

Leucine incorporation assay

Radioactive L-[3,4,5-3H]-leucine (NET460A001MC, PerkinElmer) was included in culture medium at a concentration of 2 μCi/mL. PE (50 μM) was added for 24 hours. After treatment, NRVMs were first washed with ice-cold PBS and incubated with trichloroacetic acid (LC262302, 2 mL, LabChem) for 30 min at 4°C with gentle agitation. After two washes with 95% ice-cold ethanol, NaOH (1 mL, 0.5 N) was added and the plates were sealed at 37°C overnight. Equal volume of HCl (0.5 N) was then used to neutralize and all content was transferred to a scintillation vial for radioactivity detection (LS5000TA, Beckman).

RNA isolation and PCR

RNA was isolated from heart tissues and NRVMs with a Total RNA Fatty and Fibrous Tissue kit (Bio-Rad) and a Quick-RNA MicroPrep kit (Zymo Research), respectively. Approximate 250 ng total RNA was used for reverse transcription with the iScript Supermix (Bio-Rad). Quantitative PCR (qPCR) was performed with a LightCycler 480 (Roche). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the 18s RNA as an internal control. Primers are provided in Table S1.

Supplementary references:

1. Bi X, Zhang G, Wang X, Nguyen C, May HI, Li X, Al-Hashimi AA, Austin RC, Gillette TG, Fu G, Wang ZV and Hill JA. Endoplasmic Reticulum Chaperone GRP78 Protects Heart From Ischemia/Reperfusion Injury Through Akt Activation. *Circ Res*. 2018;122:1545-1554.
2. Rothermel BA, Berenji K, Tannous P, Kutschke W, Dey A, Nolan B, Yoo KD, Demetroulis E, Gimbel M, Cabuay B, Karimi M and Hill JA. Differential activation of stress-response signaling in load-induced cardiac hypertrophy and failure. *Physiol Genomics*. 2005;23:18-27.
3. Wang ZV, Deng Y, Gao N, Pedrozo Z, Li DL, Morales CR, Criollo A, Luo X, Tan W, Jiang N, Lehrman MA, Rothermel BA, Lee AH, Lavandero S, Mammen PP, Ferdous A, Gillette TG, Scherer PE and Hill JA. Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway. *Cell*. 2014;156:1179-92.
4. Wang X, Bi X, Zhang G, Deng Y, Luo X, Xu L, Scherer PE, Ferdous A, Fu G, Gillette TG, Lee AS, Jiang X and Wang ZV. Glucose-regulated protein 78 is essential for cardiac myocyte survival. *Cell Death Differ*. 2018. doi: 10.1038/s41418-018-0109-4. Epub 2018 Apr 17.

Gene	Species	Primer	Test
18s	mouse	AGGGTTCGATTCCGGAGAGG	qPCR
		CAACTTTAATATACGCTATTGG	
18s	rat	AAACGGCTACCACATCCAAG	qPCR
		CCTCCAATGGATCCTCGTTA	
Cre		GATTTCGACCAGGTTCGTTC	genotyping
		GCTAACCAGCGTTTTCGTTC	
GRP78	mouse	CCTCTCTGGTGATCAGGATA	qPCR/
		CGTGGAGAAGATCTGAGACT	GRP78-TG
			genotyping
		TGTCATATGTTCTGAAGAGGGAATC	
Anf	mouse	CTTCTTCCTCGTCTTGGCCT	qPCR
		CTGCTTCCTCAGTCTGCTCA	
Bnp	mouse	CATGGATCTCCTGAAGGTGC	qPCR
		CCTTCAAGAGCTGTCTCTGG	
Ctgf	mouse	TGCACTTGCCTGGATGG	qPCR
		GGCAGTTGGCTCGCATCATA	
Caspase 1	mouse	TTGGCACGATTCTCAGCATA	qPCR
		AGATGCCCACTGCTGATAGG	
IL18	mouse	GGCAAGCAAGAAAGTGTCCT	qPCR
		GCTGTGACCCTCTCTGTGAA	
IL1β	mouse	GTTGAAGACAAACCGTTTTTCC	qPCR
		CCAAGCAACGCAAAATACC	
IL6	mouse	GGTCTGTTGGGAGTGGTATC	qPCR
		TCCATCCAGTTGCCTTCTTG	
NLRP3	mouse	TCTTCCTGGACGCTTCTAA	qPCR
		GTGGTGACCCTCTGTGAGGT	
TNFα	mouse	GGCTACAGGCTTGTCACTCG	qPCR
		CCTCCCTCTCATCAGTTCTATGG	
TGFβ1	mouse	GCCCTGTATTCCGTCTCCTTG	qPCR
		CTGCTGACCCCCACTGATAC	
XBP1s	mouse	GGTCTGCTGAGTCCGCAGCAGG	qPCR
		GAAAGGGAGGCTGGTAAGGAAC	
Anf	rat	CTTCTTCCTCTTCCTGGCCT	qPCR
		TTCATCGGTCTGCTCGCTCA	

Table S1. Primers used in this study.

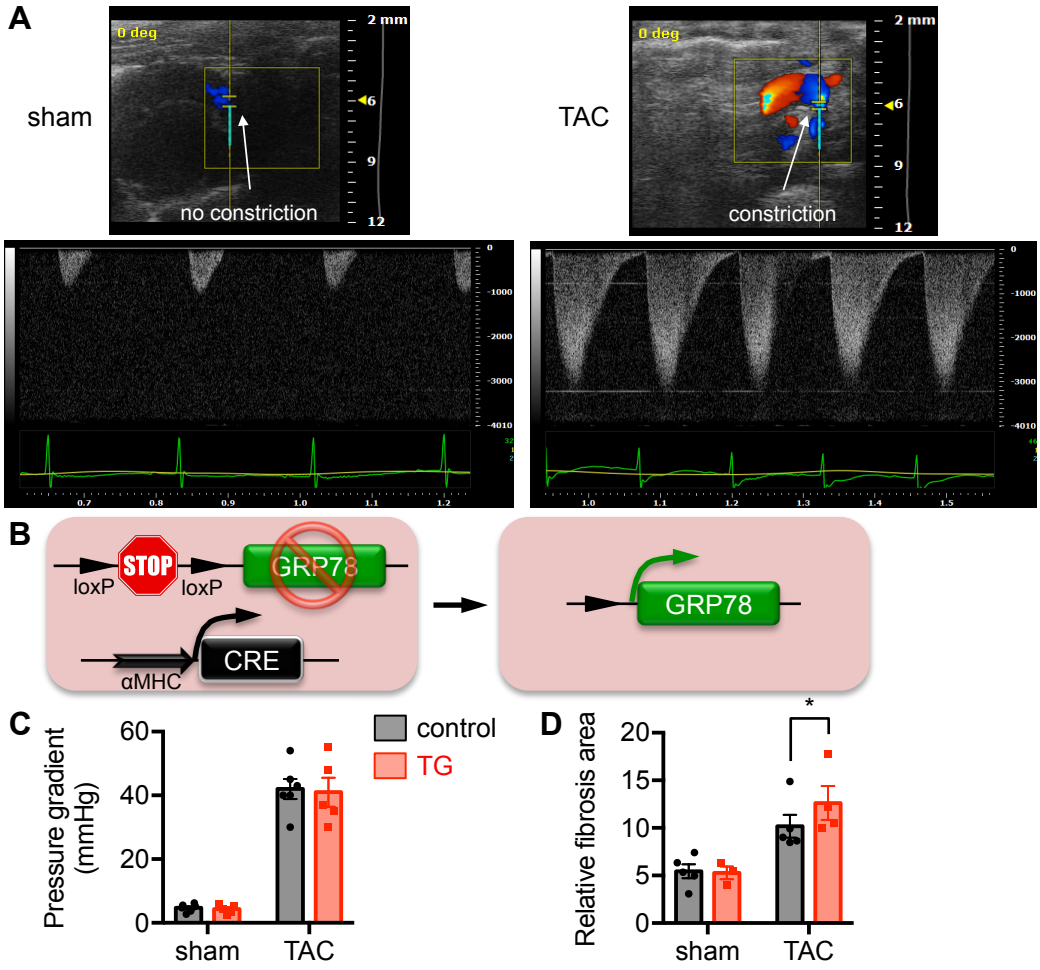

Figure S1. GRP78 overexpression mouse model.

A. Thoracic aortic constriction (TAC) created augmentation of pressure gradient. After sham or TAC surgery for 24 hours, Doppler imaging was performed to assess blood pressure across the aortic constriction site. Peak aortic velocity was imaged as shown. Pressure gradient was calculated by 4 x V_{max}^2 according to the modified Bernoulli equation. **B.** The GRP78 transgenic mouse model. In the transgenic construct, GRP78 was placed under the control of the universal CAG promoter. A loxP flanked transcriptional/translational stop cassette was inserted between GRP78 and CAG promoter. We then crossed the transgenic mice to the cardiomyocyte-restricted αMHC-Cre mouse model. In the presence of Cre in cardiac myocytes, Cre-mediated excision of the stop region led to GRP78 expression. In other tissues or single transgenic mice, GRP78 was not induced. **C.** TAC surgery was similarly performed in control and transgenic (TG) mice. No difference in pressure gradient was detected. Note the average pressure gradient in sham mice was 4 mmHg, while TAC mice had pressure gradient of 40 mmHg. N = 5-6. **D.** Fibrotic response was augmented in GRP78 transgenic mice after TAC. Relative fibrosis area was calculated and compared between groups. N = 3-5. *, $p < 0.05$.

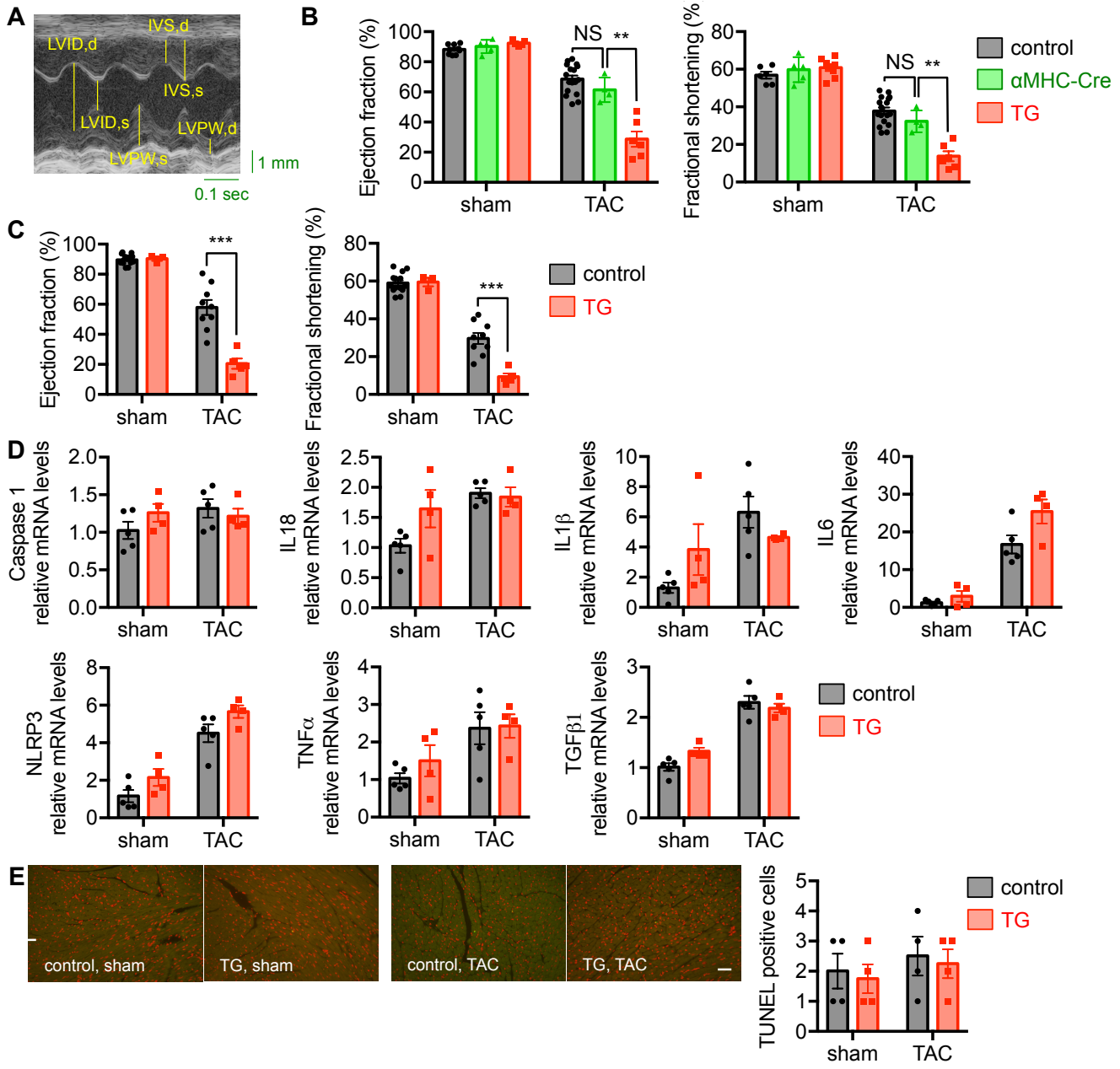

Figure S2. GRP78 overexpression leads to more profound response by pressure overload.

A. Representative echocardiography M-mode image. Calculation of various parameters was shown. **B.** The transgenics αMHC-Cre only did not cause exacerbated response to pressure overload. There is no significant difference between control (all single transgenic mice and wild type animals) and αMHC-Cre only mice at functional levels. In contrast, GRP78 overexpression in TG mice led to significant decline in both ejection fraction and fractional shortening. N = 4-17. **C.** Cardiac function showed further deterioration after 3 weeks of TAC. N = 3-14. **D.** GRP78 overexpression in the heart did not affect inflammatory response after pressure overload. Expression of various genes from the inflammatory pathway was determined by qRT-PCR. N = 4-5. **E.** Overexpression of GRP78 in the heart did not cause elevation in apoptotic cell death, as

revealed by TUNEL staining. Red indicates nuclei and green signal is for TUNEL positive cells. Scale bar, 50 μm. N = 4. **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

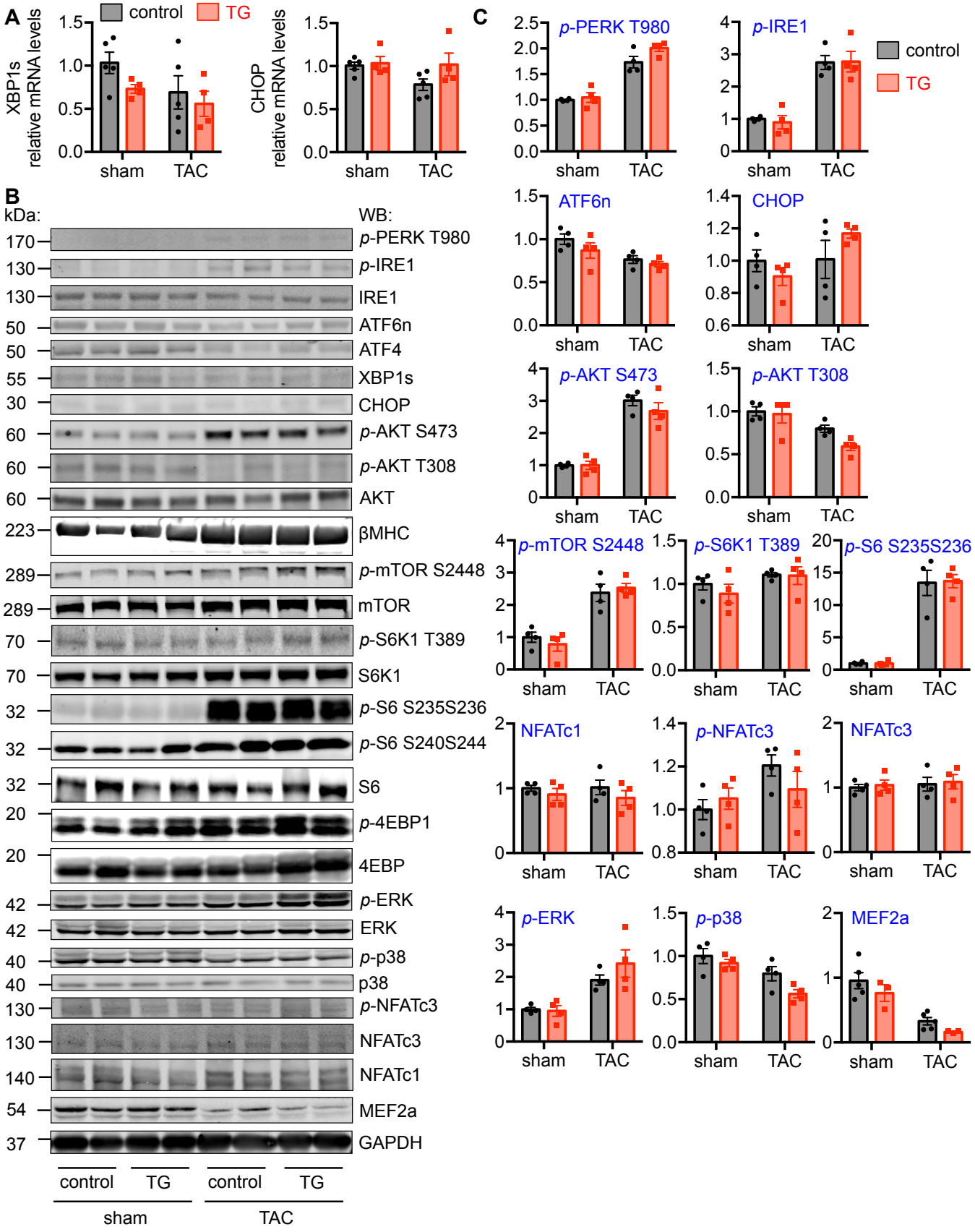
A
control
TG
XBP1s relative mRNA levels
CHOP relative mRNA levels
sham
TAC
B
kDa:
WB:
170 p-PERK T980
130 p-IRE1
130 IRE1
50 ATF6n
50 ATF4
55 XBP1s
30 CHOP
60 p-AKT S473
60 p-AKT T308
60 AKT
223 βMHC
289 p-mTOR S2448
289 mTOR
70 p-S6K1 T389
70 S6K1
32 p-S6 S235S236
32 p-S6 S240S244
32 S6
20 p-4EBP1
20 4EBP
42 p-ERK
42 ERK
40 p-p38
40 p38
130 p-NFATc3
130 NFATc3
140 NFATc1
54 MEF2a
37 GAPDH
control
TG
control
TG
sham
TAC
C
p-PERK T980
p-IRE1
control
TG
ATF6n
CHOP
p-AKT S473
p-AKT T308
p-mTOR S2448
p-S6K1 T389
p-S6 S235S236
NFATc1
p-NFATc3
NFATc3
p-ERK
p-p38
MEF2a
sham
TAC

Figure S3. GRP78 overexpression does not affect mTOR signaling *in vivo*.

A. Control and GRP78 transgenic (TG) mice were used for sham or TAC surgery. Gene expression of XBP1s and CHOP was not affected by GRP78 overexpression at mRNA levels *in vivo*. N = 4-5. **B.** After a week of TAC, cardiac tissues were subjected to immunoblotting for the pathways of mTOR, ERK, p38, etc. Representative immunoblots were shown with GAPDH as loading control. **C.** Quantification of B showed no significant changes between control and TG mice in response to pressure overload. N = 4.

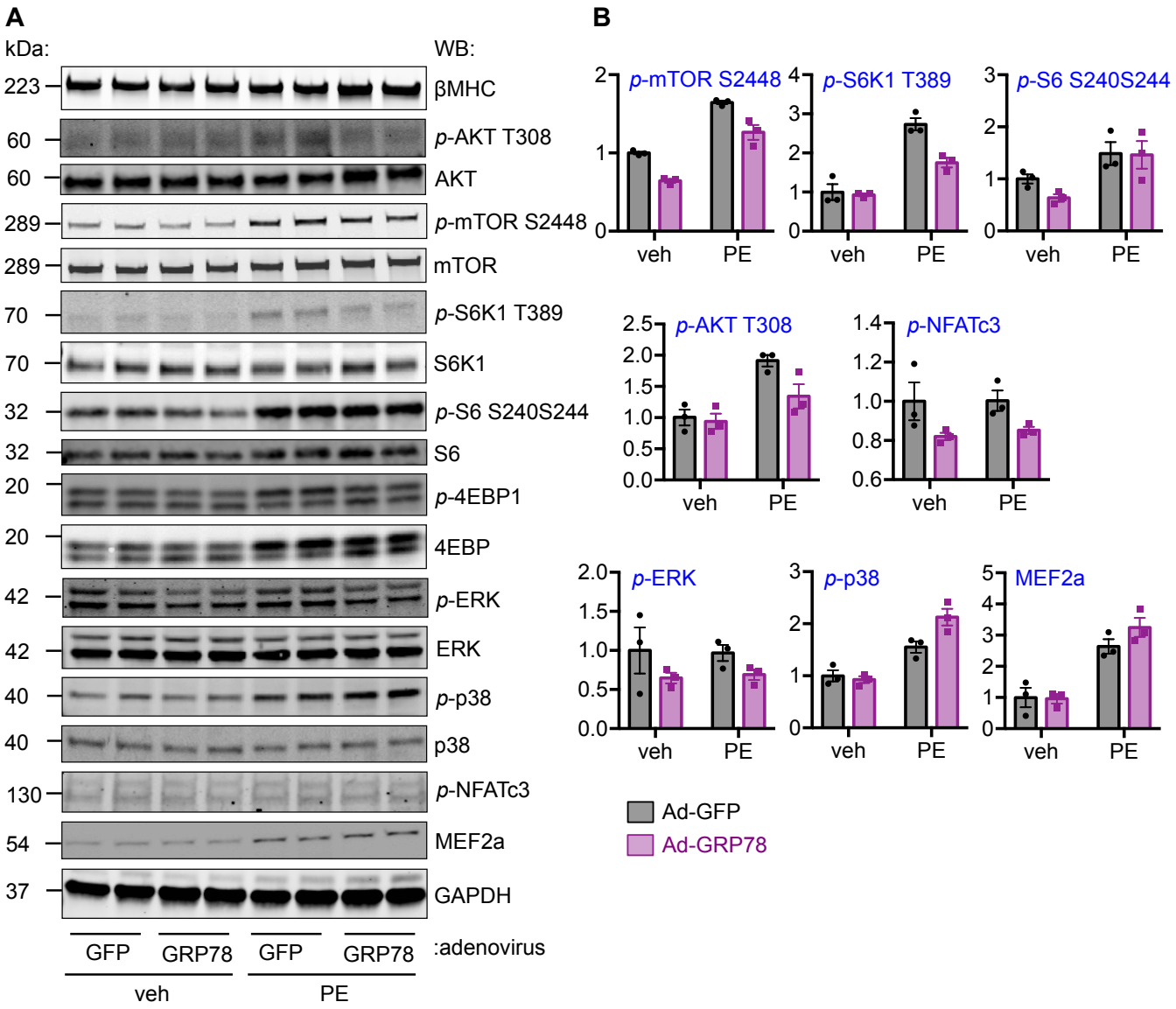

Figure S4. GRP78 overexpression does not affect mTOR signaling *in vitro*.

A. NRVMs were infected by adenovirus expressing either GFP or GRP78. PE treatment was then conducted for 24 hours. Protein lysates were extracted for western blotting to detect the mTOR signaling, ERK, p38, NFAT, and MEF2a. GAPDH was used as a loading control. **B.** Quantification of A showed no significant changes between GFP and GRP78 overexpressing NRVMs post PE treatment. N = 3.

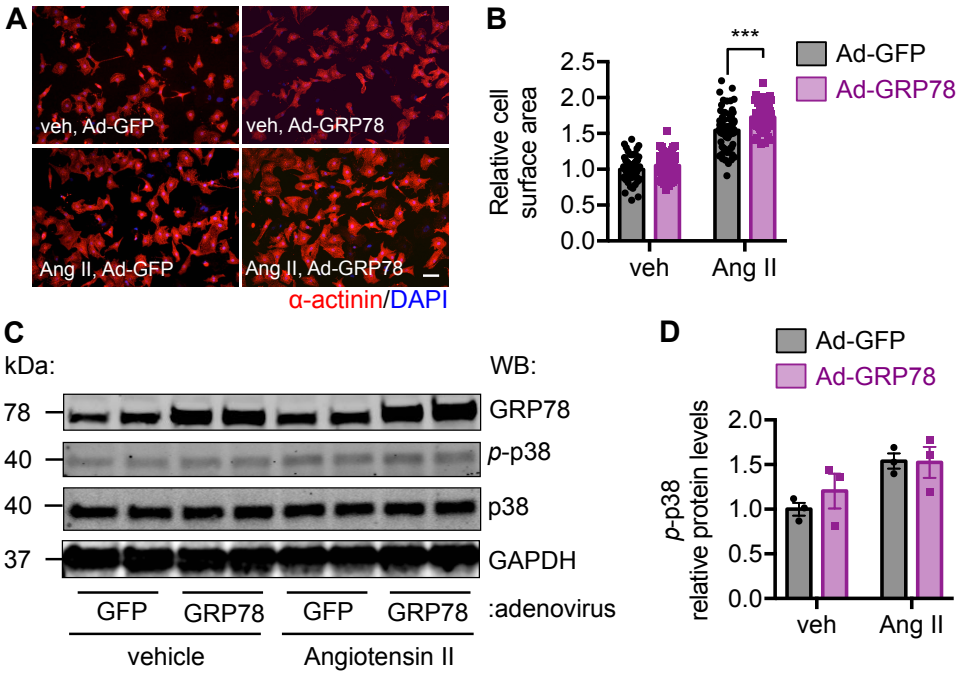

Figure S5. GRP78 overexpression increases cardiomyocyte hypertrophic growth in response to angiotensin II.

A. NRVMs were infected by adenovirus expressing either GFP or GRP78. Angiotensin II treatment (1 μM) was then conducted for 24 hours. Cardiac cells were harvested for immunofluorescence staining for α–actinin. **B.** Quantification of A showed GRP78 overexpression led to more profound hypertrophic growth. N = 62-66. **C.** Protein lysates were extracted for western blotting to detect phosphorylation of p38. GAPDH was used as a loading control. **D.** Quantification of C showed no significant changes of *p*-p38 between GFP and GRP78 overexpressing NRVMs post angiotensin II treatment. N = 3. ***, $p < 0.001$.

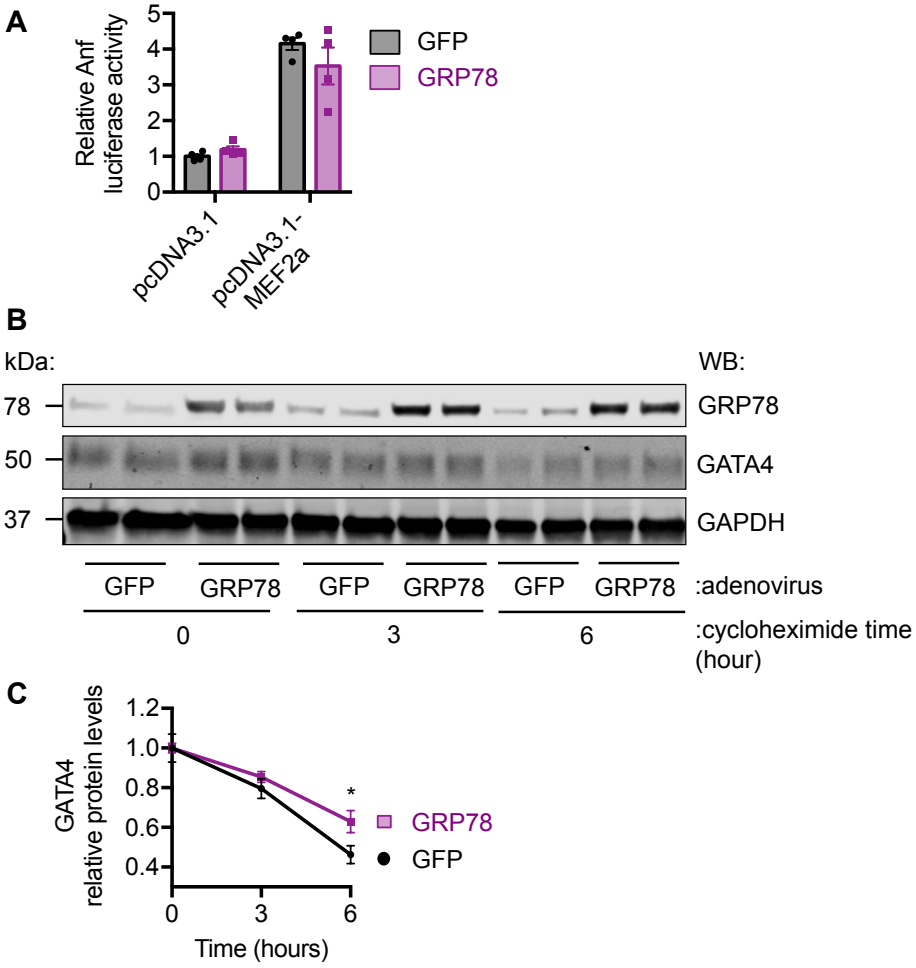

Figure S6. GRP78 overexpression increases GATA4 protein stability.

A. GRP78 overexpression did not affect MEF2a-mediated stimulation of Anf-luciferase activity. HEK293T cells were transfected by plasmid expressing MEF2a along with Anf-luciferase construct. MEF2a increased luciferase activity by 4-fold that was not affected by co-expression of GRP78. N = 4. **B.** NRVMs were infected by adenovirus expressing either GFP or GRP78. Cycloheximide was then added to inhibit protein synthesis. The cells were harvested at indicated time points for western blotting analysis. GAPDH was used as a control. **C.** Quantification showed GRP78 overexpression significantly enhanced GATA4 protein stability. N = 3. *, $p < 0.05$.