SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents

Mouse TNFα was from R&D Systems. 5z-7-oxozeaenol, necrostatin-1, MG-132, lactacystin, PYR-41, and pruomycin dihydrochloride were from Sigma. zVad-FMK was from Abcam. Propidium iodide and Hoechst 33342 were from Invitrogen.

Animal models

The generation of *Traf2* loxP-targeted (floxed) mice, in which exon 3 was flanked by loxP, was described previously.[1] *Traf2fl/fl* mice were crossed with αMHC-Cre[2] or βMHC-Cre[3] to generate cardiac-specific *Traf2*-deficient mice. *Tnfrsf1a-/-* mice obtained from the Jackson Laboratory were crossed with *Traf2fl/fl* αMHC-Cre mice. *Ripk3-/-* mice were provided by Dr. Vishva M. Dixit from Genentech, Inc. and were crossed with *Traf2fl/fl* αMHC-Cre mice. All experiments involving animals were approved by the Institutional Animal Care and Use Committees of the University of Washington and all studies were carried out in accordance with the approved guidelines.

Echocardiography, TAC, and MI

For echocardiography, mice were anesthetized with 2% isoflurane by inhalation and scanning was performed with a VisualSonics Vevo 2100 imaging system as described previously.[4] M-mode ventricular dimensions were averaged from 3-5 cycles. Fractional shortening (FS) was calculated using ventricular dimensions in end of systole and diastole (LVES and LVED, respectively): FS = [(LVED - LVES)/LVED] x 100 (%). Transverse aortic constriction (TAC) was performed to produce cardiac pressure overload in mice using a 27-gauge needle as previously described.[5] Sham-operated mice underwent the same procedure without aortic constriction. Pressure gradients (PG; mm Hg) across the

aortic constriction were calculated from the peak blood velocity (*Vmax*) (m/s) (PG = 4 x $Vmax^2$) measured by Doppler, which was equivalent in all groups of TAC stimulated mice. The surgical procedure for myocardial infarction (MI) in the mouse with permanent ligation of the left anterior descending artery has been described previously.[6] Mice were sacrificed by CO_2 asphyxiation.

Histological analysis

Mouse hearts were fixed in 10% formalin/phosphate-buffered saline and dehydrated for paraffin embedding. Fibrosis was detected with Masson's Trichrome staining on paraffin sections. Blue collagen staining was quantified using MetaMorph 6.1 software as described previously.[4] Assessment of TUNEL from paraffin sections was performed with an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer's instructions or a TMR Red In Situ Death Detection Kit (Roche Diagnostics, Indianapolis, IN) as described in detail previously.[4,6]

Measurement of plasma HMGB1, TNFα, and cTnI

Mouse HMGB1 plasma levels were measured using an enzyme-linked immunosorbent assay kit from Chondrex, Inc. (Redmond, WA) according to the manufacturer's instructions.[4] Mouse TNFα plasma levels were measured using a TNF-alpha Quantikine ELISA kit from R&D Systems (Minneapolis, MN). Mouse cTnI plasma levels were measured using an ELISA kit from Life Diagnostics, Inc (West Chester, PA). Absorbance at 450 nm (sample) and 630 nm (reference) were measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Cell culture

Primary neonatal rat cardiomyocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rat pups as previously described.[4,5] After separation from fibroblasts, enriched cardiomyocytes were plated on 1% gelatin-coated 12-well plates for luciferase assays or on 6-cm-diameter dishes for all other

experiments. Cells were grown in M199 medium containing 100 U/ml of penicillin-streptomycin and 2 mM L-glutamine without serum for 24 h before adenoviral infection. *Traf2*+/+ and *Traf2*-/- MEFs were from Tak Mak (University Health Network, Canada). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Adenoviral and lentiviral vectors

Adβgal and AdTAK1-ΔN have been described previously.[7] Adenoviral vectors expressing RIP3 shRNA, Traf2 shRNA, or a scramble shRNA were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's protocol. Ad-shCYLD was provided by Taixing Cui, University of South Carolina. Adenoviruses encoding mouse Traf2 were generated using the ViraPower Adenoviral Expression System (Invitrogen). Traf2ΔRING adenovirus was provided by Christian Jobin, University of North Carolina. Ad-IκBαM was purchased from Vector Biolabs. Adenoviral infections were performed as described previously at a multiplicity of infection of 10 to 50 plaque forming units per ml. Lentiviral particles encoding shRNA sequences for specific target genes were obtained from Sigma.

Cell death analysis

Cell death was measured using a Cell Meter Apoptotic and Necrotic Detection kit (ATT Bioquest, Sunnyvale, CA) as previously described.[4] Briefly, cells were incubated at 37° C for 30 min with Apopxin Green for detection of phosphatidylserine on cell surface, propidium iodide (PI) or 7-ADD for labeling the nucleus of cells with membrane rupture, and CytoCalcein for labeling live cell cytoplasm. Cell death was then analyzed with an EVOS FL digital fluorescence microscope (AMG) or a FACSCalibur flow cytometer (Becton Dickson). Cells with chromatin condensation were visualized by Hoechst 33342 (Invitrogen) staining. Cell viability was also assessed using the Muse Count & Viability assay kit

(Millipore). Briefly, cells were trypsinized, washed, and incubated with the Muse Count & Viability reagent, and cell viability was quantified on a Muse cell analyzer (Millipore).

Western blot analysis

Protein extraction from mouse heart or cultured cells and subsequent Western blotting followed by enhanced chemiluminescence detection were performed as previously described.[4,7] In some experiments, cell culture supernatants were also collected for the detection of HMGB1. The following antibodies were used: Anti-TAK1 (4505), anti-phospho-TAK1 (Thr187; 4536), anti-α tubulin (3873), anti-HMGB1 (3935), anti-PARP (9532), anti-caspase 3 (9662), anti-TRADD (3694), anti-RIP1 (3493), and anti-RIP3 (15828) were from Cell Signaling Biotechnology (Beverly, MA); Anti-RIP3 (sc-135171), anti-Traf2 (sc-876), anti-CYLD (sc-74435), anti-cIAP2 (sc-7944), anti-IκBα (sc-847), anti-IκBα (Ser32; 2859), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); Ant-FADD (ADI-AAM-212-E) and anti-FLIP (XA-1008) were from Enzo; Anti-TNFR1 (AF-425-PB) was from R&D Systems.

Immunoprecipitation

Immunoprecipitation was performed as previously described.[4,7] Cells were lysed at 4°C in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 0.5% NP-40) containing protease inhibitor cocktail (Roche). Whole cell lysates were cleared by centrifugation at 18,000 x *g* for 10 min and then incubated with the indicated antibodies and protein A/G-PLUS agarose beads (Santa Cruz Biotechnologies) overnight at 4°C. The beads were washed extensively with wash buffer (0.3% NP-40 in PBS), and the proteins were resolved on an 8-12% SDS-PAGE for subsequent Western blotting.

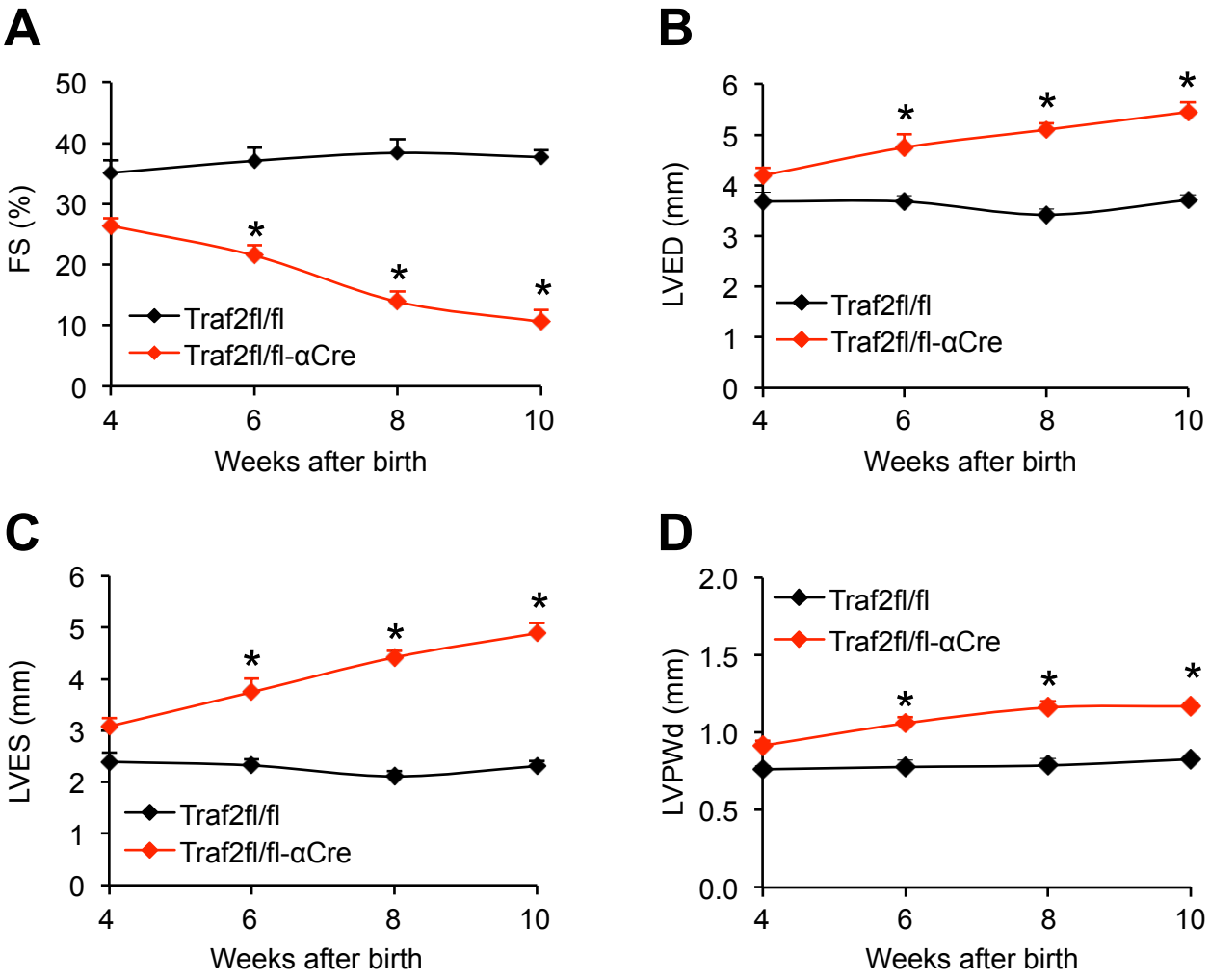
Caspase 8 activity assay

Caspase 8 activity assay was performed using the Caspase-Glo 8 Assay kit from Promega following the manufacturer's instructions. Briefly, 100 μl of Caspase-Glo 8 reagent was added to the cell culture medium in a 96-well plate. Contents of wells were gently mixed using a plate shaker at 500 rpm for 30 seconds. Luminescence was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek).

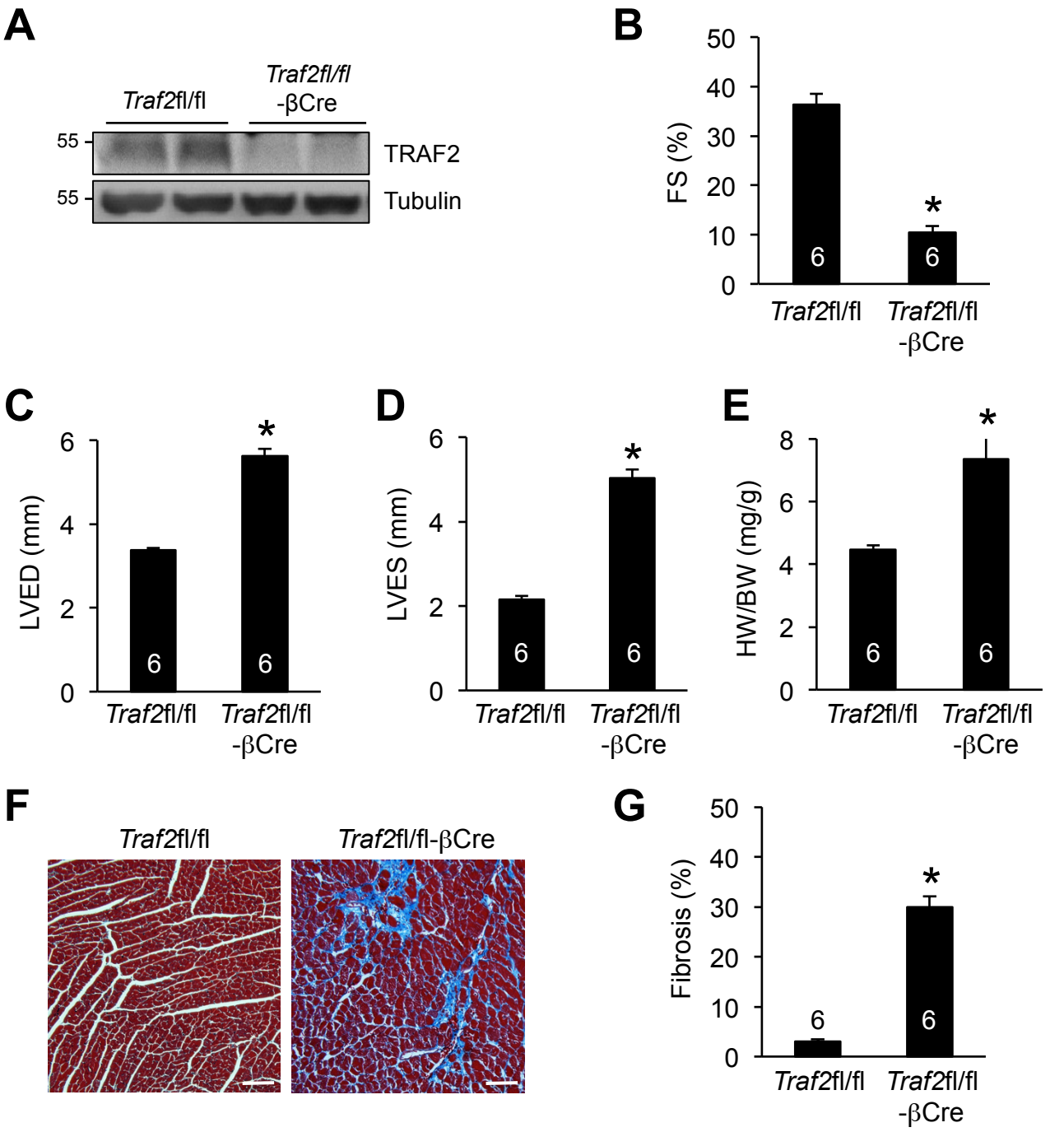
Statistics

Sample size was estimated before performing the study based on our previous experience with similar studies or by conducting pilot experiments to estimate effect size (for echocardiographic and HW/BW data, estimated effect size (d) ≥ 2, SD: 0.2-1.5; for TUNEL, fibrosis, and TNF/HMGB1 measurements, estimated effect size ≥ 2.5, SD ≤ 2; for cell death data, estimated effect size ≥3, SD around 2), followed by power analysis ($\alpha = 0.05$; power = 80%). Results are presented as mean ± s.e.m. Mann-Whitney U-test or Kruskal-Wallis test followed by *post-hoc* Mann-Whitney U-test with Bonferroni's correction was used for studies with small sample sizes. Some data were analyzed by Friedman test followed by pairwise comparisons with *post hoc* Wilcoxon signed-rank test. Data groups with normal distribution were evaluated by one-way ANOVA with the Bonferroni's *post hoc* test. $P < 0.05$ was considered statistically significant.

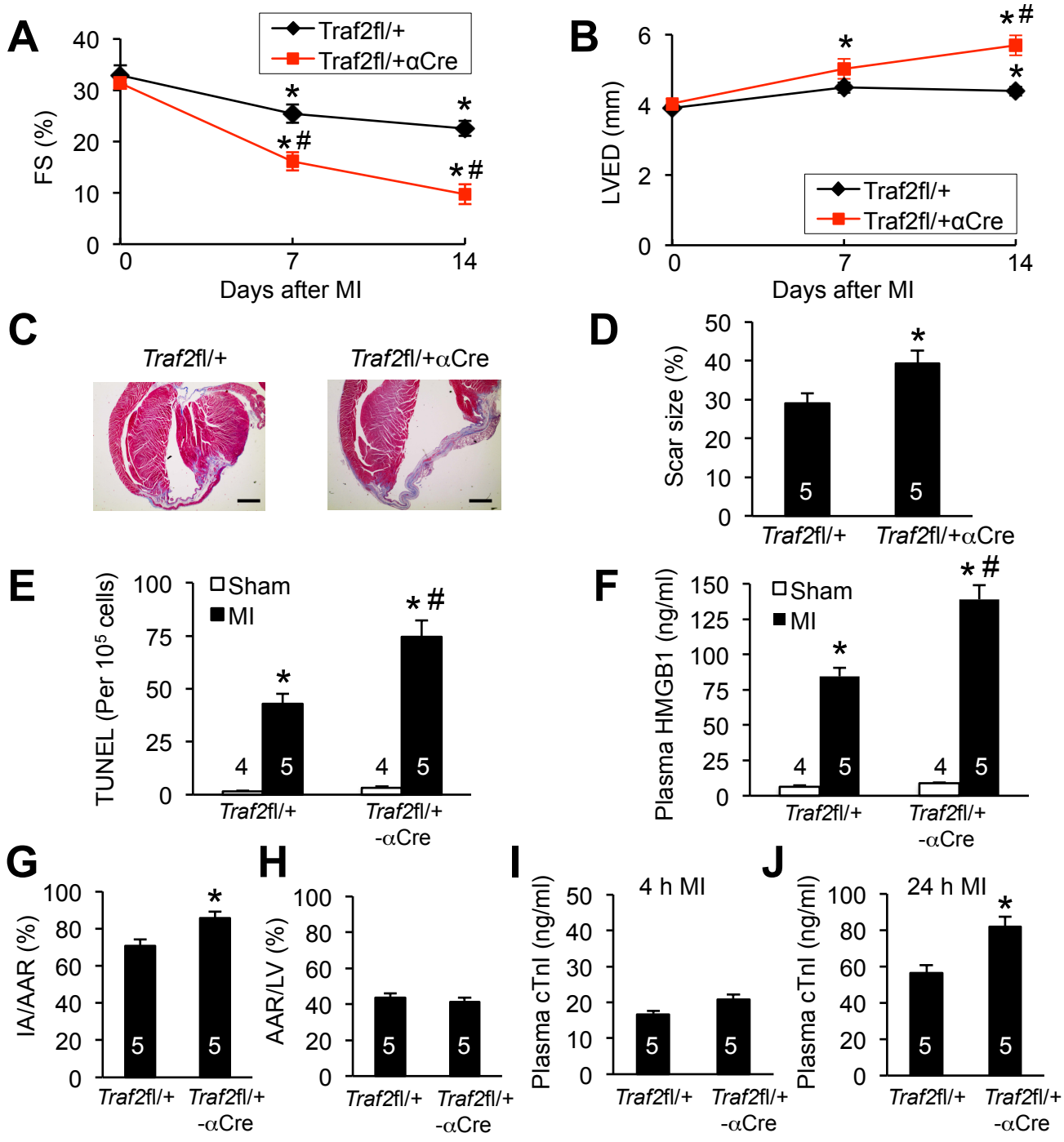
Supplemental Figures and Figure Legends



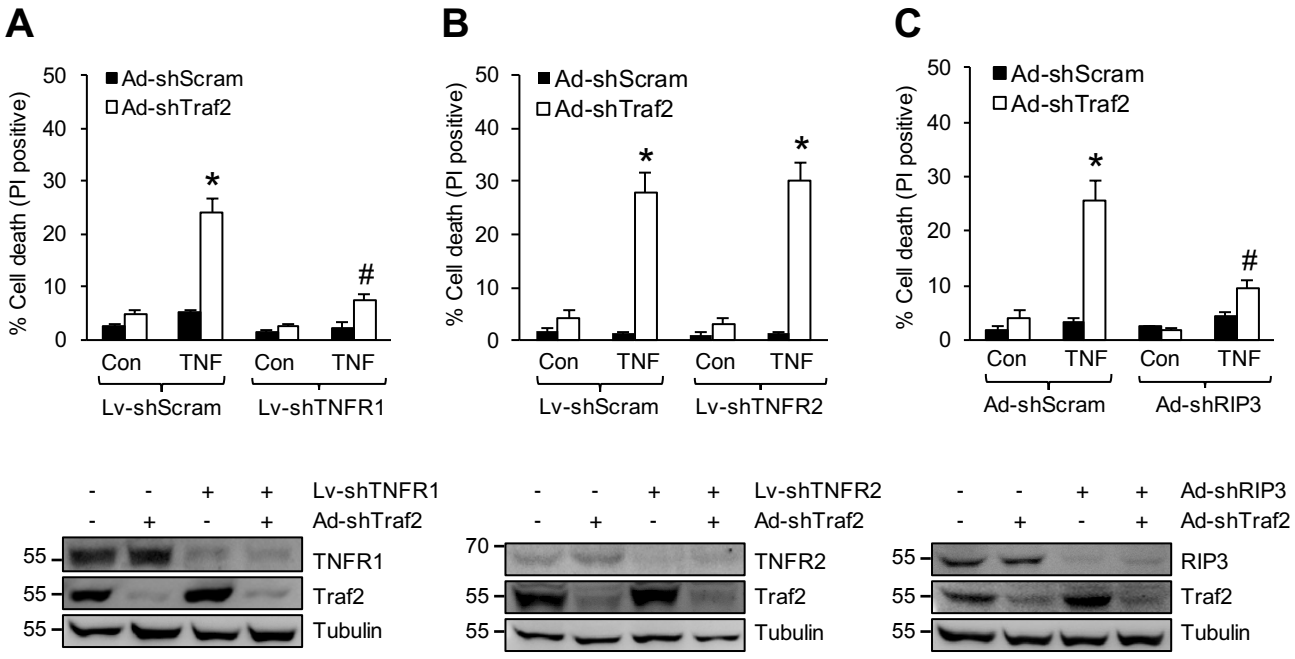
Supplemental Figure 1. *Traf2fl/fl*-αMHC-Cre mice gradually develop ventricular dilation and dysfunction. **A** through **D**, Echocardiographic assessment of FS (fractional shortening), LVED (left ventricular end-diastolic dimension), LVES (left ventricular end-systolic dimension), and LVPWd (left ventricular posterior wall thickness) in *Traf2fl/fl* and Traf2*fl/fl*-αMHC-Cre mice of the indicated ages . **P* < 0.05 versus *Traf2fl/fl* at corresponding time point or *Traf2fl/fl*-αCre at 4 weeks. n = 5. Friedman test was used followed by pairwise comparisons with *post hoc* Wilcoxon signed-rank test.



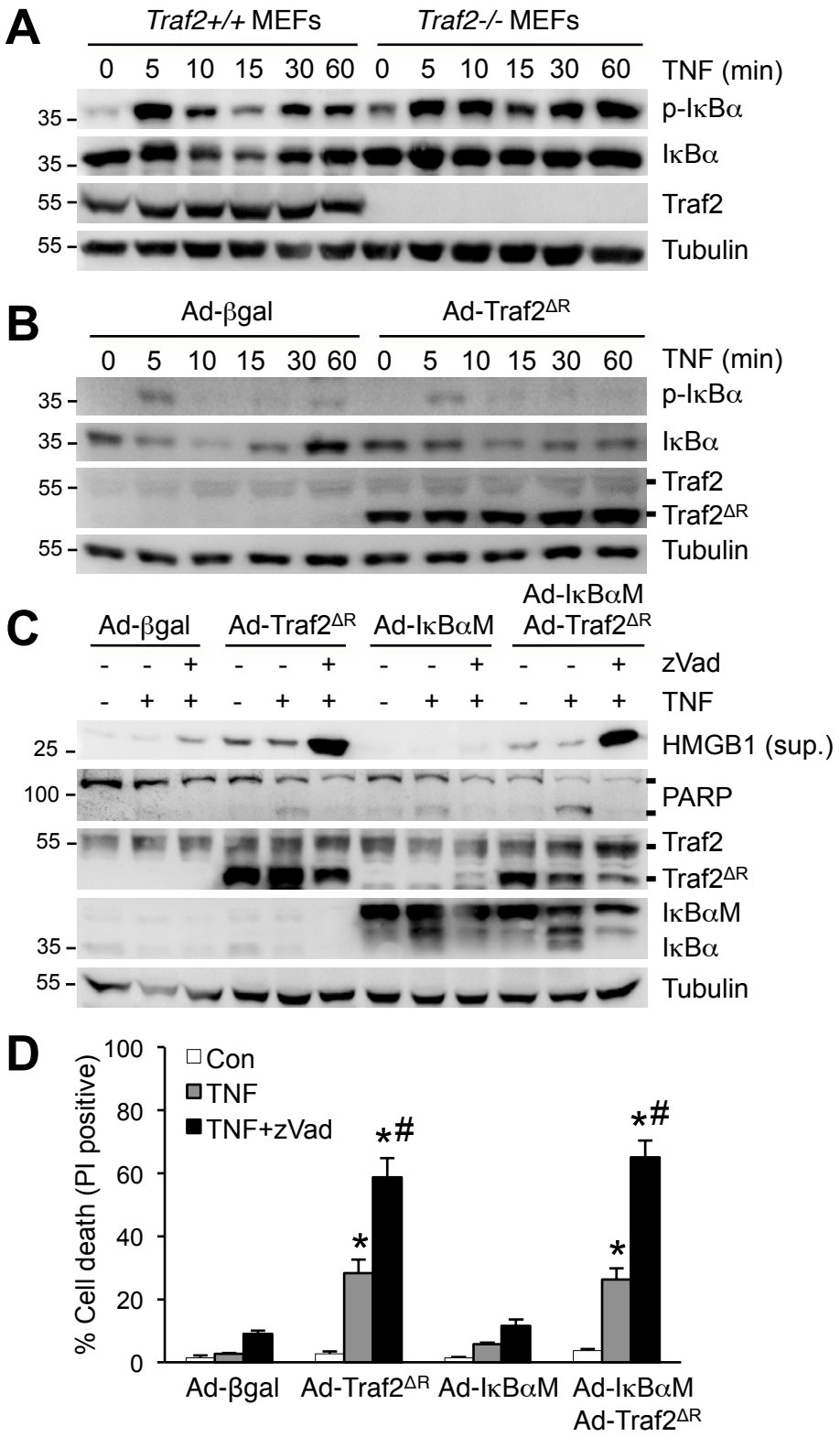
Supplemental Figure 2. Characterization of *Traf2fl/fl*-βMHC-Cre mice. **A,** Western blotting for Traf2 and α-tubulin in cardiac extracts from *Traf2fl/fl* and *Traf2fl/fl*-βMHC-Cre mice at 2 months of age. **B-D**, Echocardiographic assessment of fractional shortening (FS), and left ventricular dimension in diastole (LVED) or in systole (LVES). **P* < 0.05 versus *Traf2fl/fl*. **E**, Heart weight to body weight ratio (HW/BW) of the indicated mice. **P* < 0.05 versus Wt, αCre, or *Traf2fl/fl*. **F**, Masson's trichrome-stained, paraffin-embedded sections from the hearts of *Traf2fl/fl* and Traf2*fl/fl*-αMHC-Cre mice. Scale bars, 50 μm. **G**, Myocardial fibrosis was determined by MetaMorph software. **P* < 0.01 versus *Traf2fl/fl*. Mann-Whitney U-test was performed in B-E, and G.



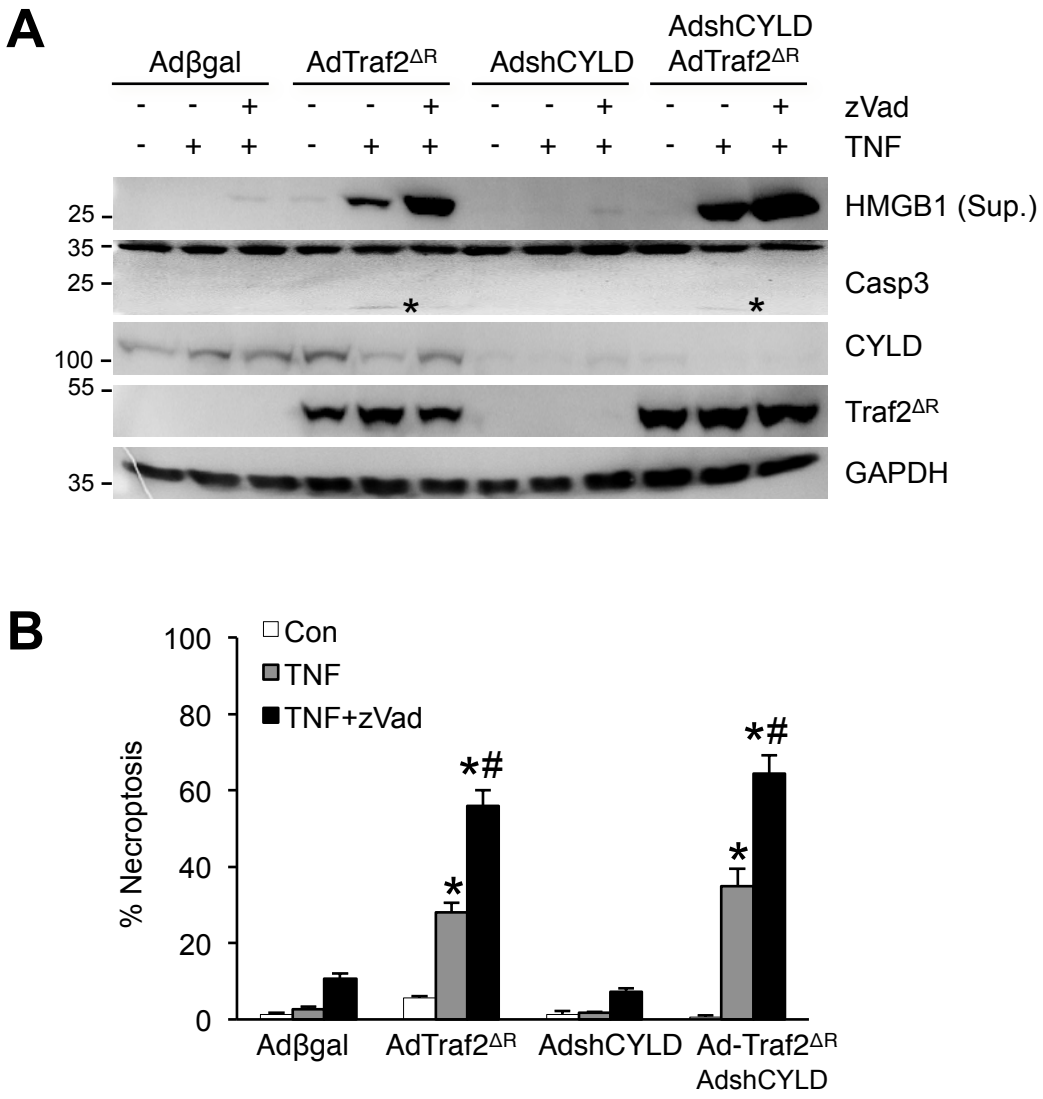
Supplemental Figure 3. *Traf2fl/+*αMHC-Cre mice were predisposed to develop pathological cardiac remodeling and dysfunction after MI. **A** and **B**, Echocardiographic measurements of *Traf2fl/fl* and Traf2*fl/fl*-αMHC-Cre mice (2 months of age) following MI. **P* < 0.05 versus day 0; #*P* < 0.05 versus *Traf2fl/+*. **C**, Masson's trichrome-stained, paraffin-embedded sections from the hearts of *Traf2fl/fl* and Traf2*fl/fl*-αMHC-Cre mice after 2 weeks of MI. Scale bars, 1 mm. **D**, Scar size of hearts from *Traf2fl/fl* and Traf2*fl/fl*-αMHC-Cre mice after 2 weeks of MI. **E**, TUNEL positive cells from cardiac sections of the indicated mice after 2 weeks of MI or sham surgery. **P* < 0.05 versus Sham; #*P* < 0.05 versus *Traf2fl/+* MI. **F**, Plasma HMGB1 from the indicated mice 12 h after MI or sham surgery. **P* < 0.01 versus Sham; #*P* < 0.05 versus *Traf2fl/+* MI. **G**, Infarct area normalized to area at risk (IA/AAR) from the indicated mice following 24 h of MI. **P* <0.05 versus *Traf2fl/+*. **H**, Area at risk (AAR) normalized to area of the left ventricle (LV) from mice indicated in G. **I** and **J**, Plasma cTnI levels from the indicated mice subjected to MI for 4 or 24 h. **P* < 0.05 versus *Traf2fl/+*. Friedman test was performed in A and B followed by pairwise comparisons with *post hoc* Wilcoxon signed-rank test. Mann-Whitney U-test was performed in D, G-J. Kruskal-Wallis test followed by *post-hoc* Mann-Whitney U-test with Bonferroni's correction was performed in E, F.



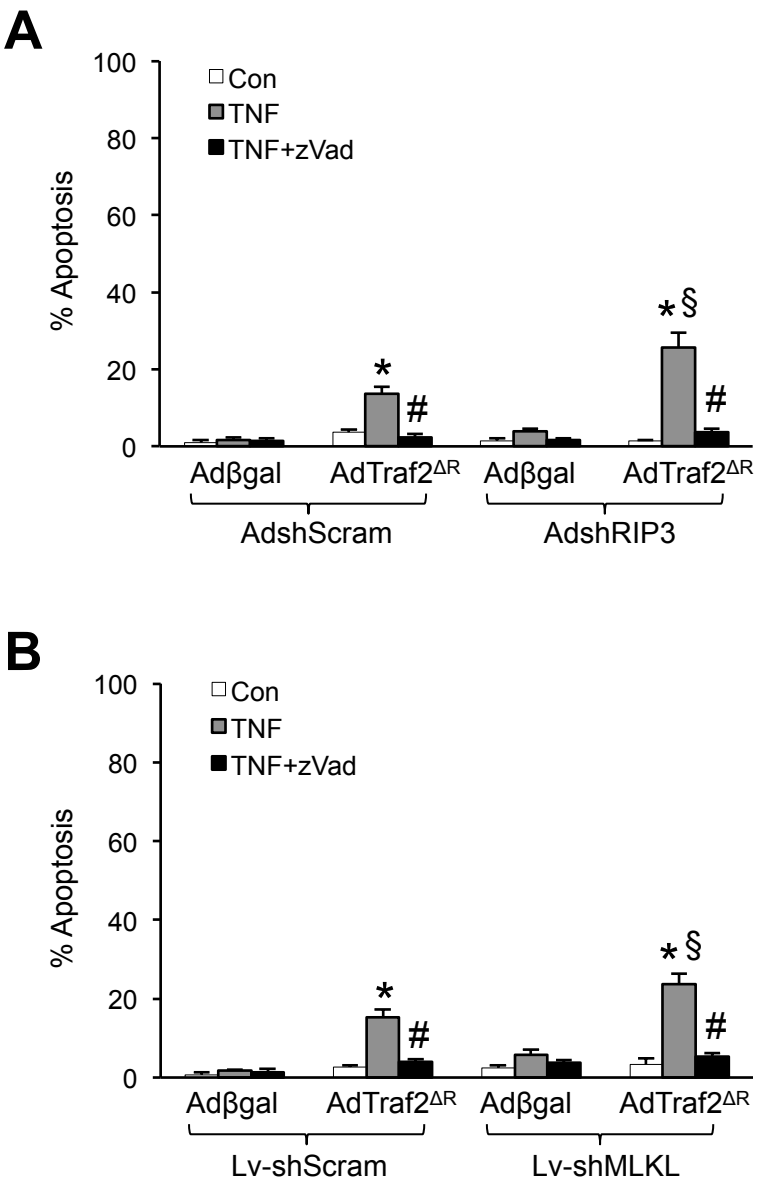
Supplemental Figure 4. Deletion of TNFR1 or RIP3 blocked necroptotosis triggered by TRAF2 ablation in cardiomyocytes. A and B, Cell death assessed by PI staining of cardiomyocytes infected with indicated lentiviral and adenoviral vectors followed by TNF or vehicle control for 4 h. Western blotting for the indicated proteins was performed from cardiomyocytes infected with the indicated viral vectors. **P* < 0.01 versus Ad-shScram Con or TNF; #*P* < 0.05 versus Lv-shScram Ad-shTraf2 TNF. **C**, Cell death assessed by PI staining of cardiomyocytes infected with indicated adenoviral vectors followed by TNF or vehicle control for 4 h. Western blotting for the indicated proteins was performed from cardiomyocytes infected with indicated adenoviral vectors. **P* < 0.01 versus Ad-shScram Con or TNF; #*P* < 0.05 versus Ad-shScram Ad-shTraf2 TNF. Data were from at least 3 independent experiments (n ≥ 900 cells per group were analyzed for cell death). One-way ANOVA with Bonferroni's *post-hoc* test was performed.



Supplemental Figure 5. TRAF2 regulates necroptotosis independent of NFκB. **A**, Western blots for the indicated proteins in *Traf2+/+* or *Traf2-/-* MEFs stimulated with 10 ng/ml TNFα for 0-60 min. **B**, Western blots for the indicated proteins from myocytes infected with Adβ-gal or Ad-Traf2$^{\Delta R}$, then stimulated with 10 ng/ml TNFα for 0-60 min. **C**, Western blots for the indicated proteins from myocytes infected with Adβ-gal or Ad-Traf2$^{\Delta R}$ along with an adenovirus encoding IκBα mutant (Ad-IκBαM) then treated as indicated for 4 h. **D**, Quantification of cell death in cells treated as in C. **P* < 0.05 versus Con; #*P* < 0.05 versus TNF. One-way ANOVA with Bonferroni's *post-hoc* test was performed in D.



Supplemental Figure 6. Ablation of the deubiquitinating enzyme CYLD had no effects on necroptotosis triggered by Traf2 inactivation. A, Western blots for the indicated proteins from cardiomyocytes infected with Adβ-gal or Ad-Traf2$^{\Delta R}$, along with an adenovirus encoding CYLD shRNA (Ad-shCYLD) for 24 h, then treated as indicated for 4 h. * indicates cleaved caspase 3. **B**, Quantification of cell death in cells treated as in A. *P < 0.05 versus Con; #P < 0.05 versus TNF. One-way ANOVA with Bonferroni's *post-hoc* test was performed.



Supplemental Figure 7. Ablation of RIP3 or MLKL mildly increased apoptotic cell death triggered by Traf2 inactivation. A, Quantification of apoptosis (PI negative with chromatin condensation) from cardiomyocytes infected with the indicated adenoviral vectors then treated with vehicle control, TNF, or TNF+zVad for 4 h. **P* < 0.05 versus Con; #*P* < 0.05 versus TNF in the corresponding group; §*P* < 0.05 versus Ad-shScram AdTraf2$^{\Delta R}$ TNF. **B**, Quantification of apoptosis from cardiomyocytes infected with the indicated lentiviral (Lv) and adenoviral (Ad) vectors then treated with vehicle control, TNF, or TNF+zVad for 4 h. **P* < 0.05 versus Con; #*P* < 0.05 versus TNF in the corresponding group; §*P* < 0.05 versus Lv-shScram AdTraf2$^{\Delta R}$ TNF. One-way ANOVA with Bonferroni's *post-hoc* test was performed.

Supplemental References

1. Grech AP, Amesbury M, Chan T, Gardam S, Basten A, Brink R. TRAF2 differentially regulates the canonical and noncanonical pathways of NF-kappaB activation in mature B cells. *Immunity*. 2004;21:629-642.
2. Parsons SA, Millay DP, Wilkins BJ, Bueno OF, Tsika GL, Neilson JR, Liberatore CM, Yutzey KE, Crabtree GR, Tsika RW, Molkentin JD. Genetic loss of calcineurin blocks mechanical overload-induced skeletal muscle fiber type switching but not hypertrophy. *J Biol Chem.* 2004;279:26192-261200.
3. Palermo J, Gulick J, Colbert M, Fewell J, Robbins J. Transgenic remodeling of the contractile apparatus in the mammalian heart. *Circ Res.* 1996;78:504-509.
4. Li L, Chen Y, Doan J, Murray J, Molkentin JD, Liu Q. Transforming growth factor β-activated kinase 1 signaling pathway critically regulates myocardial survival and remodeling. *Circulation.* 2014;130:2162-2172.
5. Liu Q, Chen Y, Auger-Messier M, Molkentin JD. Interaction between NFκB and NFAT coordinates cardiac hypertrophy and pathological remodeling. *Circ Res.* 2012;110:1077-1086.
6. Li L, Chen Y, Li J, Yin H, Guo X, Doan J, Molkentin JD, Liu Q. TAK1 Regulates Myocardial Response to Pathological Stress via NFAT, NFκB, and Bnip3 Pathways. Sci Rep. 2015;5:16626.
7. Liu Q, Busby JC, Molkentin JD. Interaction between TAK1-TAB1-TAB2 and RCAN1-calcineurin defines a signalling nodal control point. *Nat Cell Biol.* 2009;11:154-161.