SUPPLEMENTAL MATERIAL

Expanded Methods

Reagents

5z-7-oxozeaenol, cycloheximide, necrostatin-1, and tamoxifen were purchased from Sigma. Mouse TNFα was from R&D Systems. zVad-FMK and z-IETD-FMK were obtained from Abcam. Propidium iodide, Hoechst 33342, and G418 were from Invitrogen. pcDNA3-casp8 and pcDNA3-casp8 C360A constructs were obtained from Addgene.

Animal models

The generation of *Map3k7* (*TAK1*) loxP-tageted (fl) mice, in which exon 2 (encoding the ATP-binding site) was flanked by loxP, was described previously[15]. *Map3k7fl/fl* mice were crossed with *Nkx2.5*-Cre, αMHC-Cre, βMHC-Cre, or MerCreMer mice to generate cardiac-specific TAK-deficient mice. In some experiments, mice were treated with tamoxifen (1 mg per 20 g body weight, i.p.) or necrostatin-1 (3 mg per kg body weight, i.p.) for 5 consecutive days. *Tnfrsf1a-/-* mice (the Jackson Laboratory) were crossed with *Map3k7fl/fl* βMHC-Cre or *Map3k7fl/fl* αMHC-Cre mice. All experiments involving animals were approved by the Institutional Animal Care and Use Committees of the University of Washington.

Echocardiography, TAC, and plasma HMGB1

For echocardiography, mice were anesthetized with 2% isoflurane by inhalation and scanning was performed with a VisualSonics Vevo 2100 imaging system as described previously[35]. M-mode left ventricular dimensions were averaged from 3-5 beats. Fractional shortening was calculated as described previously[35]. Transverse aortic constriction (TAC) was performed to produce cardiac pressure overload in mice using a 27-gauge needle as previously described[47]. Sham-operated mice underwent the same procedure without 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. Mouse plasma HMGB1 levels were measured using an enzyme-linked immunosorbent assay kit (Chondrex) according to the manufacturer's instructions. Absorbance at 450 nm (sample) and 630 nm (reference) were measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek).

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[48]. Assessment of TUNEL from paraffin sections was performed with an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions or a TMR Red In Situ Death Detection Kit (Roche Diagnostics) as described in detail previously[49].

Cell lines

Primary neonatal rat cardiomyocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rat pups as previously described[50]. *Tnfrsf1a*-/-, *Tnfrsf1b*-/-, *casp8*-/- MEFs were kindly provided by David Vaux (Walter and Eliza Hall Institute Biotechnology Centre, Australia). *Map3k7-/-* MEFs was a gift from Shizuo Akira (Osaka University, Japan), *Ripk1-/-* and *Ripk3*-/- MEFs were obtained from Zheng-gang Liu (National Institutes of Health) and Francis Chan (University of Massachusetts), respectively. *Fadd*-/- MEFs were provided by Astar Winoto (University of California, Berkley). *Casp8*-/- MEFs were transfected with pcDNA3-Casp8, pcDNA3-Casp8 C360A, or the empty pcDNA3 vector (Addgene) using Lipofectamine 2000 reagent. Stably transfected cells were selected with 400 μg/ml G418 (Invitrogen) and expanded. MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell death analysis

Cell death was measured using a Cell Meter Apoptotic and Necrotic Detection kit (ATT Bioquest, Sunnyvale, CA) according to the manufacturer's instructions. Cells were incubated at room temperature for 30 min with Apopxin Green (for detection of phosphatidylserine on the cell surface), propidium iodide 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 a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) and luminescence was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek).

Adenoviral constructs and shRNA

Adβgal, AdTAK1, Ad TAB1, AdTAK1-KW, and AdTAK1-ΔN have been described previously[31]. Adenoviral vector expressing RIP3 shRNA or a scramble shRNA were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's protocol. The sequence for RIP3 shRNA is 5'-CACCGCTGCTGTCTCCAAGGTAAAGCGAACTTT-ACCTTGGAGACA-GCAGC-3'. The sequence for the scrambled shRNA is 5'-CACCGCCTTAGGTTGGTCGAGAAACGAATTT-CTCGACCAACCTAAGG-3'. Adenoviral infections were performed as described previously at a multiplicity of infection of 10 to 50 plaque forming units per ml[50].

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[50]. In some experiments, cell culture supernatants were also collected for the detection of HMGB1. The following antibodies were

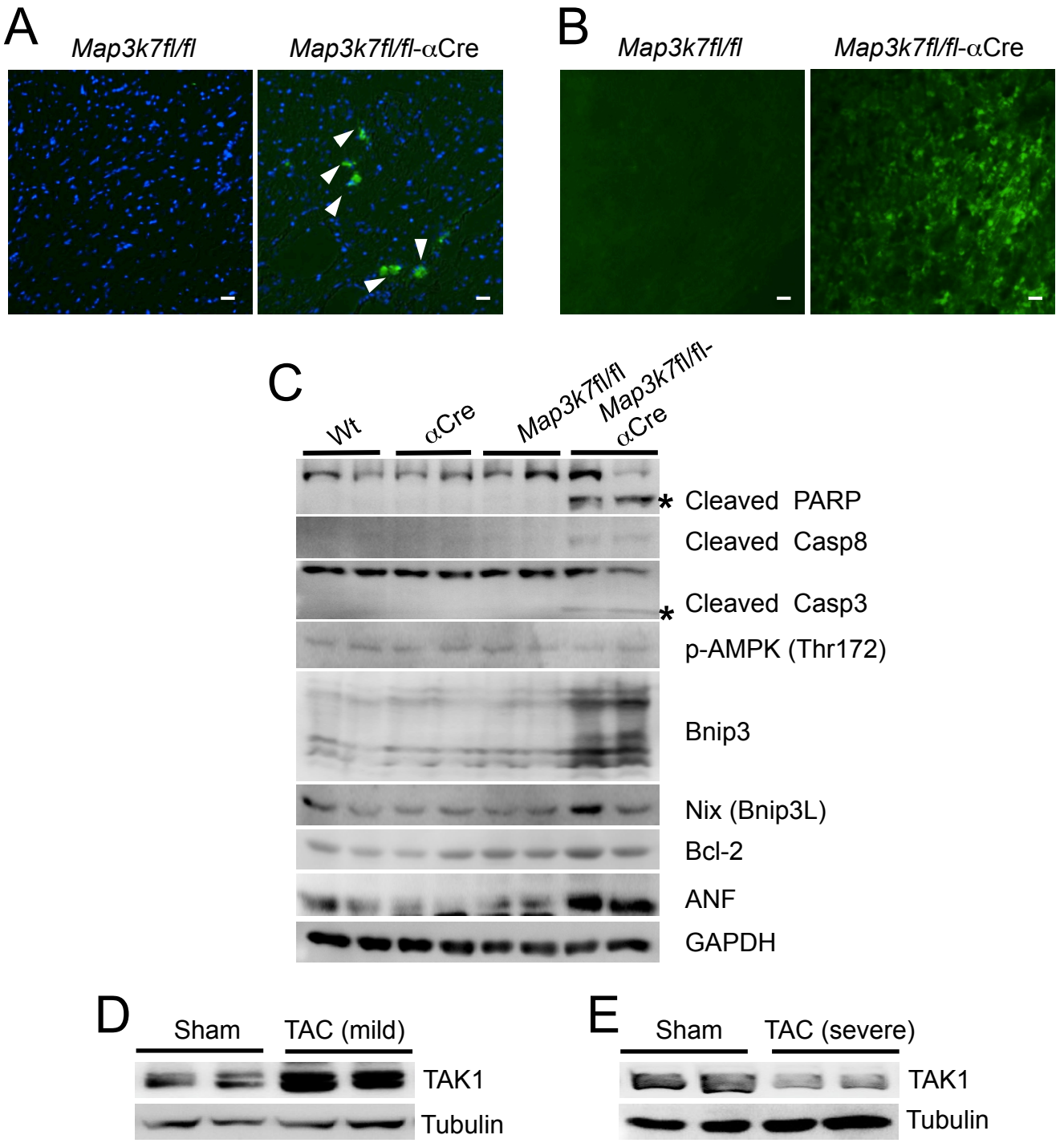
used: Anti-TAK1 (4505), anti-phospho-TAK1 (Thr187; 4536), anti-α tubulin (3873), anti-HMGB1 (3935), anti-PARP (9532), anti-caspase 8 (4790), anti-cleaved caspase 8 (9429), anti-caspase 3 (9662), anti-p-JNK1/2 (Thr183/Tyr185; 4668), anti-RIP1 (3493), anti-Bnip3 (3769), anti-Bnip3L/Nix (12396), and anti-p-AMPK (Thr172; 4188) were from Cell Signaling Biotechnology (Beverly, MA); Anti-RIP3 (sc-135171), anti-Fadd (sc-6036), anti-CYLD (sc-74435), anti-IκBα (sc-847), Ub (sc-8017), Bcl-2 (sc-7382), 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-RIP3 (2283) was from ProSci; Anti-TNFR1 (AF-425-PB) was from R&D Systems.

Immunoprecipitation

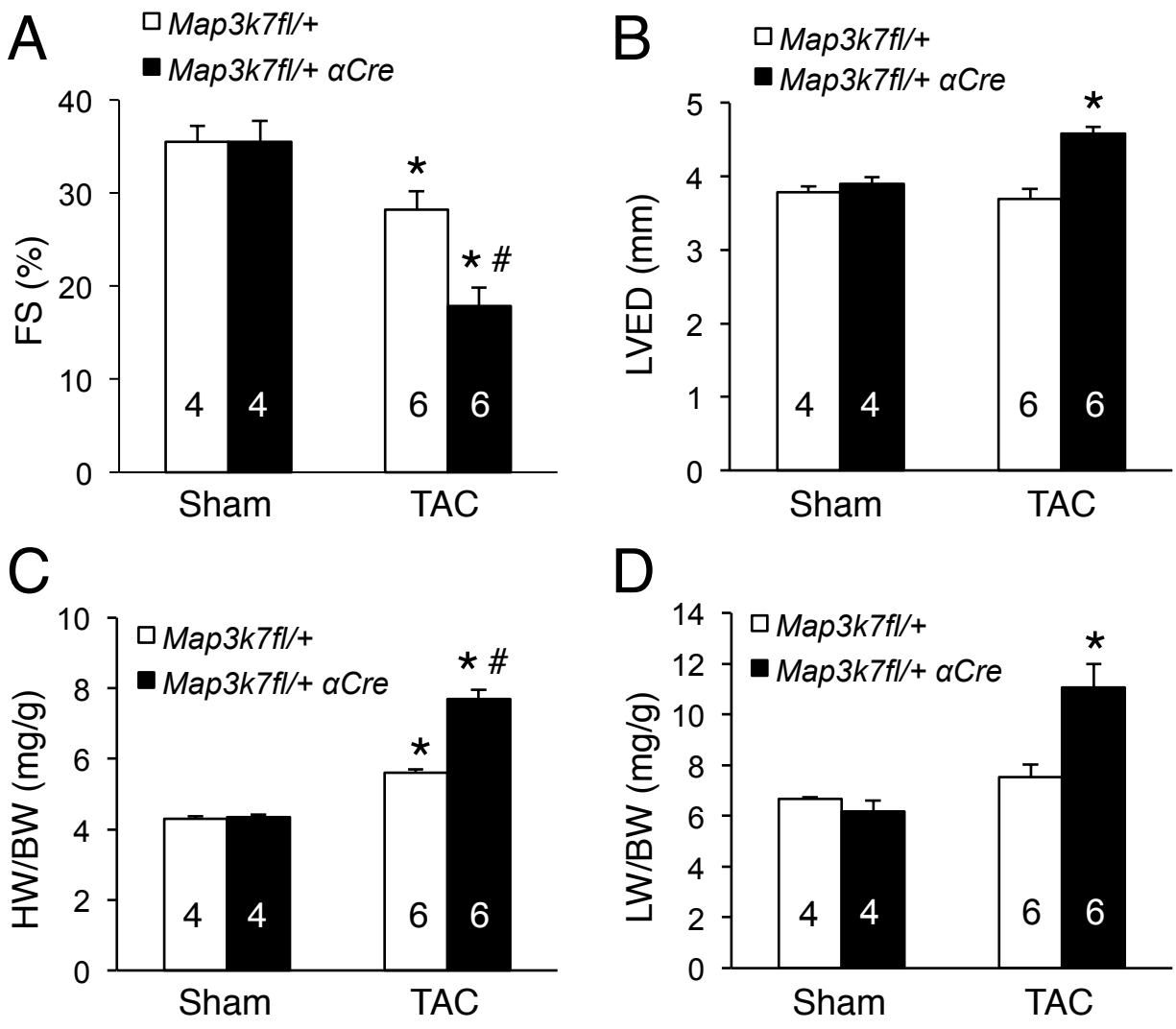
Cells were lysed at 4°C in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 0.5% NP-40) containing protease inhibitor cocktail (Roche). Immunoprecipitation was performed as described previously[50]. Whole cell lysates were cleared by centrifugation at 18,000 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 binding buffer, and the proteins were resolved on an 8-12% SDS-PAGE for subsequent Western blotting.

Statistics

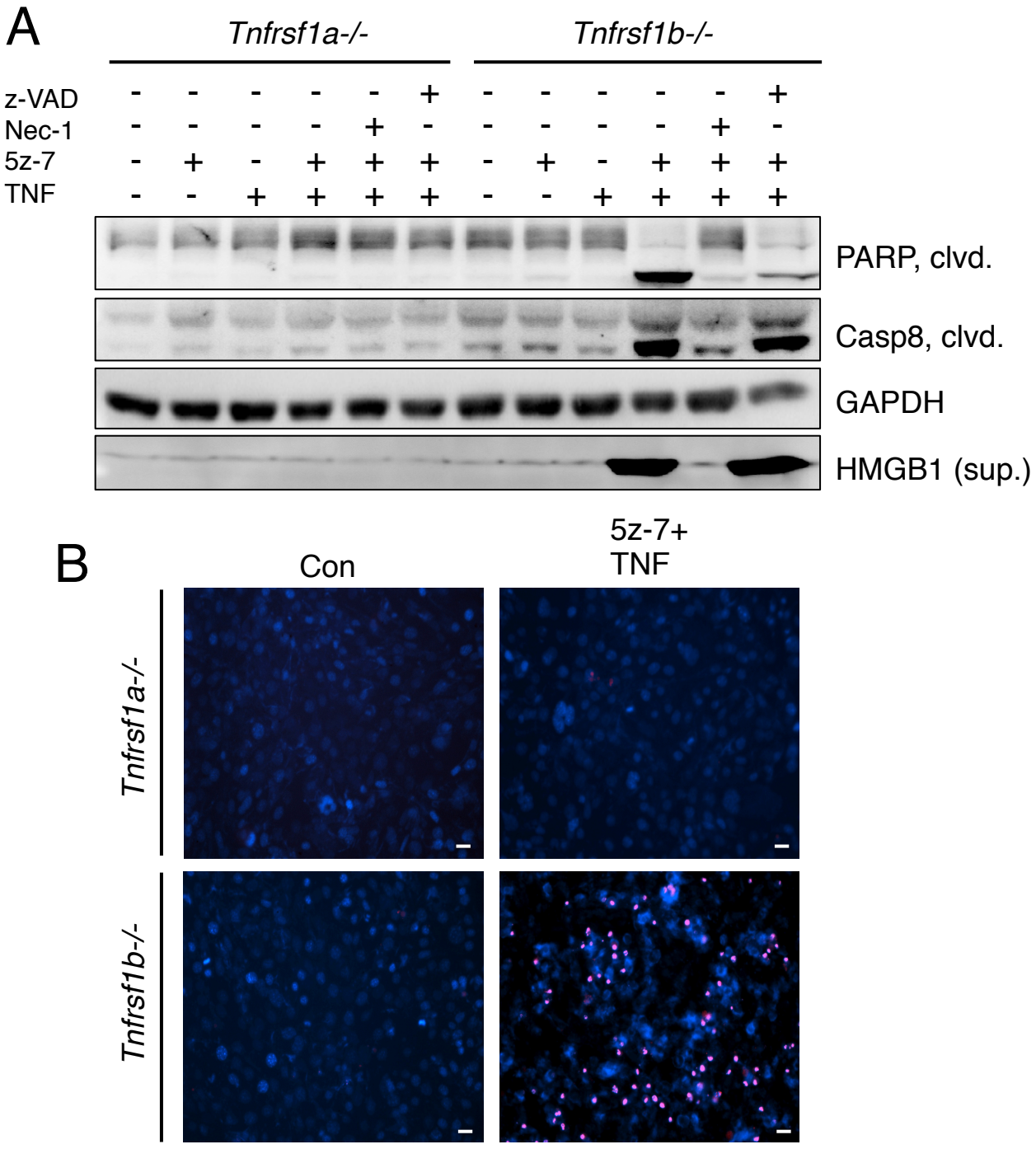
Sample size was estimated by conducting pilot experiments and power analysis. Investigators were blinded to group allocation when performing drug treatment or surgery on animals. Exact testing (Exact Wilcoxan Rank-sum or Kruskal-Wallis test) was used for studies with small sample sizes as well as whenever feasible for large sample sizes. Data with normal distribution were evaluated by one-way ANOVA with the Bonferroni's post hoc test or repeated-measures ANOVA. The log-rank test was used for the comparison of survival data. $P < 0.05$ was considered statistically significant.



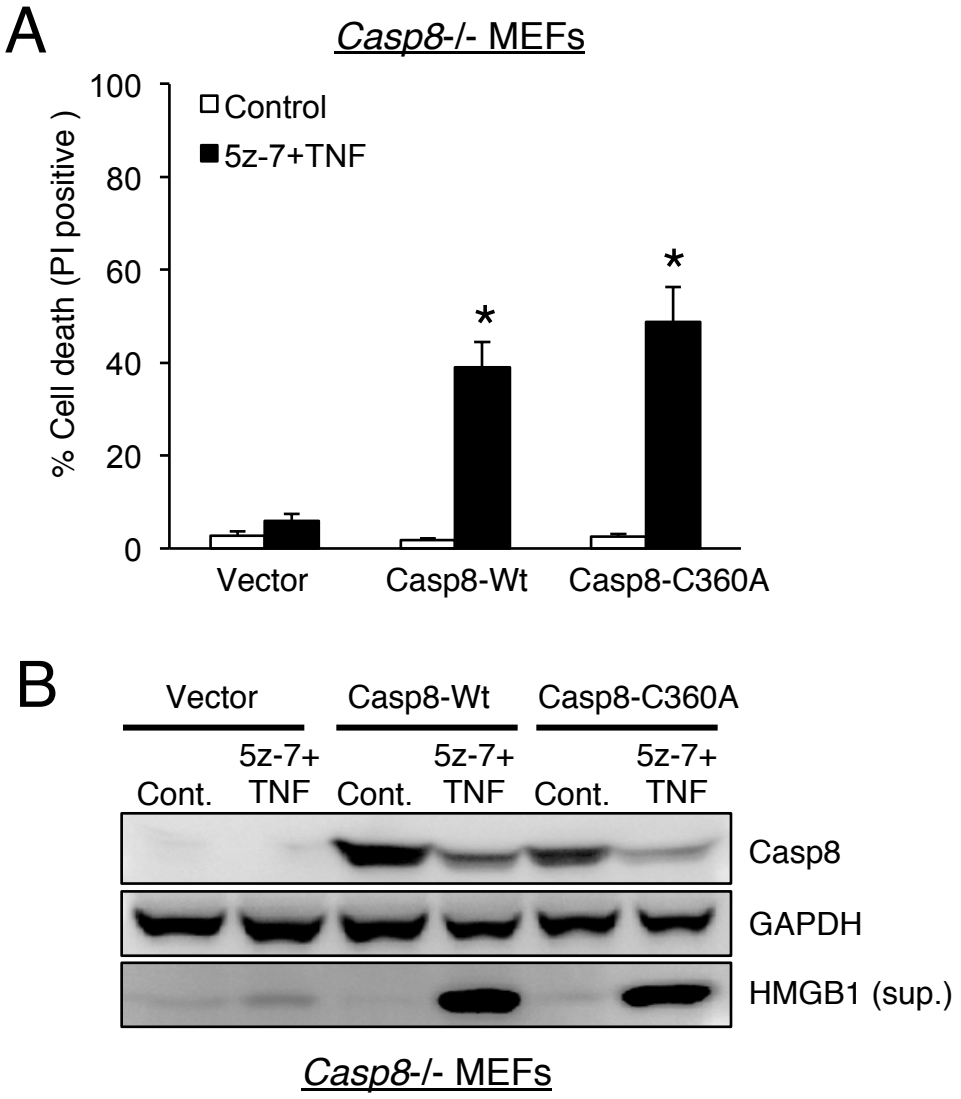
Supplemental Figure 1. TAK1 ablation in the heart promotes apoptotic signaling and ROS production. (**A**) Immuno-staining for cleaved caspase 3 of cardiac sections from mice of the indicated genotypes. Arrow heads denote apoptotic cells. (**B**) ROS accumulation detected by CM-H2DCFDA staining of frozen cardiac sections. (**C**) Immunoblots for the indicated proteins in heart extracts from mice of the indicated genotypes. Asterisks denote cleaved proteins. (**D, E**) Immunoblots for TAK1 and α-tubulin from heart extracts of wild-type mice subjected to mild TAC (27G, 2 weeks) or severe TAC (28G, 4 weeks). Scale bars, 25 μm.



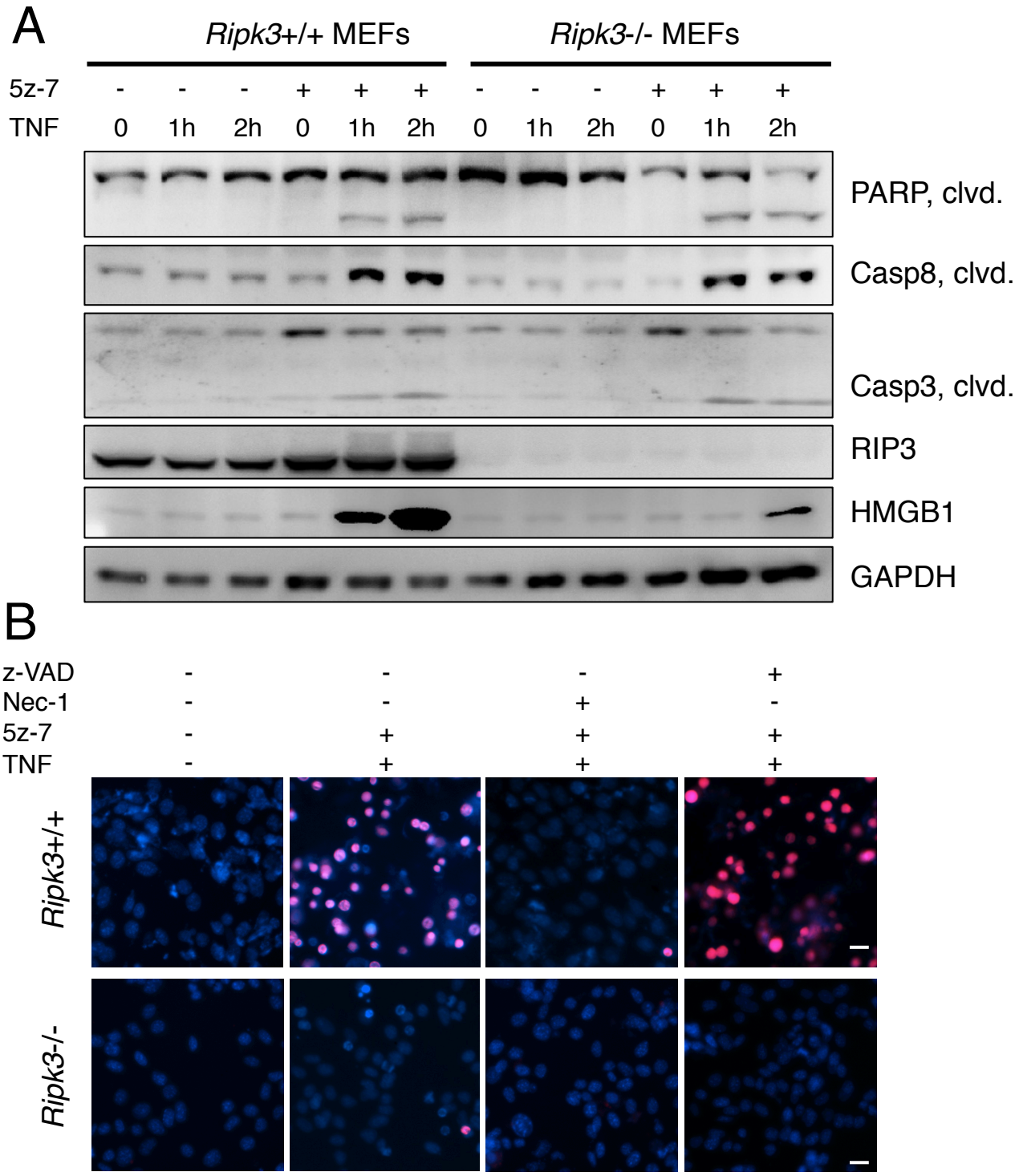
Supplemental Figure 2. TAK1 protects against pressure overload-induced cardiac dysfunction and failure. (**A and B**) Fractional shortening and left ventricular end diastolic dimension in *Map3k7fl/+* and *Map3k7fl/+*αCre mice 2 weeks after sham or TAC surgery. (**C and D**) Heart weight to body weight and lung weight to body weight ratios in mice as described in A and B. The number of mice per group is indicated within the bar. **P* < 0.05 versus Sham; #*P* < 0.05 versus *Map3k7fl/+* TAC.



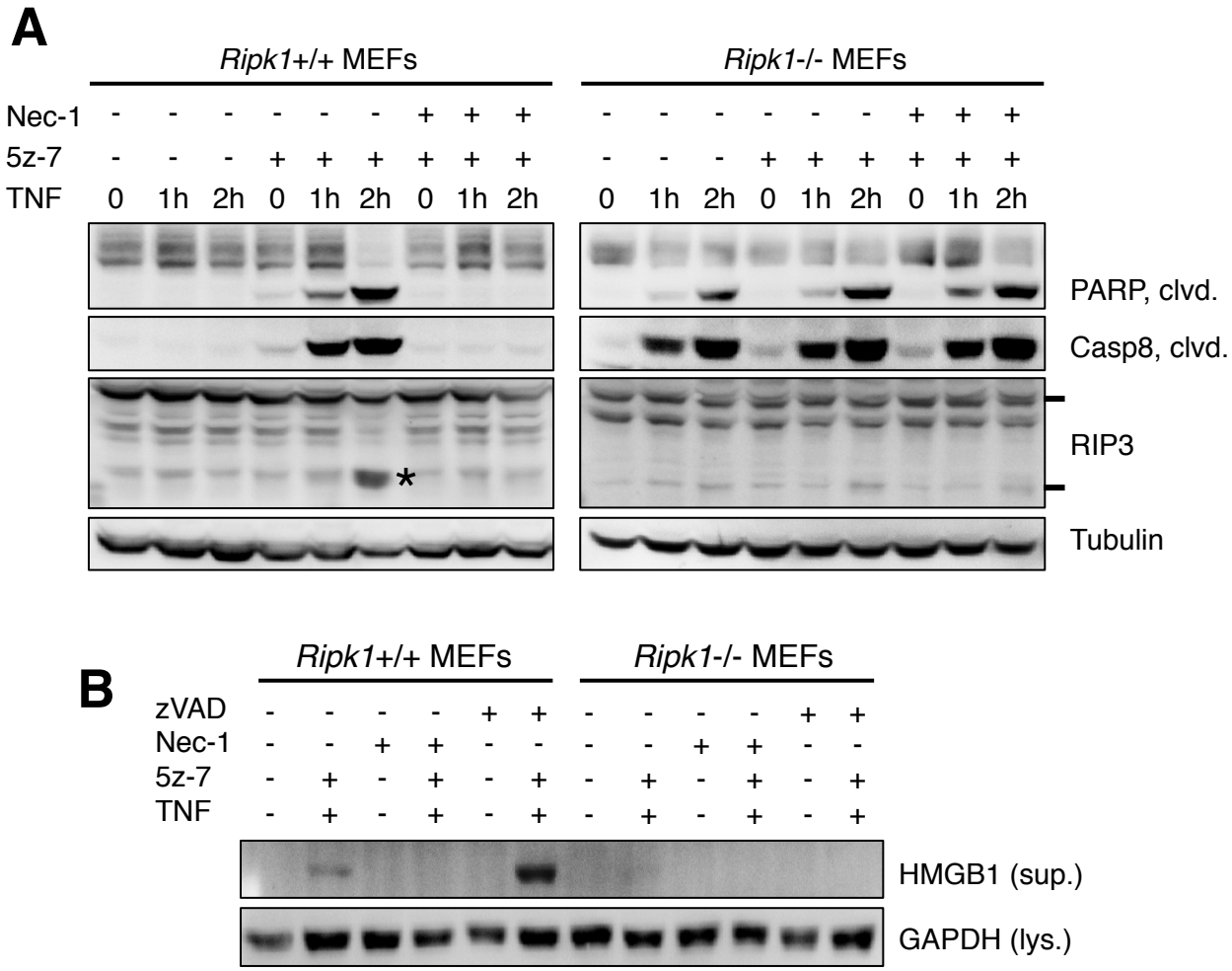
Supplemental Figure 3. TNFR1, but not TNFR2, mediates cell death associated with TAK1 inactivation. (**A**) Immunoblots for the indicated proteins in cellular extracts from *Tnfrsf1a*-/- and *Tnfrsf1b*-/-MEFs treated with vehicle control or 10 ng/ml TNFα for 2 hours in the presence or absence of 5z-7, Nec-1, or zVAD. (**B**) Cell death assessed by PI (red) and Hoechst 33342 (blue) staining of *Tnfrsf1a*-/- and *Tnfrsf1b*-/-MEFs treated with vehicle control or TNFα plus 5z-7 for 4 hours. Scale bars, 25 μm.



Supplemental Figure 4. Capase 8 plays a scaffolding role in necroptosis signaling. (**A**) Cell death assessed by PI staining of *Casp8*-/- MEFs reconstituted with wild-type caspase 8 (casp8-wt), the C360A mutant (casp8-CA), or empty vector, and treated with vehicle control or TNFα plus 5z-7 for 4 hours. *$P < 0.05$ versus Vector 5z-7+TNF. (**B**) Immunoblots for the indicated proteins in extracts of the indicated cells treated with vehicle control or TNFα plus 5z-7 for 2 hours.



Supplemental Figure 5. Genetic deletion of RIP3 blocks necroptosis, but not apoptosis signaling. (**A**) Immunoblots for the indicated proteins in cellular extracts from *Ripk3*+/+ and *Ripk3*-/- MEFs treated with vehicle control or 10 ng/ml TNFα for 2 hours in the presence or absence of 5z-7. (**B**) Cell death assessed by PI (red) and Hoechst 33342 (blue) staining of *Ripk3*+/+ and *Ripk3*-/- MEFs treated with vehicle control or TNFα plus 5z-7 for 4 hours in the presence or absence of Nec-1 or zVAD. Scale bar, 10 μm.



Supplemental Figure 6. Necrostatin-1 inhibits both TNFα-induced apoptotic and necrotic signaling through an RIP1-dependent mechanism. (**A**) Immunoblots for the indicated proteins in cellular extracts from *Ripk1*+/+ and *Ripk1*-/-MEFs treated with vehicle control or 10 ng/ml TNFα for 1 and 2 hours in the presence or absence of 5z-7 and/or Nec-1. Asterisk denotes cleaved RIP3. (**B**) Immunoblots for the indicated proteins in culture supernatant or whole cell lysates from *Ripk1*+/+ and *Ripk1*-/-MEFs treated with vehicle control or 10 ng/ml TNFα for 2 hours in the presence or absence of 5z-7, nec-1, or zVAD.

Supplemental references

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