SUPPLEMENTAL MATERIAL

Supplemental MATERIALS & METHODS

Materials: Monoclonal antibodies to c-Cbl and ubiquitin were obtained from BD Biosciences, and polyclonal antibody for FAK was from Santa Cruz Biotechnology. Phospho-c-Cbl Y^{774}, EGFR receptor (EGFR), Bcl2, phospho-Bad-S^{112}, Bad, c-IAP1, and XIAP were from Cell Signaling. Monoclonal antibodies for phosphotyrosine and ubiquitin Lys^{48} were from Upstate Biotechnology and anti-troponin I was from Sigma. All other chemicals were from standard suppliers.

Generation of cardiac myocyte-specific c-Cbl knockout mice: Conditional mice bearing floxed c-Cbl alleles (c-$Cbl^{flox/flox}$) have previously been described.[1] Transgenic mice overexpressing Cre-recombinase protein fused to 2 mutant estrogen receptor ligand-binding domains under the control of the α-myosin heavy chain (α-MHC) promoter (α-MHC-MerCreMer)[2] were received from The Jackson Laboratory (JAX Mice and Services, Bar Harbor, Me). Homozygous mice with the floxed c-Cbl alleles were crossed with αMHC-MerCreMer mice and bred back to c-$Cbl^{flox/flox}$ to produce αMHC-MerCreMer×c-$Cbl^{flox/flox}$ mice. c-$Cbl^{flox/flox}$ littermate were taken as controls. All animals were bred and maintained on a C57Bl/6 background.

To induce Cre-dependent recombination, tamoxifen (20 mg/kg body weight, Sigma-Aldrich) was injected into 8-weeks-old animals intraperitoneally (i.p.) for 5 days. Tissue-specific c-Cbl deficiency was achieved 4 weeks after injection as verified by western blot analysis.[2]

Genotyping of mice: The c-$Cbl^{flox/flox}$ and c-$Cbl^{wt/wt}$ alleles and the Cre transgene were detected in mouse tail DNA by PCR. Primers for c-$Cbl^{wt/wt}$ and c-$Cbl^{flox/flox}$ result in a 600bp and a 700bp product, respectively. The primer sets used are: Cre (Forward 5'-

TCCAATTTACTGACCGTACACCAA -3'; Reverse, 5'- CCTGATCCTGGCAATTTCGGCTA -3'); c-Cbl$^{flox/flox}$ (Forward 5'-GTGGTGGCTTGCAATTATAATCCTACCACTTAGG-3'; Reverse 5'-GTTTGAGATGTCTGGCTGTGTGTACACGCG-3'), c-Cbl$^{-/-}$ (Forward 5'-TCCCCTCCCCTTCCCATGTTTTTAATAGACTC-3'; Reverse 5'-TGGCTGGACGTAAACTCCTCTTCAGACCTAATAAC-3'), and c-Cbl$^{wt/wt}$ (Forward 5'-GACGATAGTCCCGTGGAAGAGCTTTCGACA-3', Reverse 5'-CCTAAGTGGTAGGATTATAATTGCAAGCCACCAC-3').

Assessment of area at risk and infarct size: After 24 hours of reperfusion, the slipknot was retied and the right carotid artery was cannulated to allow injection of KCL (40 mEq/L) followed by 1% Evan's blue dye for identification of the area at risk (AR). The hearts were excised, rinsed briefly in PBS, and sliced transversely. Slices were then incubated with 1% triphenyltretrazolium chloride (TTC, pH 7.4 in phosphate buffer) at 37°C for 30 minutes, fixed in 10% formalin, photographed, and the images were used to quantify IR-induced myocardial infarction using Bioquant software. The Evan's blue stained area defined the perfused area, whereas the Evan's blue unstained area defined the area at risk (AAR). The area lacking the red TTC staining within the AAR was considered as the infarct area (IA). Both the surgeon and the evaluator of infarct size were blinded to mouse genotypes.

Heart function: Echocardiographic measurements were taken before surgery and at 24 hours after IR injury or 4 weeks after MI to determine the baseline heart function and ventricular dimensions in the experimental groups. Briefly, following light sedation with 1% Isoflurane, the mice were placed on a heated platform in the left lateral decubitus position for imaging. A Visualsonic Ultrasound System (Vevo770) containing a 40 Mhz variable frequency probe was used to capture the echocardiogram. Standard long and short axis M-Mode views were recorded

when the mouse possessed a target heart rate between 450 and 550 beats per minute. End-diastolic and end-systolic interventricular septum (IVSd, IVDs), posterior wall thickness (PWTd, PWTs) and left ventricular internal diameters (LVEDD, LVESD) were calculated and averaged from 4 consecutive contractions using manufacturer's software. Percent fractional shortening was calculated using: % FS= [(LVEDD-LVESD)/LVEDD] x 100. LVEF was calculated by the cubed method as follows: LVEF = $[(LVEDD)^3 – (LVESD)^3]/(LVEDD)^3$.

Hemodynamic measurements were performed using a 1.0 F catheter tip micromanometer (Millar PVR-1035). Following calibration, the catheter (connected to a Powerlab 40/3 data acquisition box; ADInstruments) was inserted through the right carotid artery of anesthetized mice. The first derivative of left ventricular pressure (dP/dT) was assessed using PVAN 3.6 software (Millar).

Neonatal rat cardiomyocyte isolation: Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described.[3] After 30 minutes of preplating (to eliminate non-myocyte cell contamination), myocytes were plated in collagen precoated dishes or in fibronectin (BD bioscience) precoated glass coverslips at a density of 160,000/cm^2 in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine, antibiotic/antimycotic solution, and 100 μmol/L 5-bromo-2-deoxyuridine (BrdU). Under these high density conditions, the myocytes form cell-cell contacts and display spontaneous contractile activity within 24 hours of plating.

Cardiac troponin-I ELISA assay: Mice were exsanguinated after IR injury and the serum levels of the cardiac-specific isoform of troponin-I were assessed using ELISA assay (Life Diagnostics, PA).

Immunoprecipitation and immunoblot analysis: Extraction of proteins from heart tissue samples was performed as described previously.[3] Briefly, lysates were cleared by centrifugation at

12,000 rpm and the supernatants (800 μg of protein/ml) were subjected to immunoprecipitation with corresponding antibodies. After overnight incubation at 4 °C, protein A- or G-agarose beads were added and left for an additional 3 hours. Immunocomplexes were then subjected to SDS-PAGE followed by Western blot analysis according to methods published previously or to the manufacturer's instructions.[3] Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

c-Cbl auto-ubiquitination assay: c-Cbl ubiquitin ligase activity was performed using auto-ubiquitination assay kit from Enzo Life Sciences with minor modification. Briefly, c-Cbl immunoprecipitates were washed twice with lysis buffer, once with ubiquitination buffer and then were incubated with 30 μL of ligase reaction buffer mixture containing ubiquitination buffer, 1 mmol/L DTT, 20 U/ml inorganic pyrophosphatase, 5 mmol/L Mg-ATP, 2.5 μM ubiquitin, 100 nmol/L E1, and 2.5 μM at 37°C for 1 h. The reaction was quenched by adding 30 μl of 2x non-reducing gel loading buffer and heating at 95°C for 5 minutes. Finally, the samples were assessed for immunoblotting with anti-ubiquitin for detecting ubiquitinated *c*-Cbl and for c-Cbl as a loading control.

Expression of adenoviral vectors: Production of recombinant adenovirus expressing Lac-Z, ligase deficient c-Cbl (70Z-Cbl) , a mutant that lacks 17 amino acid in the RING finger domain necessary for ubiquitin ligase activity, was described elsewhere.[3,4] The 70Z Cbl plasmids were a gift from Tsyganov A (Temple University, USA). Adenoviral vectors were purified using a kit from Virapur and titrated using BD Adeno-X rapid titer kit (BD Bioscience). After 24 hours of plating, NRCMs were infected with adenoviruses expressing Lac-Z (10 pfu/cell), or 70Z-Cbl-Cbl (5 pfu/cell) in DMEM for 2 h, then 5% fetal bovine serum DMEM was added, and cells were

incubated for an additional 48 h. Serum-free DMEM/F-12 medium was changed 1 hour before the start of the experiments.

Preparation of adenoviral shRNA: The generation of adenovirus harboring shRNA constructs of c-Cbl and control has been described previously.[5] Briefly, DNA sequences that encoded short hairpin RNA to c-Cbl (Ad-shCbl) 5′-CACCGCAGATGGCTCAAGAGACACGAATGTCTCTTGAGCCATCTGC-3′ or an inactive randomized control RNA (Ad-shCtrol) 5′-CACCGCCTCCAGCACTGTCGTTAACGAATTAACGACAGTGCTGGAGG-3′ were cloned into pENTRU6 vector, recombined into pAd/BLOCKit vector (Gateway system; Invitrogen), and packaged into recombinant adenoviruses expressing Ad-shCbl or Ad-Ctrl by using U293 cells (Invitrogen) following the manufacturer's instructions. Adenovirus was added to neonatal rat cardiomyocytes after 1 day of culture on collagen at 10 plaque forming unit (PFU)/cell, which resulted in a 75-85% down-regulation of c-Cbl relative to uninfected or control-shRNA-infected myocytes.

Immunostaining. Paraffin heart sections were deparaffinized in xylene and re-hydrated. Antigen retrieval was achieved by boiling the slides in citrate solution for 12-15 minutes and slides were then washed with phosphate-buffered Saline (PBS). After quenching endogenous tissue peroxidase activity with 3% H_2O_2 for 20 minutes, slides were then washed in PBS and samples blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 minutes. Primary antibodies to detect c-Cbl (BD Biosciences), CD31 (Santa-Cruz Biotechnology), SM α-actin (Sigma), and VEGFr2 (Cell Signaling) were applied overnight at 4°C in PBS containing 2% BSA. The next day, samples were washed in PBS and then sequentially incubated with Vectastain Elite ABC Kit (Avidin/Biotin/Horseradish Peroxidase-

System (Vector Laboratories) or with fluorophore-secondary antibody conjugates (Molecular Probes). The peroxidase reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Hematoxylin.

Cathepsin G activity in LV tissue lysates. Snap frozen LV tissues were homogenized in ice-cold buffer containing 100 mmol/L Hepes, pH 7.5, 1 mol/L NaOH, 50 mmol/L $CaCl_2$, and 0.01% Igepal CA-630 in presence or absence of Cat.G inhibitor. After centrifugation, supernatants containing 100 μg proteins were used for Cat.G activity assay by measurement of the rate of cleavage of fluorogenic conjugated substrate Suc-Ala_2-Pro-Phe-Amc (R&D Systems).

Measurement of myeloperoxidase activity. MPO activity in LV homogenates was determined using MPO peroxidation assay kit from Cayman Chemicals. Briefly, LV samples were suspended in lysis buffer provided in the kit and homogenates were cleared by centrifuging at 10,000 rpm at 4°C. The samples were incubated with hydrogen peroxide and the substrate ADHP (10-acetyl-3,7-dihydroxyphenoxazine). Fluorescence was then analyzed with an excitation wavelength of 540 nm and emission wavelength of 595 nm. The specificity of the assay was confirmed by addition of a MPO inhibitor (4-aminobenzhydrazide) in the reaction mixture prior to the assay.

Terminal deoxynucleotidyl transferase (TdT) and tropomyosin immunolabeling. Three sections from each LV cut perpendicularly to the major axis of the heart were sampled. TdT assay was performed using kit from Promega. Positive cells were counted throughout the LV and were expressed as percentage of the total number of nuclei as determined by DAPI (Molecular probes) staining.

Apoptotic Cell Death ELISA: A cell death detection ELISA kit (Roche Applied Science) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes fragments associated with apoptotic cell death.

Supplemental Table 1. Demographic and clinical data.

	Non-failing (n=5)	DCM (n=5)	ICM (n=5)
Age (years)	62 ± 9	49 ± 11	52 ± 9
Gender (male/female)	1/4	2/3	5/0
Heart Weight (g)	418 ± 68	561 ± 218	514 ± 165
Ejection Fraction (%)	60 ± 5	14 ± 6 *	24 ± 22 *
Inotropes (yes/no)	1/4	2/3	2/3
ACE inhibitors (yes/no)	0/5	5/0	3/2
β-blockers (yes/no)	1/4	2/3	3/2

DCM: dilated cardiomyopathy; ICM: ischemic cardiomyopathy; ACE: angiotensin II converting enzyme. *$p<0.001$ compared to non-failing group.

Supplemental Table 2. Echocardiographic measurements in CM-Cbl KO and $Cbl^{flox/flox}$ littermates subjected or not to IR injury.

	Sham		**IR**	
	$Cbl^{flox/flox}$ (n=5)	**CM-Cbl KO (n=6)**	**$Cbl^{flox/flox}$ (n=5)**	**CM-Cbl KO (n=6)**
HR (bpm)	465 ±18	453 ±14	473 ±24	485 ±32
HW (mg)	111 ±10	108 ±10	110 ±8	104 ±7
BW (g)	21 ±2	21 ±3	21 ±2	19 ±2
HW/BW (mg/g)	5.3 ±0.2	5.1 ±0.1	5.4 ±0.2	5.5 ±0.3
LVEDD (mm)	3.66 ±0.13	3.43 ±0.12	3.97 ±0.11*	3.63 ±0.12†
LVESD (mm)	2.34 ±0.12	2.18 ±0.14	3.12 ±0.13*	2.60 ±0.15*†
LVPWTd (mm)	0.78 ±0.01	0.83 ±0.02	0.76 ±0.04	0.86 ±0.06
LVPWTs (mm)	1.12 ±0.05	1.17 ±0.08	0.94 ±0.06	1.07 ±0.10

HR indicates heart rate; HW, heart weight; BW, body weight; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. WT shams, †P<0.05 vs. WT IR

Supplemental Table 3. Echocardiographic measurements in WT and c-Cbl KO mice subjected or not to 7d MI injury.

	Sham		**7d MI**	
	WT (n=5)	**c-Cbl KO (n=5)**	**WT (n=6)**	**c-Cbl KO (n=6)**
HR (bpm)	428 ±13	471 ±24	482 ±26	484 ±32
HW (mg)	137 ±5	117 ±10	199 ±10*	173 ±11*†
BW (g)	24 ±2	25 ±2	21 ±1	22 ±2
HW/BW (mg/g)	5.7 ±0.3	5.5 ±0.3	9.5 ±0.3*	7.9 ±0.4*†
LVEF (%)	65 ±2	64 ±2	20 ±3*	44 ±3*†
LVFS (FS)	36 ±2	34 ±3	10 ±2*	21 ±2*†
LVEDD (mm)	3.39 ±0.10	3.15 ±0.15	4.93 ±0.27*	3.81 ±0.2*†
LVESD (mm)	2.19 ±0.1	2.00 ±0.12	4.48 ±0.26*	2.98 ±0.17*†
LVPWTd (mm)	0.98 ±0.01	0.94 ±0.01	0.59 ±0.04*	0.85 ±0.04*†
LVPWTs (mm)	1.45 ±0.04	1.49 ±0.03	0.70 ±0.03*	1.25 ±0.09*†

HR indicates heart rate; HW, heart weight; BW, body weight; LVEF, LV ejection fraction; LVFS, LV fractional shortening; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. WT shams, †P<0.05 vs. WT MI

Supplemental Table 4. Echocardiographic measurements in WT and c-Cbl KO mice subjected or not to 30d MI injury.

	Sham		**30d MI**	
	WT (n=6)	**c-Cbl KO (n=6)**	**WT (n=8)**	**c-Cbl KO (n=8)**
HR (bpm)	439 ±13	471 ±24	477 ±16	484 ±32
HW (mg)	135 ±5	117 ±10	209 ±10*	193 ±21*†
BW (g)	25 ±2	25 ±2	23 ±1	25 ±2
HW/BW (mg/g)	5.2 ±0.2	5.6 ±0.4	9.17 ±0.3*	8.14 ±0.3*†
LVEF (%)	63 ±2	64 ±2	30 ±2*	45 ±3*†
LVFS (FS)	34 ±2	35 ±2	15 ±1*	23 ±2*†
LVEDD (mm)	3.41 ±0.10	3.23 ±0.16	4.83 ±0.27*	4.09 ±0.2*†
LVESD (mm)	2.10 ±0.1	2.04 ±0.20	4.19 ±0.26*	3.30 ±0.05*†
LVPWTd (mm)	0.99 ±0.01	0.93 ±0.02	0.74 ±0.04*	0.98 ±0.04†
LVPWTs (mm)	1.58 ±0.04	1.38 ±0.04	0.92 ±0.03*	1.26 ±0.09*†

HR indicates heart rate; HW, heart weight; BW, body weight; LVEF, LV ejection fraction; LVFS, LV fractional shortening; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. WT shams, †P<0.05 vs. WT MI

SUPLLENTARY FIGURE LEGEND

Supplemental Figure S1. Targeting strategy for specific disruption of c-Cbl in cardiac myocytes. (A) Generation of c-Cbl floxed mutant mice has been previously described.[1] In brief, the floxed Cbl allele was generated by introducing two loxP sequences (triangles) into the introns flanking one of the exons of c-Cbl (corresponding to nucleotides 681−837 of the mouse c-Cbl cDNA) by gene targeting. Removal of the floxed exon by Cre-loxP-mediated DNA recombination generated transcripts out-of-frame for translation. PTB, phosphotyrosine binding domain; RF, RING-finger domain; LZ, leucine-zipper domain; S, SphI site. (B) Cardiac myocyte-specific deletion of c-Cbl mediated by αMHC-MerCreMer transgene expression. Genomic DNA samples from heart tissues obtained from $Cbl^{flox/flox}$ and CM-Cbl KO mice (α-MHC-MerCreMer×c-$Cbl^{flox/flox}$) were digested with SphI and hybridized with the probe in (A). (C) Immunoblot analysis of c-Cbl deletion in the heart. Heart lysates were immunoblotted with c-Cbl antibodies. The same membrane was reprobed for GAPDH as a loading control.

Supplemental Figure S2: Cardiac myocyte-specific deletion of c-Cbl improves cardiac function. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. Cardiac function was measured by echocardiography after IR injury. The data demonstrate improved left ventricular ejection fraction (A) and fractional shortening (B) in CM-Cbl KO compared to c-$Cbl^{flox/flox}$ mice (n=7 in sham groups; n=8 in MI groups). (C and D) Cardiac-myocyte deletion of c-Cbl attenuated IR-induced increase in LV end-systolic (C) and end-diastolic dimension (D). (E) Quantification of infarct area (IA) vs. area at risk (AAR) after IR injury shows significantly reduced infarct in CM-Cbl KO compared to c-$Cbl^{flox/flox}$ mice (n=6 per group). *P<0.05 vs. c-$Cbl^{flox/flox}$ shams. $^{\dagger}P$<0.05 vs. c-$Cbl^{flox/flox}$ IR.

Supplemental Figure S3: Cardiac myocyte-specific deletion of c-Cbl reduces apoptotic cell death. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. (A) LV tissue sections from $Cbl^{flox/flox}$ (a, c) or CM-Cbl KO (b, d) were assessed for apoptosis using TUNEL assay (green), tropomyosin (red), and DAPI (4',6-diamidino-2-phenylindole) (blue) staining. Arrows indicate apoptotic myocytes. Bar=40 μm (a, b) or 20 μm (c, d). (B) The number of TUNEL-positive myocytes in the ischemic area was expressed as a percentage of total nuclei detected by DAPI staining. (C) Quantification of caspase-3 activity in the LV using caspase-3 specific fluorogenic substrate. **P*<0.05 vs. $Cbl^{flox/flox}$ shams. †*P*<0.05 vs. $Cbl^{flox/flox}$ IR.

Supplemental Figure S4. c-Cbl deletion attenuates H2O2-induced AKT , but not JNK, phosphorylation. Neonatal rat cardiac myocytes were transduced with adenoviruses expressing shRNA c-Cbl or shRNA control for 48 hours and then untreated or treated with 100 ng/ml EGF or 100 μM H2O2 for 10 minutes. Cell lysates were assayed for immunoblot analysis. Experiments were performed at least three times from three different cultures and the data values were scaled to untreated Ad-shCtrl. *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

Supplemental Figure S5. c-Cbl ubiquitin ligase activity is involved in H2O2-induced myocyte apoptosis. (A) Neonatal rat cardiac myocytes were transduced with adenoviruses expressing Lac.Z or ligase deficient c-Cbl mutant 70Z-Cbl for 48 hours and then untreated or treated with 100 ng/ml EGF or 100 μM H2O2 for 10 minutes. Cell lysates were assayed for EGFR immunoprecipitation assays (A) or immunoblot analysis (B). Experiments were

performed at least three times from three different cultures and the data values were scaled to untreated Ad-shCtrl. (C-D) Myocyte apoptosis induced by H2O2 as assessed by percentage of TUNEL-positive myocytes in culture (C) or DNA fragmentation assay (D). Results are expressed as (OD410-OD500)/mg DNA (D) for triplicate determinations from a single experiment (mean ± SE). *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

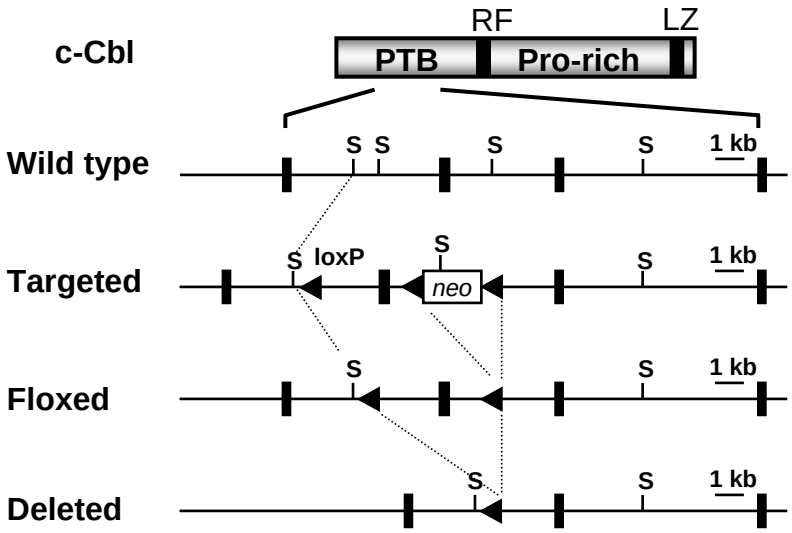
Supplemental Figure S6. Deletion of c-Cbl reduces myocyte hypertrophy 4 weeks after MI. c-Cbl or WT mice were subjected to MI for 4 weeks. (A) Histological assessment of cellular pathology of c-Cbl KO or WT mice by Masson's trichrome staining. Scale Bar: 40 μm. (B) Histological analysis of myocyte cross-sectional area from sham and infarcted LV. Values are mean ±SE. *p<0.05 vs. WT sham; †P<0.05 vs. WT MI.

Supplemental Figure S7. Effect of c-Cbl deletion on $Erk_{1/2}$ and AKT phosphorylation in infarcted LV. *Top:* Immunoblot analysis indicates a similar increase in $Erk_{1/2}$ and AKT phosphorylation in the infarcted region of the c-Cbl KO mice and the WT 7 days post-MI. *Bottom*: Data are represented as fold change compared to WT animals (n=6 per group; means±SEM). *P<0.05 compared with the WT sham group, †P<0.05 compared with the WT MI group.

Supplemental Figure S8. Leukocyte infiltration and activation induced after IR injury is minimally affected by c-Cbl deletion. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. (A) Representative immunolabeling of paraffin-embedded heart sections stained for MPO and counterstained with

hematoxylin. Scale Bar: 40 μm. (B) Quantification of MPO positive cells in the ischemia reperfused area. (C-D) LV homogenates from shams or mice subjected to IR for 24 hours were assessed for myeloperoxidase (MPO) (C) or cathepsin G (Cat.G)(D) activity assay using specific fluorogenic substrates. Results are expressed as relative fluorescence unit (RFU)/min/g protein. Values are mean ±SEM. *$p<0.05$; **$p<0.01$ vs. WT sham.

A



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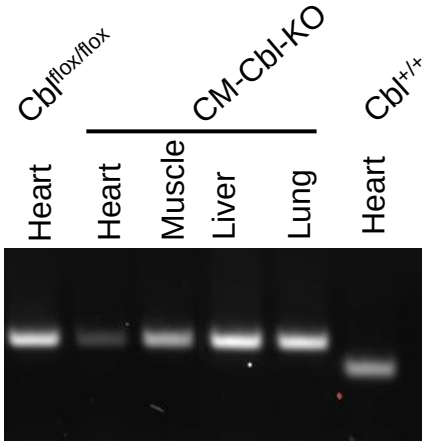

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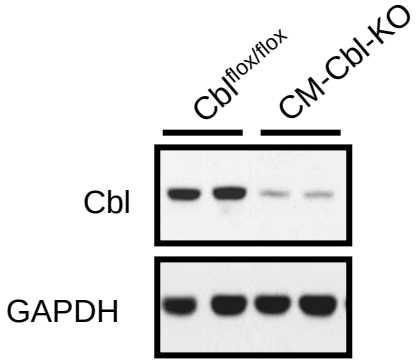
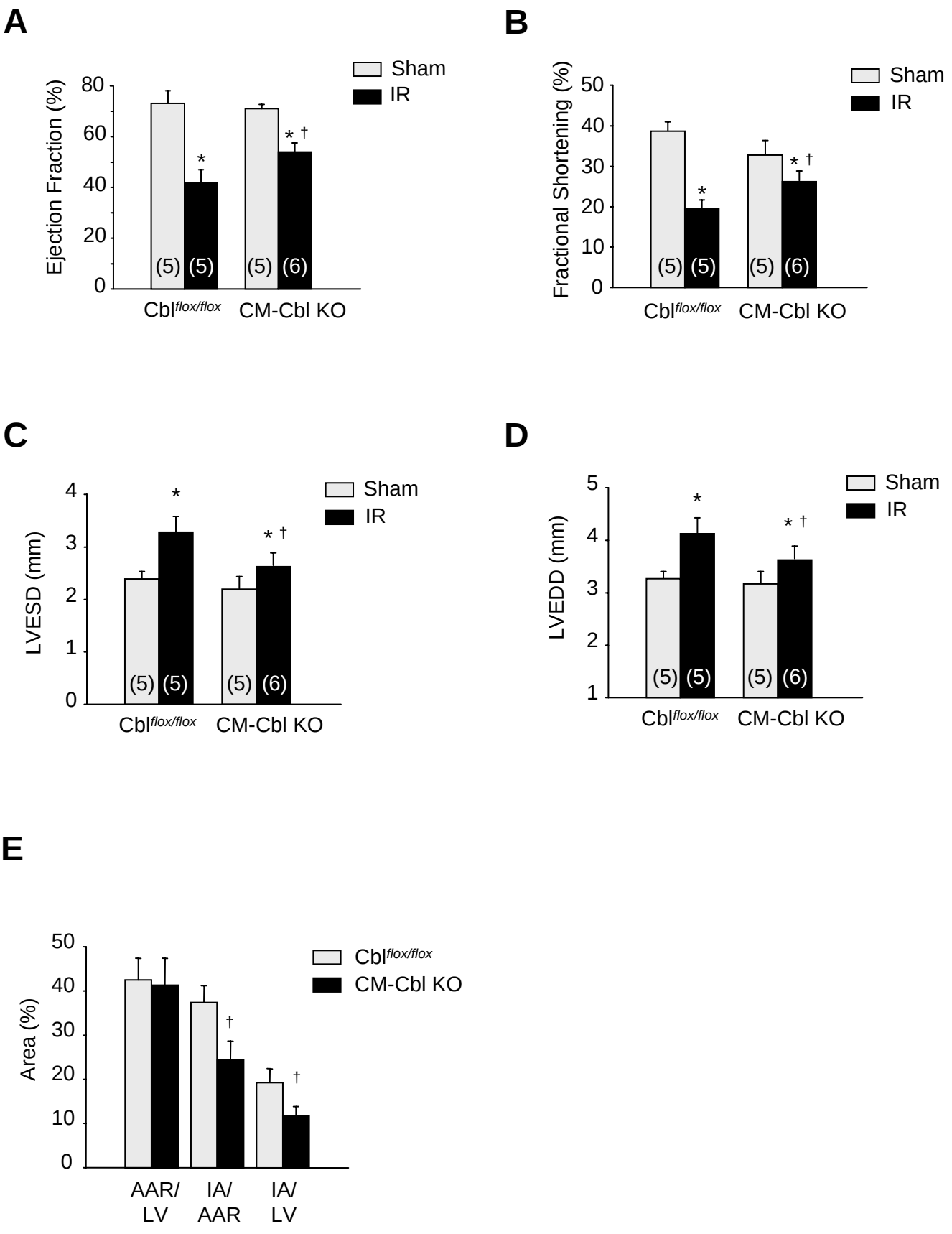


Supplemental Figure S1



Supplemental Figure S2

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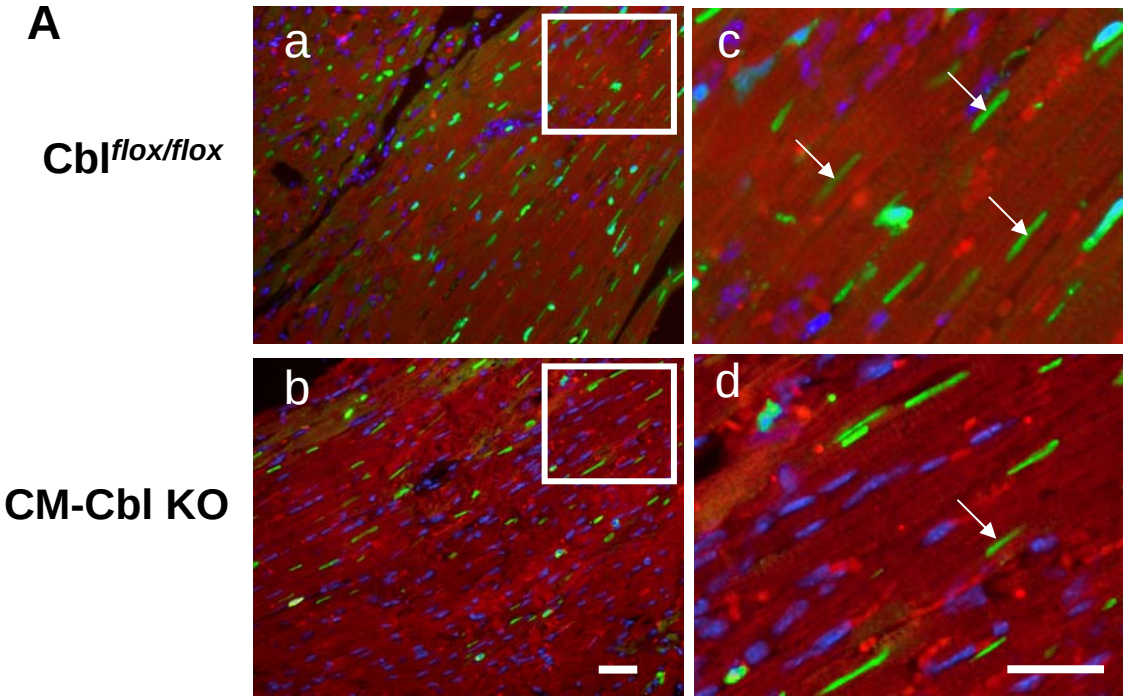

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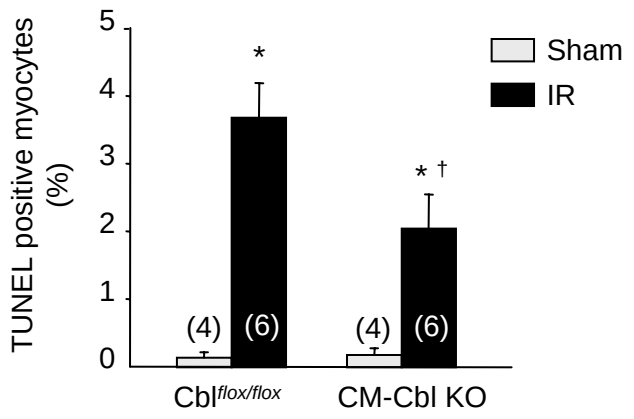

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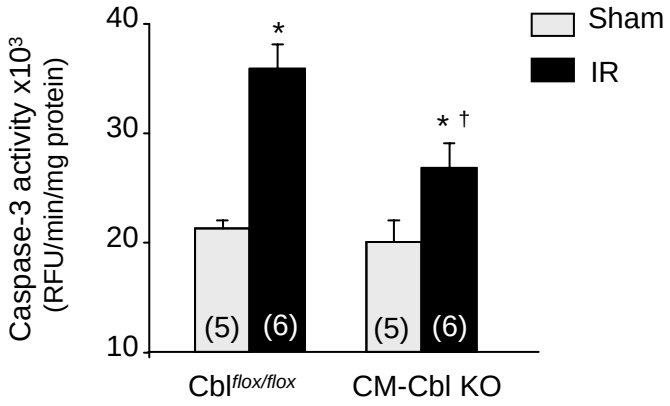
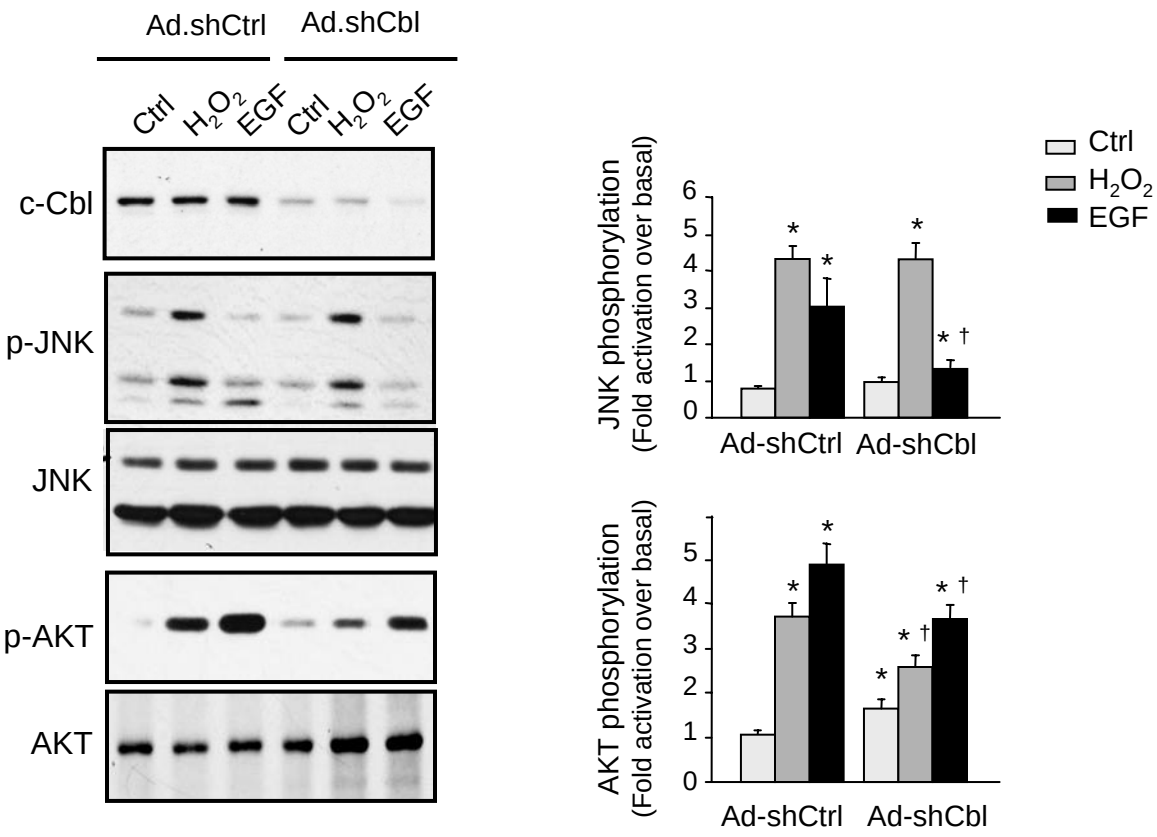


Supplemental Figure S3



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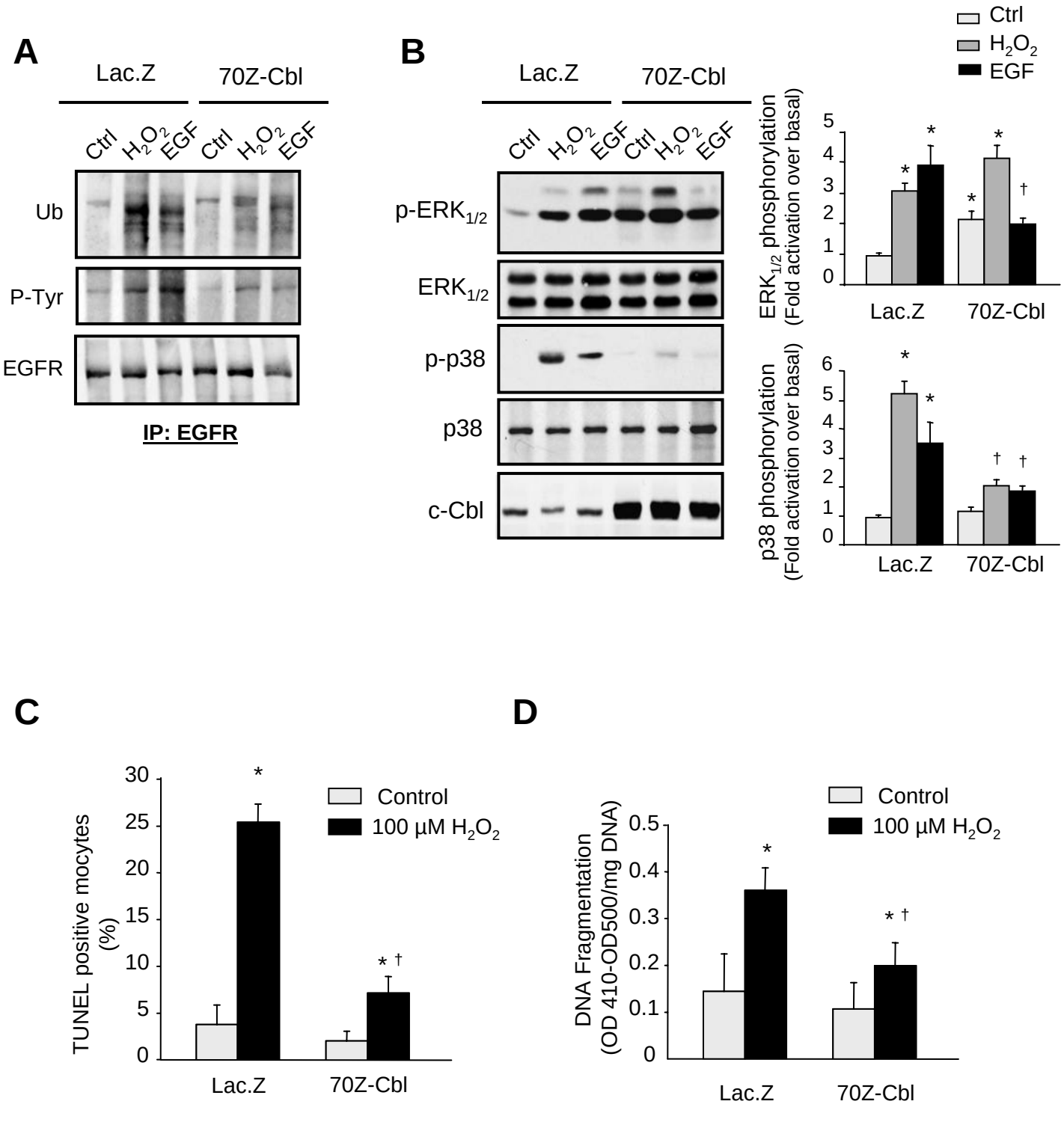

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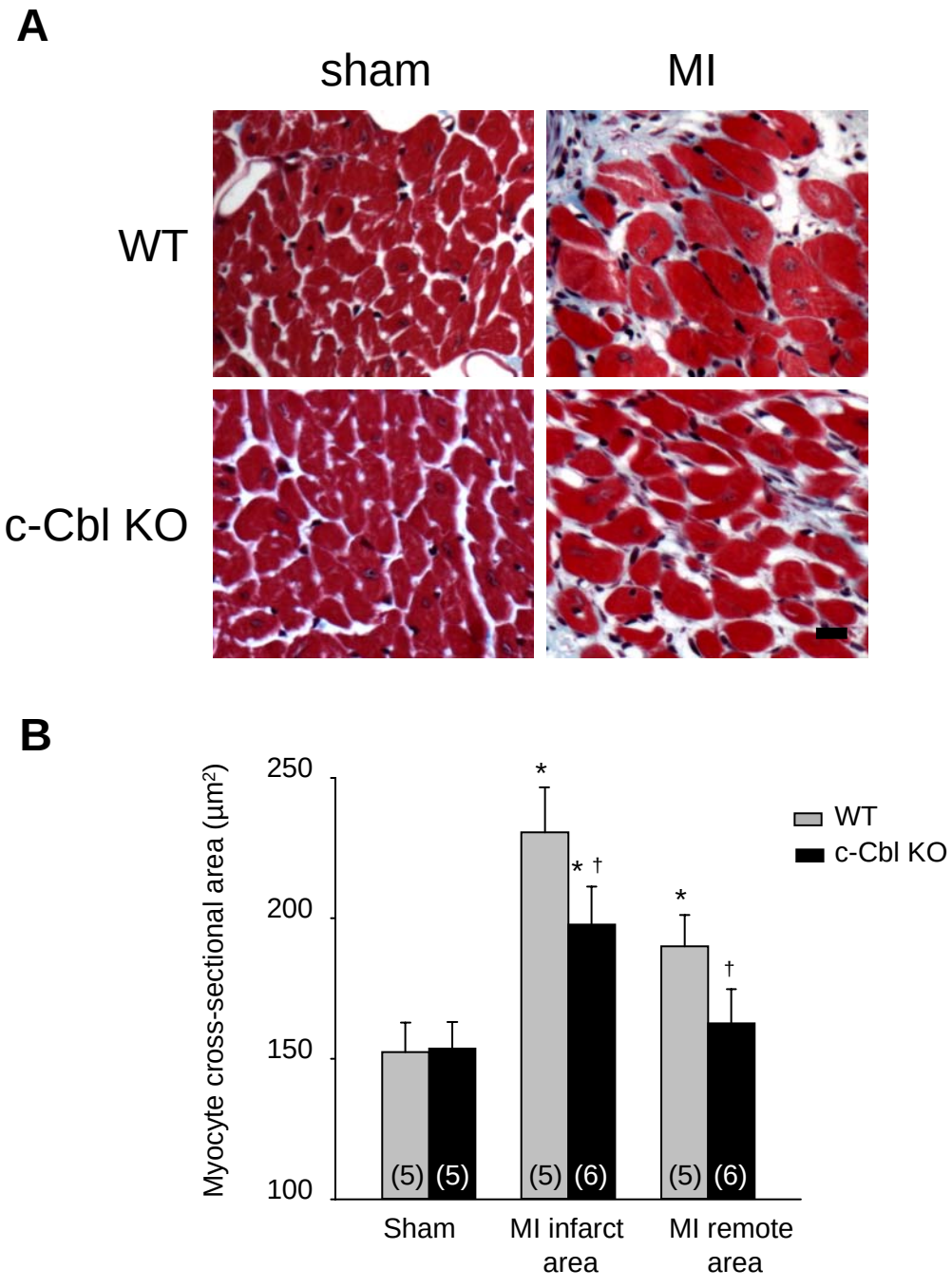

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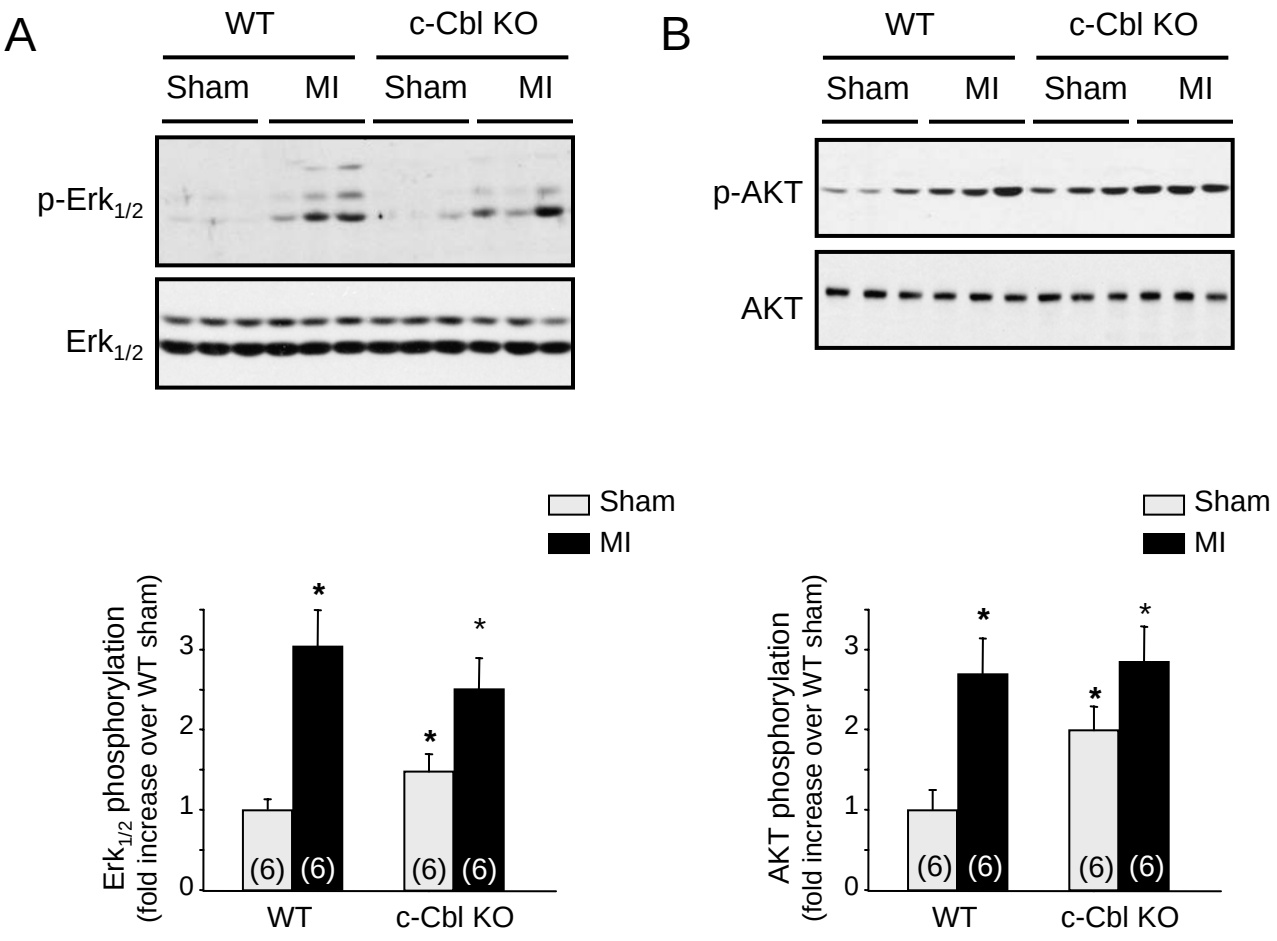

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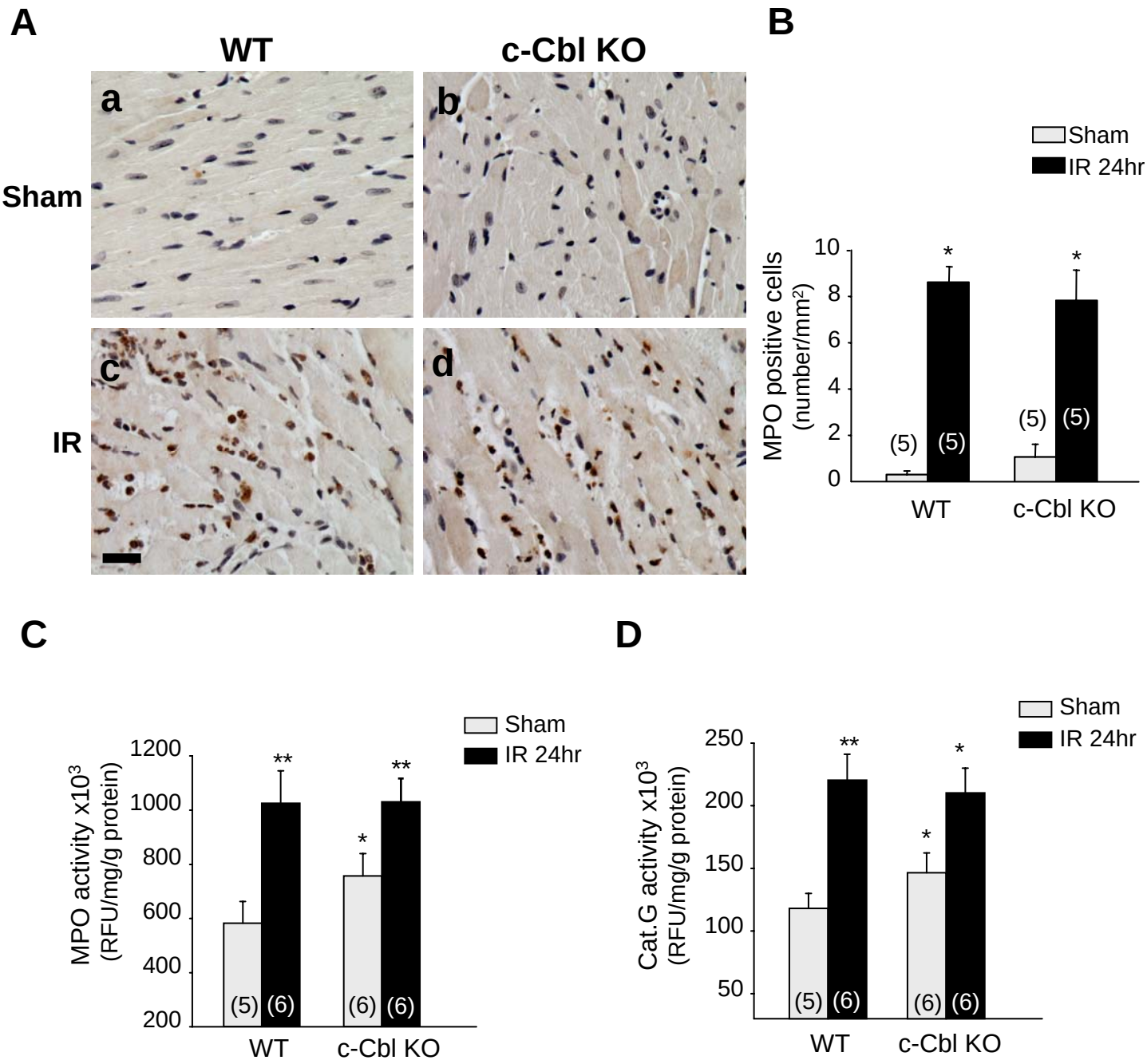

Supplemental Figure S8

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