SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIALS & METHODS

Materials: Polyclonal antibody for PAR4 used for immunohistochemistry was from MBL International (MC-1309). Phospho-JNK, phospho-Erk$_{1/2}$, phospho-p38 MAPK, S^{473}-AKT, Bcl2 and XIAP were from Cell Signaling (9251, 9106, 9211, 9271, 9942 and 2042, respectively). PAR1/2 and PAR4 agonist peptide, SFLLRN and AYPGKF, were synthetized by Invitrogen. JNK inhibitor (SP600125), Erk$_{1/2}$ inhibitor (PD98059), p38 MAPK inhibitor (SB203580), Src family kinase inhibitor (PP1), EGFR kinase inhibitor (AG1478) and PKC inhibitor (GF109203X) were from Calbiochem. All other chemicals were from standard suppliers.

Heart function: Echocardiographic measurements were taken before surgery and at 24 h after IR injury to determine the baseline heart function and ventricular dimensions in the experimental groups [1]. 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 = $[(LVEDD)^3 – (LVESD)^3]/(LVEDD)^3$.

Isolated perfused (ex vivo) IR: Hearts from 12 weeks old male mice were removed quickly and perfused in a retrograde manner with modified Krebs-Henseleit buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11 mM glucose, 1.5 mM Na-Pyruvate, and 2 mM $CaCl_2$) in a Langendorff apparatus at constant pressure (80 mmHg). The buffer was continuously gassed with 95% O_2 + 5% CO_2 (pH~7.4) and warmed by a heating bath/circulator. The heart temperature was continuously monitored and maintained at 37±0.5°C. To record cardiac contractile function, a water-filled balloon made of plastic film connected to a pressure transducer was inserted into the LV after the right atrium was removed. Hearts were paced at 480 beats min^{-1} via silver chloride electrode on the LV and were stabilized for 15 min as we described previously [1]. After stabilization, hearts were subjected to 45 minutes of global ischemia, followed by 60 minutes of reperfusion. The LVDP (peak systolic pressure minus LV end-diastolic pressure [LVEDP]), +dP/dt, and -dP/dt were monitored and recorded. To measure infarct size, hearts subjected to IR were frozen and then cut transversely into 5 slices of equal thickness. The slices were then incubated in 1% TTC and fixed in 10% formalin-PBS for 24 h. Fixed slices were then scanned, and Bioquant was used to measure and calculate the size of the infarct area and the total area.

Assessment of area at risk and infarct size: After 24 h 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 area 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.

Neonatal rat cardiomyocyte isolation: Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described [2,3]. 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 h of plating.

Immunoblot analysis: Extraction of proteins from heart tissue samples was performed as described previously [2,3]. Briefly, hearts were homogenized in Triton X-100 extraction buffer with inhibitors and partially purified plasma membrane was prepared. Lysates were cleared by centrifugation and the supernatants were subjected to immunoblot analysis according to methods published previously or to the manufacturers' instructions [2,3]. 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.

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).

Expression of adenoviral vectors: Production of recombinant adenovirus expressing β-galactosidase (Lac.Z) and human wild-type PAR4 (WT-PAR4) was described elsewhere [1,2].

The WT-PAR4 plasmid was provided by Dr. Kunapuli S (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 h of plating, NRCMs were infected with adenoviruses expressing Lac-Z or WT-PAR4 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 h before the start of the experiments.

Preparation of adenoviral shRNA: For knockdown of PAR4, DNA sequences that encoded short hairpin RNA to PAR4 (Ad-shPAR4) 5′-CCCUCAGGACAUGACCUUA-3′ or an inactive randomized control RNA (Ad-shCtrl) 5′-UAAGGUCAUGUCCUUGAGGG-3′ were cloned into pENTRU6 vector, recombined into pAd/BLOCKit vector (Gateway system; Invitrogen), and packaged into recombinant adenoviruses expressing Ad-shPAR4 or Ad-Ctrl by using U293 cells (Invitrogen) following the manufacturer's instructions. Adenovirus was added to neonatal rat cardiomyocytes after 1 d of culture on collagen at 10 pfu/cell, which resulted in a 75-85% down-regulation of PAR4 relative to uninfected or control-shRNA-infected myocytes.

RNA isolation and real-time quantitative PCR (RT qPCR): Total RNA was isolated using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. RNA (1 μg) was used to generate cDNA using Superscript III (Invitrogen) according to the protocol of the manufacturer and detected using SYBR Green PCR Master Mix (Applied Biosystems). Primers for RT qPCR were the following: for PAR4 - forward 5′-GCCAATGGGCTGGCGCTGTG-3′, reverse 5′-GCCAGGCAGATGAAGGCCGG-3′; for PAR1 - forward 5′-CAGCCAGAATCAGAGAGGACAGA-3′, reverse 5′-CCAGCAGGACGCTTTCATTT-3′; for PAR2 - forward 5′-AGCCGGACCGAGAACCTT-3′, reverse 5′-GGAACCCCTTTCCCAGTGATT-3′; for PAR3 – forward 5′-

CGGTAAGGCTGAGCAAGAAAACC-3′, reverse – 5′-TGAGGCGTGAGCACTATGAGGTAG-3′, for TNFα - forward 5′-TGAACTTCGGGGTGATCGG-3′, reverse 5′-GGCTTGTCACTCGAATTTTGAGA-3′; for IL1ß - forward 5′-CAACCAACAAGTGATATTCTCCATG-3′, reverse 5′-GATCCACACTCTCCAGCTGCA-3′, for IL6 - forward 5′-ATGGATGCTACCAAACTGGAT-3′, reverse 5′-TGAAGGACTCTGGCTTTGTCT-3′; for GAPDH - forward 5'-CATGGCCTTCCGTGTTCCTA-3′, reverse 5′-CCTGCTTCACCACCTTCTTGAT-3′. Gene expression levels relative to GAPDH were determined using the $2^{-\Delta Ct}$ method [4] and expressed relative to controls.

Immunohistochemistry: Tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm intervals. Paraffin heart sections were deparaffinized in xylene and re-hydrated. Antigen retrieval was achieved by boiling the slides in citrate solution for 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 blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 minutes. Primary antibodies to detect PAR4 or myeloperoxidase (Thermo, *RB-373-A*) 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 (Cat.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 MPO 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 (G3250). Positive myocytes were counted throughout the LV and were expressed as percentage of the total number of cardiomyocyte nuclei as determined by DAPI (Molecular probes, D1306) and tropomyosin (Sigma, T9283) staining.

Caspase-3 Assay: Caspase 3 activity was measured with CaspACE assay system (Promega). In brief, LV lysates were prepared by homogenization in lysis buffer provided with the kit. The lysates were centrifuged at 15,000g for 20 minutes at 4 ºC, and the supernatants containing 100 μg protein were used for caspase-3 activity assay using specific fluorogenic conjugated substrate.

SUPPLEMENTAL REFERENCES

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[2] Rafiq K, Kolpakov MA, Seqqat R, Guo J, Guo X, Qi Z, et al. c-Cbl inhibition improves cardiac function and survival in response to myocardial ischemia. Circulation. 2014. 129(20):2031-43.

[3] Rafiq K, Hanscom M, Valerie K, Steinberg S, Sabri A. Novel mode for neutrophil protease cathepsin G-mediated signaling: Membrane shedding of epidermal grwoth factor is required for cardiomyocyte anoikis. Circ Res. 2008;102:32-41.

[4] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protocols. 2008;3:1101-8.

Supplemental Table 1. Summary of heart weight, body weight and echocardiographic measurements in WT and PAR4 KO mice subjected or not to IR injury.

	Sham		**IR**	
	WT (n=8)	**PAR4 KO (n=8)**	**WT (n=8)**	**PAR4 KO (n=8)**
HR (bpm)	460±11	466±10	488±18	494±37
HW (mg)	120±1	133±10	131±4	137±10
BW (g)	23±0.9	24.0±0.9	24.2±0.6	24.4±0.9
HW/BW (mg/g)	5.3±0.2	5.46±0.3	5.5±0.2	5.6±0.2
LVEDD (mm)	3.66±0.19	3.38±0.10	4.07±0.14	3.83±0.31
LVESD (mm)	2.45±0.13	2.25±0.05	3.35±0.15*	2.9±0.23*†
LVPWTd (mm)	0.97±0.03	1.09±0.04	0.97±0.06	0.95±0.05
LVPWTs (mm)	1.49±0.05	1.37±0.04	1.38±0.05	1.39±0.05

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

SUPPLEMENTARY FIGURE LEGEND

Supplemental Fig. S1: Specificity of PAR4 antibody. (A) Representative immunostaining of paraffin-embedded heart sections of PAR4 KO sham or after ischemia reperfusion (IR) injury stained for PAR4 and counterstained with hematoxylin. (B) Representative immunostaining of paraffin-embedded heart sections of WT sham or after IR injury stained with polyclonal IgG and counterstained with hematoxylin. Note that no PAR4 immunostaining was detected in both A or B. Scale bar: 40 μm.

Supplemental Fig. S2: PAR4 deletion reduces apoptosis after ex vivo IR. Isolated hearts from WT (n=5) and PAR4 KO (n=5) mice were subjected to global ischemia for 45 minutes and reperfusion for 60 minutes (IR). Quantification of caspase-3 activity in the LV using caspase-3 specific fluorogenic substrate. Values are expressed as relative fluorescence unit (RFU)/min/mg protein and are presented as mean ± SEM, $^{\dagger}P<0.05$ vs. WT IR.

Supplemental Fig. S3: Acute PAR4 stimulation induces JNK activation. Neonatal rat cardiomyocytes (NRCMs) were untreated or treated with 10 U/ml thrombin (Thr.) or 500 μM PAR4-agonist peptide (AYPGKF) for the indicated time. Cell lysates were processed for immunoblot analysis. Results are representative of 3 independent experiments. Data are mean ± SEM; *P<0.05 vs. control.

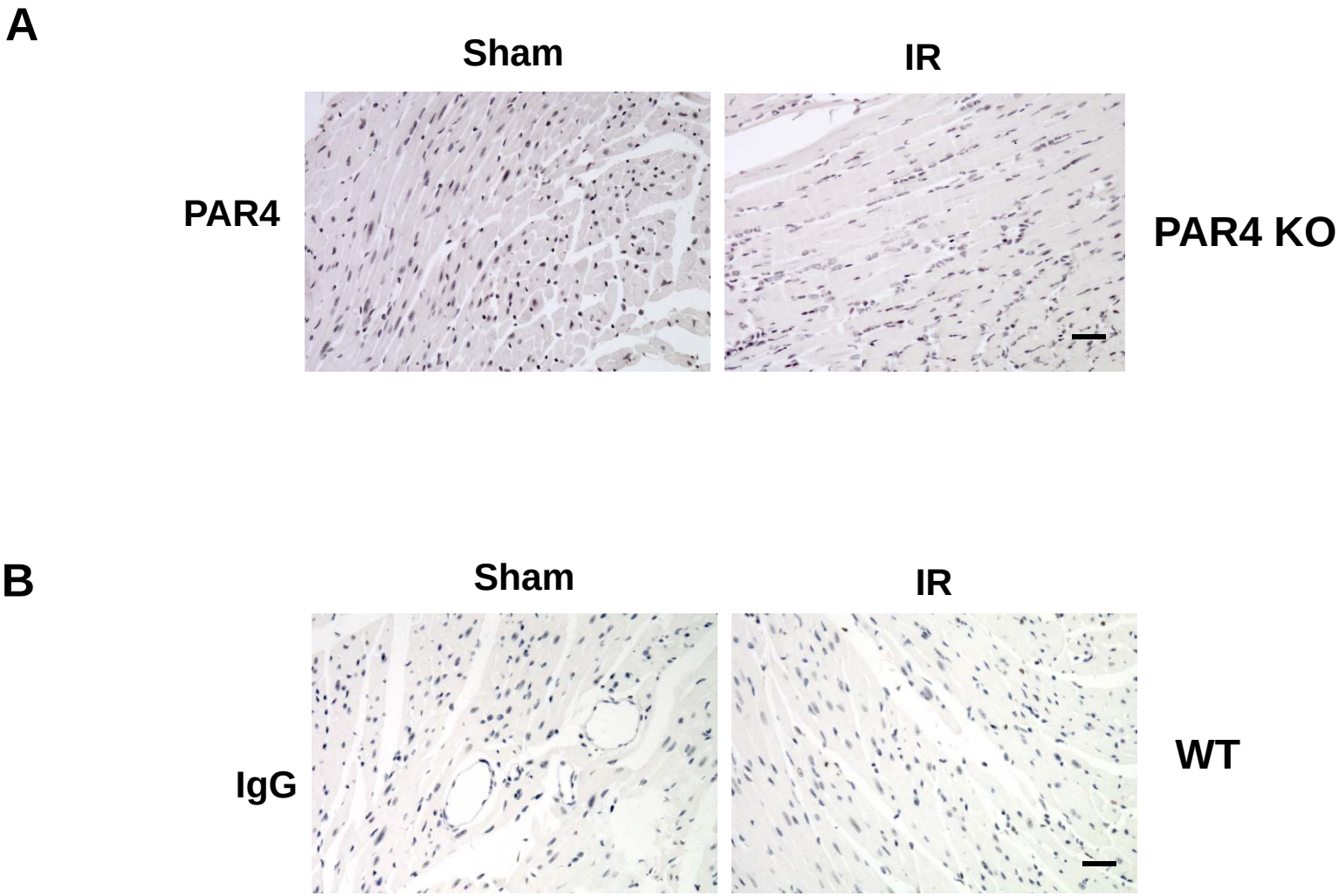
Supplemental Fig. S4: Effects of PAR4 expression on $Erk_{1/2}$ and p38 MAP kinase phosphorylation in cardiomyocytes. Neonatal rat cardiomyocytes (NRCMs) were infected with Ad-shCtrl, Ad-shPAR4 (**A-C**), Lac.Z or WT-PAR4 (**D-F**) adenoviruses and were either untreated

or treated with 10 U/ml thrombin, 300 μM PAR1/PAR2- (SFLLRN) or 500 μM PAR4-agonist peptide (AYPGKF) for 24 h. Cell lysates were processed for immunoblot analysis. Results are representative of 3 independent experiments. Data are mean ± SEM; *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

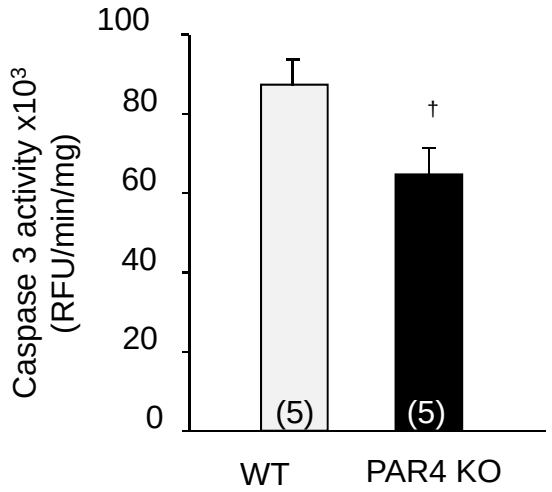
Supplemental Fig. S5: Effects of $Erk_{1/2}$ or p38 MAPK inhibition on PAR4-induced myocyte apoptosis. NRCMs were pretreated with vehicle, 5 μM PD98059 or 10 μM SB203580 for 30 minutes prior to treatment with 10 U/ml thrombin (Thr.), 300 μM PAR1/PAR2- (SFLLRN) or 500 μM PAR4-agonist peptide (AYPGKF) for 24 h. Myocyte apoptosis was assessed by quantification of the percentage of TUNEL-positive myocytes in culture (**A**) or by caspase-3 activity assay (**B**). Results are expressed as relative fluorescence unit (RFU)/min/mg of proteins for triplicate determinations from a single experiment (mean ± SEM). *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

Supplemental Fig. S6: PAR4-induced myocyte apoptosis involves Src and EGFR kinase activity. NRCMs were pretreated with vehicle, 5 μM PP1 or 5 μM AG1478 for 30 minutes prior to treatment with 10 U/ml thrombin (Thr) or 500 μM PAR4-agonist peptide (AYP GKF) for 24 h. Myocyte apoptosis was assessed by caspase-3 activity assay. Results are expressed as relative fluorescence unit (RFU)/min/mg of proteins for triplicate determinations from a single experiment (mean ± SEM). *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

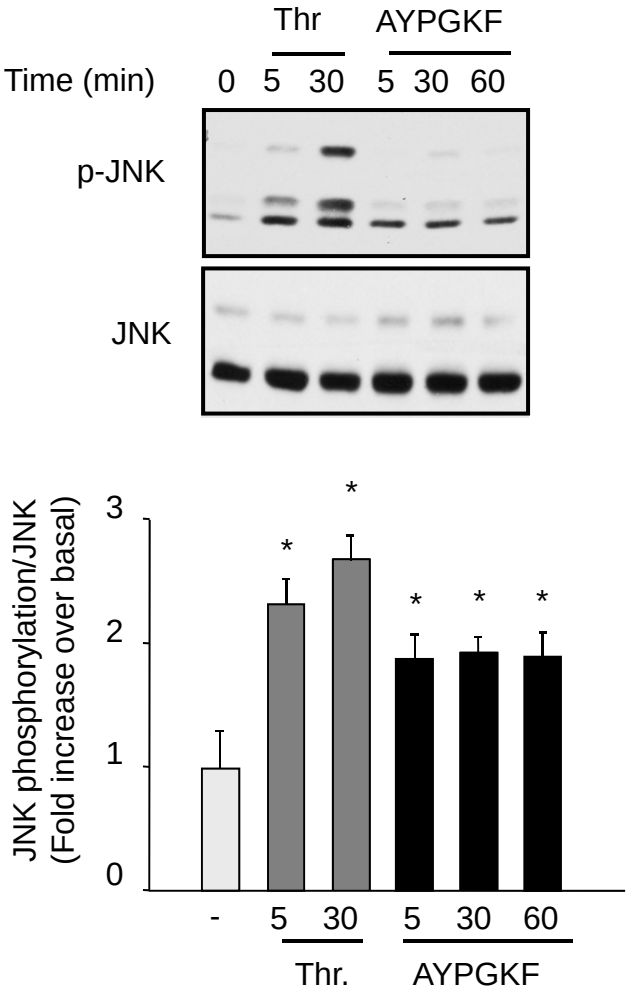
Supplemental Fig. S7: Schematic representation of PAR4 signaling pathways involved in myocyte apoptosis.



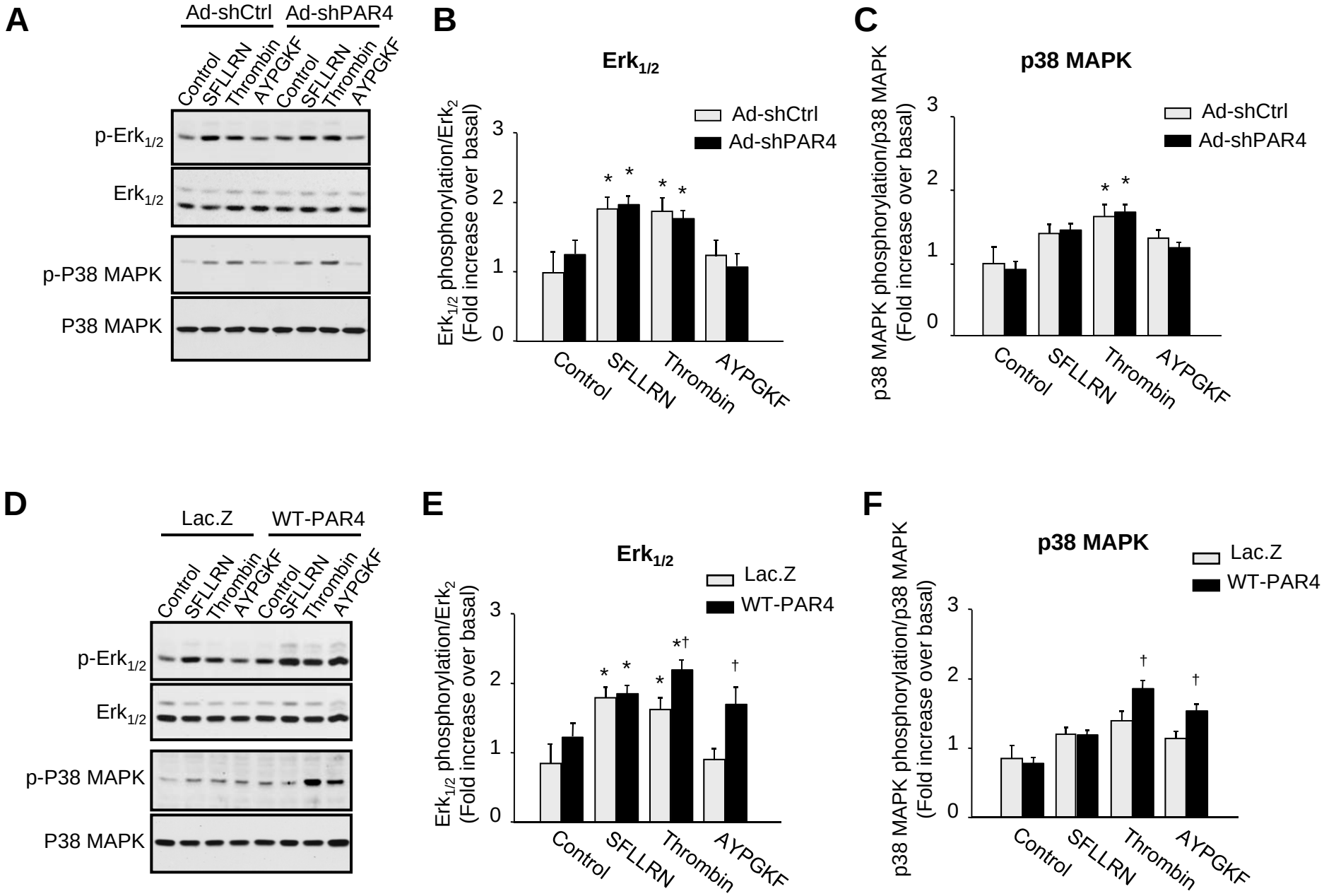
Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3



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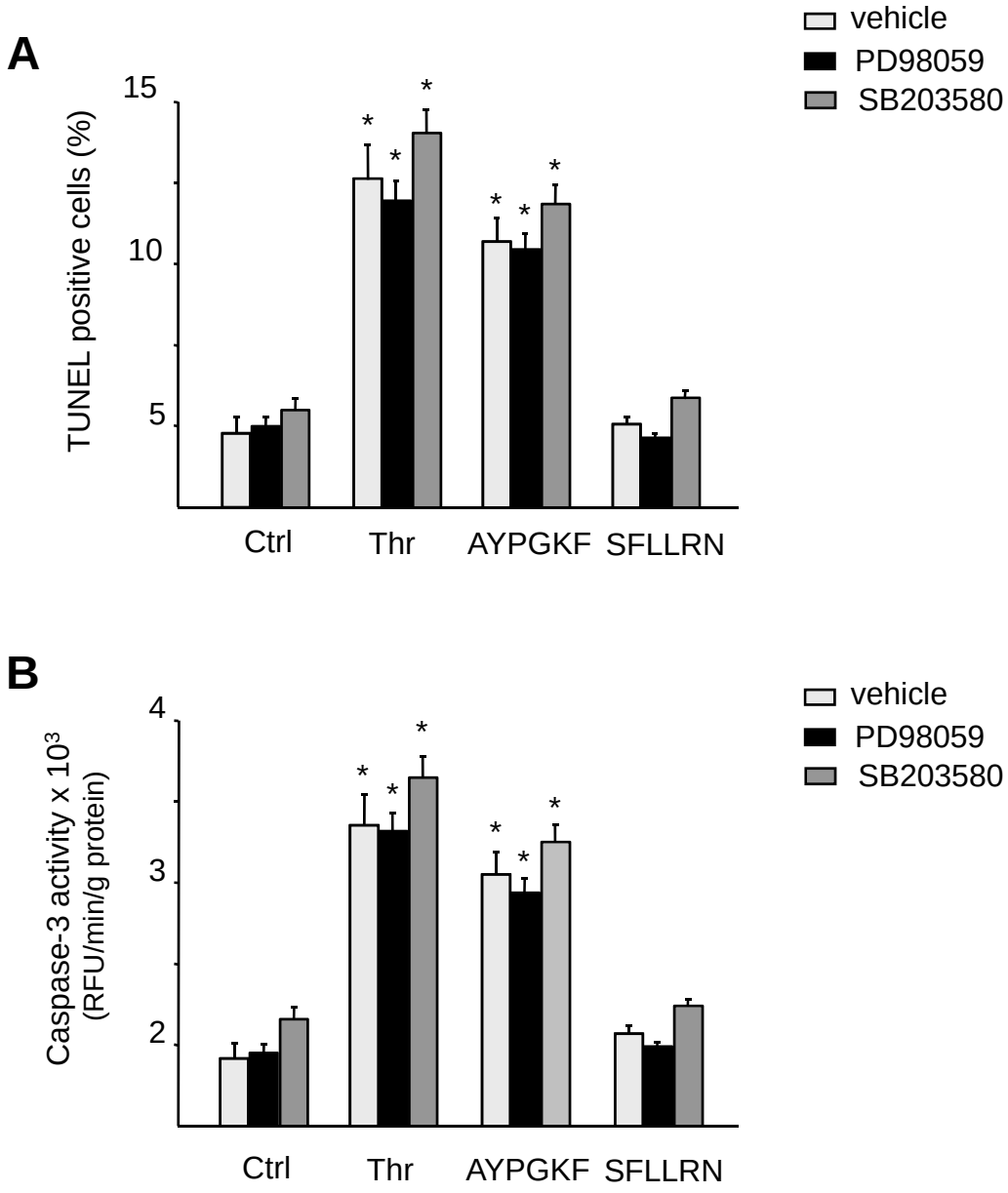

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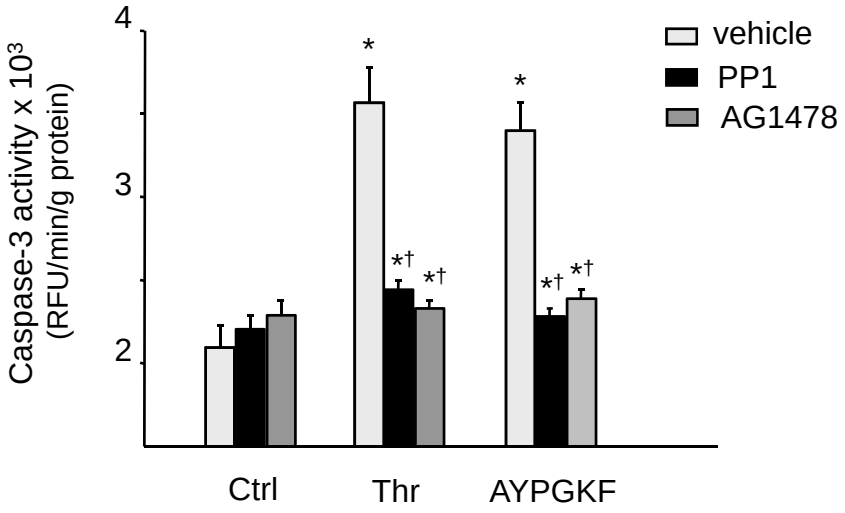
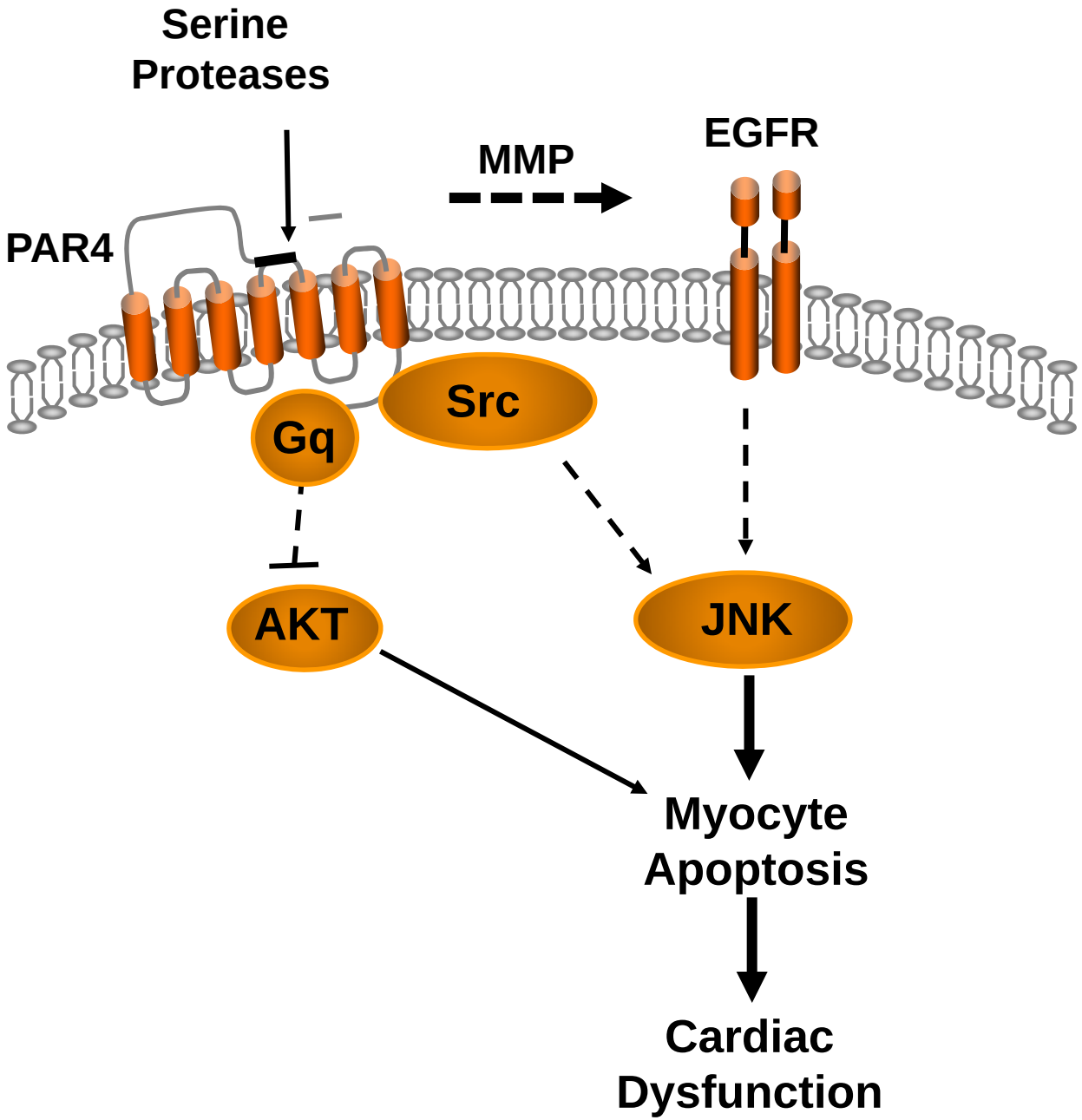


Supplemental Figure S6



Supplemental Figure S7