Supplemental Material

Supplemental Methods:

Antibodies and reagents

TRAP and Thrombin (Cayman Chemicals), ADP (Tocris, Bristol, UK), Convulxin (Santa Cruz Biotechnology), hydrogen peroxide (Millipore), U46619 (Cayman Chemical), PD184325 (Promega Biosciences), gelatin (Fisher Scientific). ERK5 antibody, p-ERK5 antibody, p-P70S6K antibody, P70S6K antibody, RAC antibody, p-RAC antibody, MMP2 antibody, ubiquitin antibody, actin antibody, and tubulin antibody were all purchased from Cell Signaling Technology, GAPDH antibody (Santa Cruz Biotechnology), MMP9 antibody (Millipore), TIMP1 antibody (Abcam). Anti-mouse and anti-rabbit secondary antibody (GE healthcare, UK).

Protein Biochemistry

Platelet Isolation: For human platelets, whole blood was collected into citrate plasma tubes and thoroughly mixed. The sample was centrifuged at 1100 rpm for 15 minutes using a bench top centrifuge. The supernatant was then added in a 1:1 (vol/vol) mix of supernatant/Tyrodes solution with final concentration 10 μM prostaglandin I_2 (Cayman Chemical) and centrifuged at 2600 rpm for 5 minutes using a bench top centrifuge. The supernatant was discarded, and the washed platelet pellet was carefully resuspended in fresh Tyrodes solution, and the experiments were immediately commenced. For mouse platelets, two drops of retro-orbital venous blood was collected using a capillary tube into heparinized Tyrodes solution: 950 μL Tyrodes and 50 μL 1000U/mL heparin in PBS (Sigma) which was divided into 330 μL aliquots. The final sample was centrifuged at

1000 rpm for 5 minutes using a bench top centrifuge. The erythrocyte pellet was discarded and the supernatant then centrifuged again at 1000rpm for 5 minutes using a bench top centrifuge. The pellet was discarded and the supernatant was then added in a 1:1 (vol/vol) mix of supernatant/Tyrodes with final concentration 10μM prostaglandin I_2 and centrifuged at 2600 rpm for 5 minutes using a bench top centrifuge. The final supernatant was discarded and the washed platelet pellet from each mouse was carefully resuspended in 50 μL fresh Tyrodes solution, and immediately used for cell signaling studies.

Protein studies: Cell lysis and cell protein extraction, SDS PAGE, and Western blotting were conducted using buffers and techniques as described previously [1]. Blocking buffer was 3% BSA (Sigma Aldrich)) dissolved in Tris-buffered saline at pH 8.0 (Fisher Scientific) with 0.1% Tween-20 (TBS-T) at room temperature for 1 hour. Primary antibody was 1:1000 overnight at 4 °C in 3% BSA/TBS-T. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/TBS-T for 1 hour at room temperature. ECL reagent used was Supersignal West Pico (Thermo Scientific) for each antibody, or supersignal West Femto (Thermoscientific) for the MMP2 and ERK5 antibodies. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (NIH).

MMP activity assay: Platelets were isolated on day 3 post-LAD coronary ligation from 120 μL whole blood as described above and ventricular apex isolates were homogenized in extraction buffer with a protease inhibitor cocktail (Roche) in the following

concentrations at pH 7.6: 1mM PMSF, 1mM sodium orthovanadate, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 10 mM Tris-HCl, 1% NP40, then final supernatants were protein normalized using a commercially-available kit (Thermo Scientific). Lysates were centrifuged for 15 mins at 4°C, and supernatants were placed in 50% vol/vol 2x non-denaturing sample buffer at the following final concentration: Tris-HCl 250 mM, 0.5% SDS, 1% glycerol, 0.05% bromophenol blue for 10 minutes without boiling. 50 μg of total cell lysate per lane was separated by SDS-PAGE (12% bis-acrylamide containing 1mg/mL final concentration gelatin within the matrix) at 125V (constant voltage, room temperature). The gel was renatured by gently rocking in 2.5% Triton-X-100 for 30 mins at room temperature, then allowed to equilibrate at room temperature with gentle rocking in zymogram buffer with the final concentrations: Tris-base 50mM, NaCl 0.2M, $CaCl_2$ 5mM, Tween-20 0.02% for 30 mins before decanting, and incubating in fresh zymogram buffer for 12 hours overnight at 37°C. The zymogram buffer was decanted, and the gel was rocked at room temperature for 4 hours in Simply Blue Safestain (Invitrogen). MMP activity was noted by clear bands in the final gel (a reverse image). Total MMP activity in each lane was quantified by densitometry using ImageJ software (NIH).

Animal models

Mouse colony: ERK5-flox mice were provided by Dr. Jun-ichi Abe (University of Rochester). These mice have loxP sites inserted at the 5' and 3' position of exon 4 in the ERK5 gene—a coding region in which the critical kinase activation domain is found. ERK5flox/flox mice on a C57Bl6 (B6) background were mated with B6 mice expressing Cre under PF4 promoter to generate platelet-specific ERK5 knockout mice (platelet

ERK5$^{-/-}$). Animal studies were performed in accordance with the University Committee on Animal Research at University of Rochester Medical Center.

Intravital microscopy (thrombosis model): Ferric chloride was used to induce mesenteric artery thrombosis in mice as described by us previously [2] . Briefly, WT or ERK5$^{-/-}$ mice were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). Platelets were labeled *in vivo* with platelet specific fluorescent antibody (Emfret). Mesentery was exteriorized arterioles (80–100 μm in diameter) were selected, and the mouse mesenteric arteriole damaged by the addition of Whatman's paper soaked in 15% $FeCl_3$ to the vessel surface for 45 sec, and images of thrombus formation were captured with a digital camera (Nikon). The time to lumen occlusion was then quantified.

Pulmonary Thromboembolism Model: Mice were briefly anesthetized with isoflurane to facilitate a retro-orbital injection of mouse anti GPIb beta (Emftret) conjugated to Alexa 750 (Invitrogen). After 20 minutes, the mice were anesthetized with ketamine/xylazine (80mg/12mg per kg i.m.). A skin incision was made over the external jugular (EJ) vein and the vessel was isolated. Collagen (Chrono-log #385, 0.8mg /kg) and epinephrine (60ug/kg) were then injected into the EJ vein to induce thromboembolism. Three minutes later, if the animal was still living, it was euthanized and the chest was opened. The right ventricle was perfused with heparinized saline followed by 10% formalin. The lungs were harvested and placed into PBS. The harvested lungs are kept at 4°C overnight and imaged on an Odyssey CLx Imager (Li-Cor Biotech.). The PE burden was expressed as mean fluorescence intensity (MFI).

Tail bleeding assay (hemostasis model): Mice were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). A length of 3 mm at the tail tip was amputated with a

scalpel and a steady stream of blood was visualized in 37°C PBS until the time of hemostasis. The experiment was terminated at 15 minutes if hemostatsis had not yet occurred. Mice were monitored for another 15 minutes post-experiment to assess for re-bleeding.

Myocardial Infarction Model: Chronic myocardial ischemia was induced by permanent ligation the left anterior descending (LAD) coronary artery: The mouse was placed on a heating pad and the airway was stabilized by endotracheal intubation and mechanical ventilation (inspiratory tidal volume of 250µl at 130 breaths/min). Maintenance anesthesia used was 1.5% isoflurane by inhalation. A left thoracotomy was performed in the fourth intercostal space. The mouse heart was exposed, and the LAD coronary artery was ligated 2 mm from its ostial origin with 9-0 silk suture. Transmural ischemia was assured by color loss on the LV wall and ST-segment elevation on the electrocardiogram (ECG). The chest was closed with 6-0 coated vicryl suture, the skin was closed using 6-0 nylon, anesthesia was stopped and the mouse recovered before extubation. A sham operation involved the same procedure, but a suture was passed under the LAD coronary artery without ligation. Aseptic technique was used throughout. Coronary artery branch points were visualized under 10x magnification prior to ligation which can be subjective and difficult to maintain consistency between animals due to variations in anatomy, and so any mouse (WT or genetically-modified) with an LVEF > 65% after 3 days of coronary artery ligation was deemed to have unsatisfactory infarction and was excluded from the study. LV Infarct assessment: Hearts were harvested at the end of the study (day 9) after perfusion and fixation in methanol/acetic acid fixative. Parasternal short axis section were cut (5 µm thickness) before mounting and staining with Masson's

trichrome reagent. Slides were analyzed and photographed using an Olympus light microscope (Model BX41). The infarcted area (blue collagen staining for scar tissue) was expressed as percentage of LV surface area using ImageJ software (NIH). Area at risk: the LAD coronary artery was ligated, and then one minute later 0.3% methylene blue was infused retro-orbitally and five minutes of perfusion was allowed prior to animal sacrifice. The hearts were excised, fixed, and sectioned. The ischemic area of LV at risk was noted by pallor, and expressed as percentage of LV surface area using ImageJ software (NIH). Echocardiography: Echocardiographic analysis using M-mode was performed using a Vevo2100 echocardiography machine (VisualSonics, Toronto, Canada) and a linear-array 40MHz transducer (MS-550D). Image capture was undertaken in mice under general isoflurane anesthesia. LV systolic and diastolic measurements were captured in M-mode from the parasternal short axis. Fraction shortening (FS) was assess as follows: %FS =(end diastolic diameter - end systolic diameter)/(end diastolic diameter) x100%. Since pronounced apical wall motion abnormalities are anticipated from LAD coronary ligation, left ventricular ejection fraction (EF) was measured and averaged in both the parasternal short axis (M-Mode) using the simplified Quinones calculation: $\%EF=Dd^2-Ds^2/Dd^2$, and by the Simpson's method of LV endocardial border tracing of the end diastolic dimension (EDD) and end systolic dimension (ESD) in the parasternal long axis: % EF=(EDD-ESD)/EDD. The pooled data were analyzed for statistical significance.

Supplemental Figures

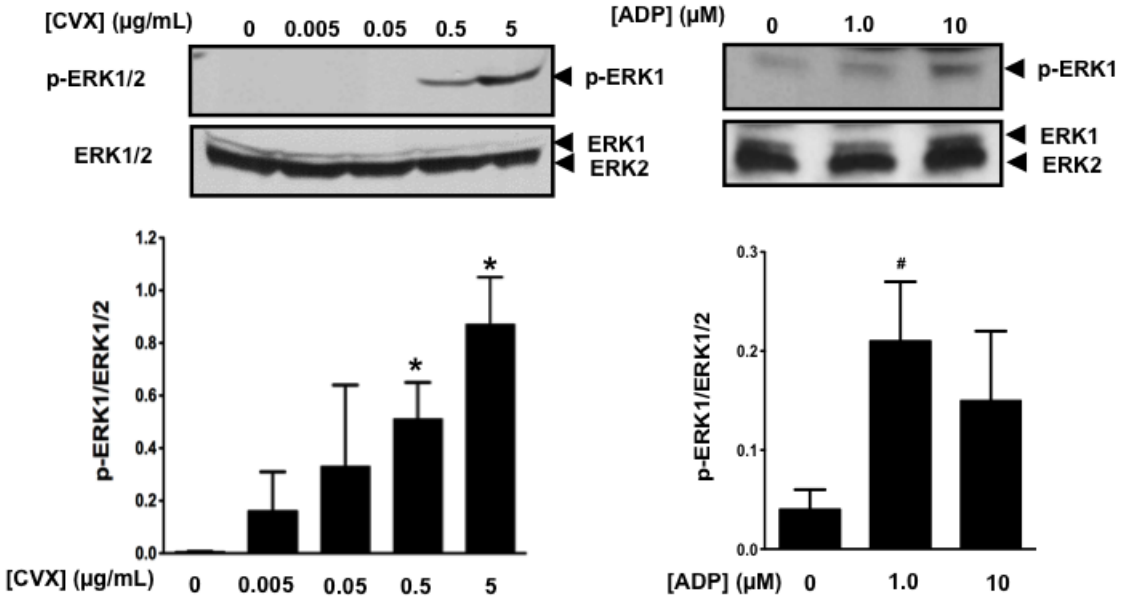


Figure S1. Human platelets were stimulated with increasing concentrations of convulxin (CVX) to stimulate the collagen receptor or 2-methyl ADP (ADP) to stimulate the P2Y12 receptor. These agonists did not strongly activate human platelet ERK5 (p-ERK5, Fig. 1), but they did activate ERK1 (P-ERK1) by Western blotting using a phospho-specific antibody (total ERK-1/2 as a loading control). ERK1 activation is expressed as ratio of P-ERK1/ERK1/2 (± SEM, n=3-4 in each group. * P=0.05 vs. 0 concentration convulxin, paired t-test; P=0.08, Friedman's test. # P=0.056 vs. 0 concentration ADP, paired t-test; P=0.19, Friedman's test)

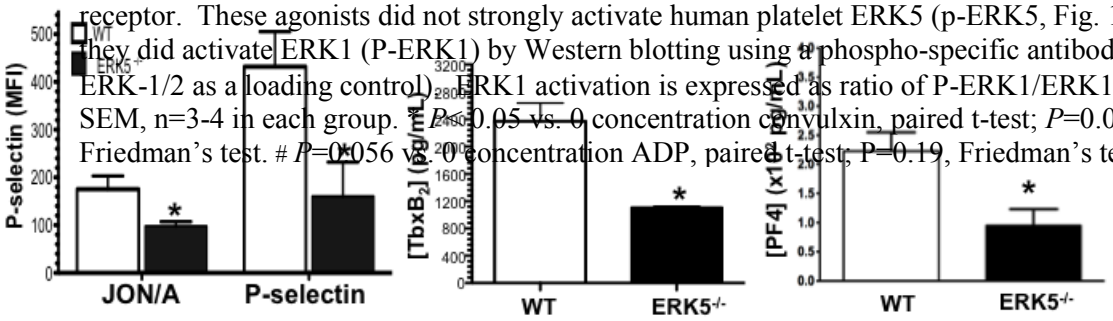


Figure S2. Ba l external P-sele t ERK5$^{-/-}$ platele r 4 (PF4) and thro =4- 5; * P < 0.05, t

	Wild-Type	ERK5$^{-/-}$
Heart Rate (beats/min)	561 ± 13	573 ± 11
Stroke Volume (μL)	33 ± 6	39 ± 6
Ejection Fraction (%)	79 ± 5	79 ± 4
Fractional Shortening (%)	47 ± 5	46 ± 4
Cardiac Output (mL/min)	19 ± 4	22 ± 4
LV Mass (mg)	69 ± 9	74 ± 5
End Systolic Volume (μL)	9 ± 3.6	11 ± 2.7
End Diastolic Volume (μL)	42 ± 8	49 ± 8
LV Anterior Wall Diameter, Dia (mm)	0.85 ± 0.05	0.83 ± 0.11
LV Anterior Wall Diameter, Sys (mm)	1.24 ± 0.05	1.3 ± 0.11
LV Internal Wall Diameter, Dia (mm)	3.27 ± 0.26	3.42 ± 0.24
LV Internal Wall Diameter, Sys (mm)	1.8 ± 0.03	1.9 ± 0.03
LV Posterior Wall Diameter, Dia (mm)	0.69 ±0.01	0.69 ± 0.08
LV Posterior Wall Diameter, Sys (mm)	1.81 ± 0.32	1.87 ± 0.27

Figure S4. Basal cardiac function in WT and ERK5$^{-/-}$ mice by TTE. Baseline LV dimensions and myocardial function is similar in WT and platelet ERK5$^{-/-}$ mice (mean ± S.D.)

Figure S3. Sustained activation of platelet ERK5 (p-ERK5) was noted by Western blotting 48 hrs following LAD coronary ligation in a mixed background strain of mice (C57Bl/6J and 129S1/SvImJ). Activation of ERK5 in washed mouse platelets was expressed as mean ratio of p-ERK5/ERK5 (mean ± S.D., n=3).

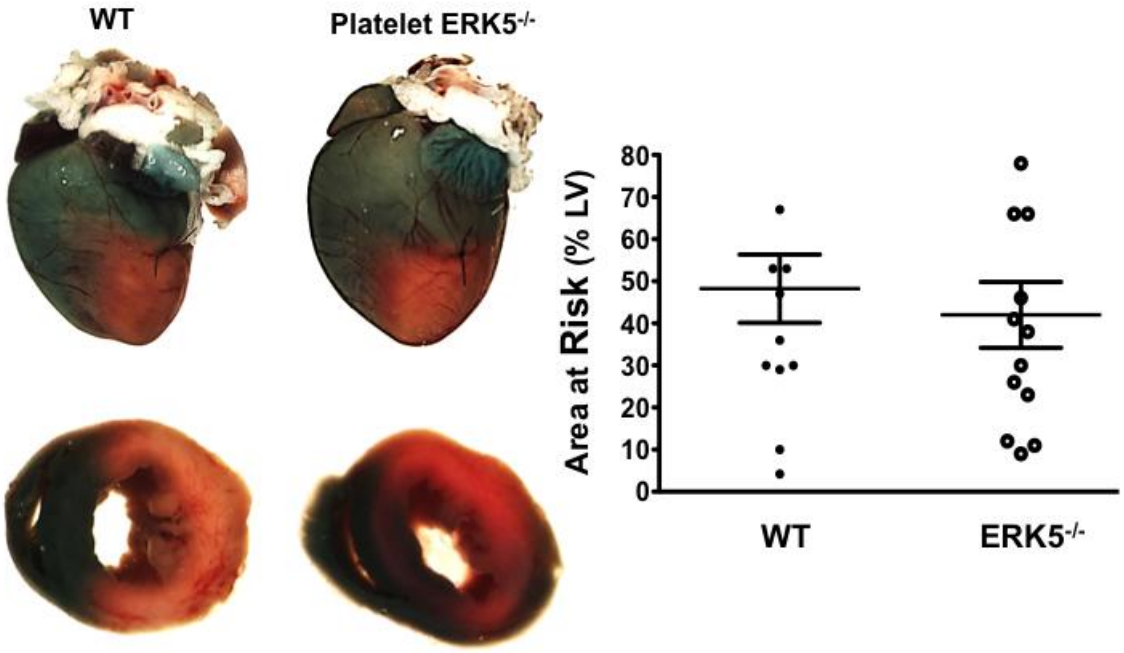


Figure S5. The LV region at risk is similar in WT and platelet ERK5$^{-/-}$ mice. 5 mins after LAD coronary ligation mice were injected intravenously (retro-orbitally) with methylene blue (2%). Perfused tissue is blue and ischemic tissue pink. LV region at risk quantified as % LV area (± SEM, n=13, and quantified using NIH ImageJ software).

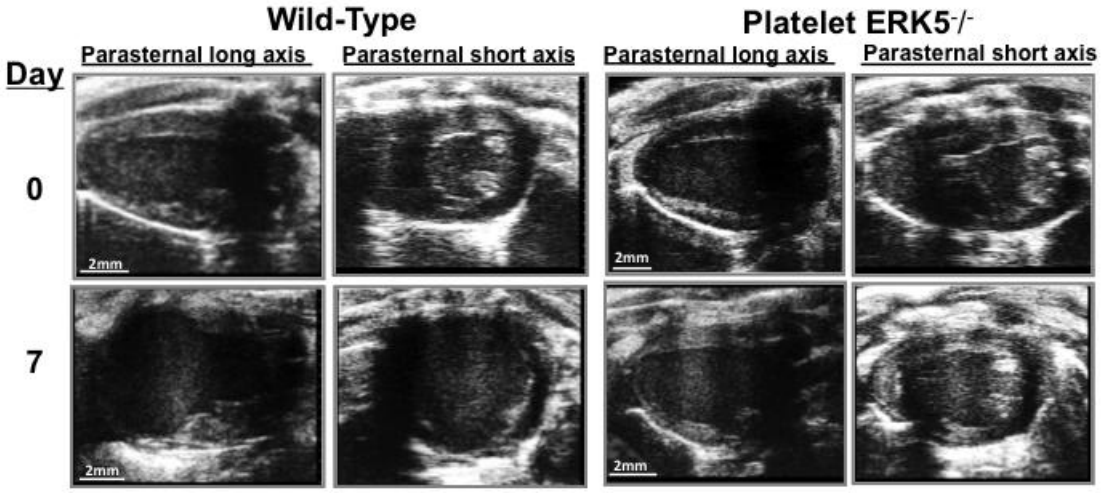


Figure S6. Representative parasternal long axis and parasternal short axis still TTE images of WT and ERK5$^{-/-}$ mice on day 0 (baseline) and 7 days post LAD coronary ligation.

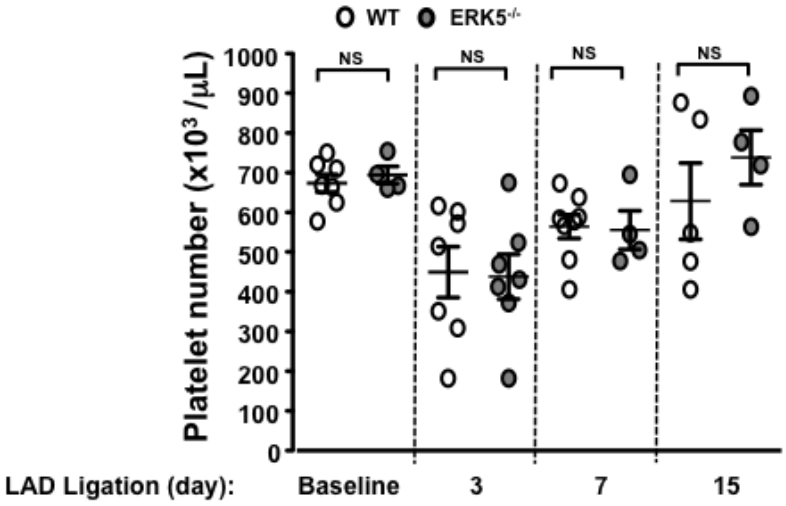


Figure S7. Platelet count in WT and ERK5$^{-/-}$ mice at baseline and following LAD coronary ligation (mean ± SEM, $P < 0.05$ day 3 vs. baseline and day 7 vs. baseline for WT and ERK5$^{-/-}$; P = not significant (NS) between WT and ERK5$^{-/-}$ on each day, t-test for unequal variances, n=4-7).

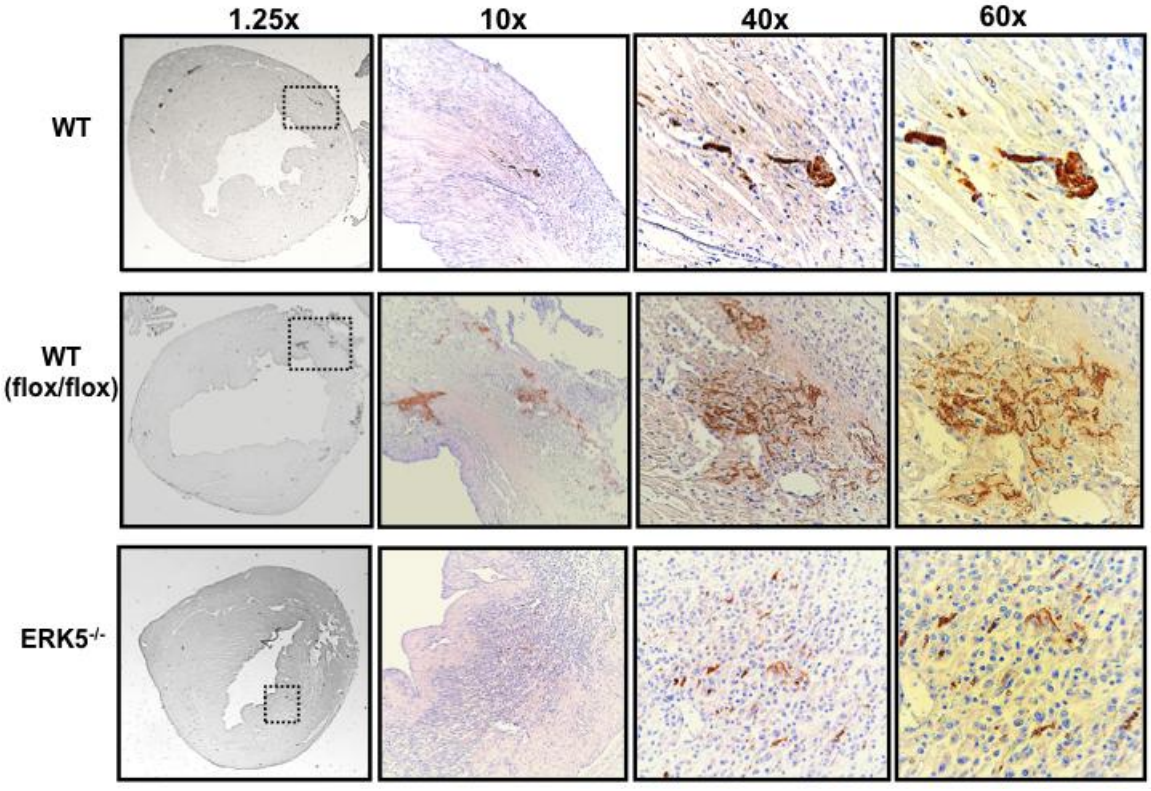


Figure S8. Representative parasternal short axis sections of the heart on day 3 after LAD coronary ligation. Staining for CD42c is shown as brown precipitate in WT, WT (flox/flox), and in platelet ERK5$^{-/-}$ mouse hearts. Less CD42c staining in platelet ERK5$^{-/-}$ mouse hearts is suggestive of less intramyocardial (microvascular) thrombus formation. These additional sections are supportive of similar data in other mice shown in Fig. 6B.

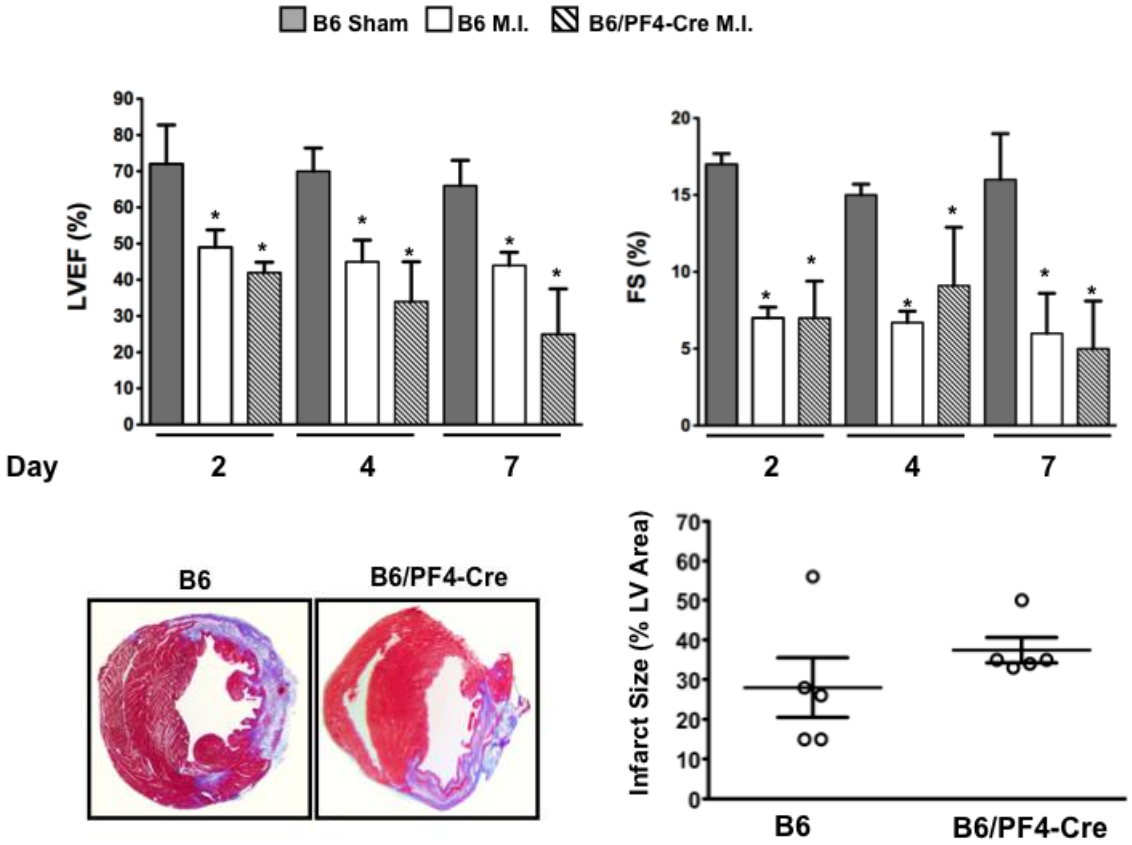


Figure S9. Cardiac function in sham WT (C57BL/6) mice after LAD coronary ligation showed similar MI function to WT-PF4-Cre (C57BL/6). Hemodynamic data (mean ± SEM, n=3-5 * $P< 0.05$ vs. B6 Sham, t-test for unequal variances). Cardiac morphology (LV infarct size) by Masson Trichrome staining (blue=infarct) is similar on day 7 following LAD coronary ligation in WT and WT-PF4-Cre mice (both C57BL/6 strain). Quantification of Masson Trichrome positive staining (mean % of LV area, N=5 ± SEM, *P*=NS, t-test for equal variances).

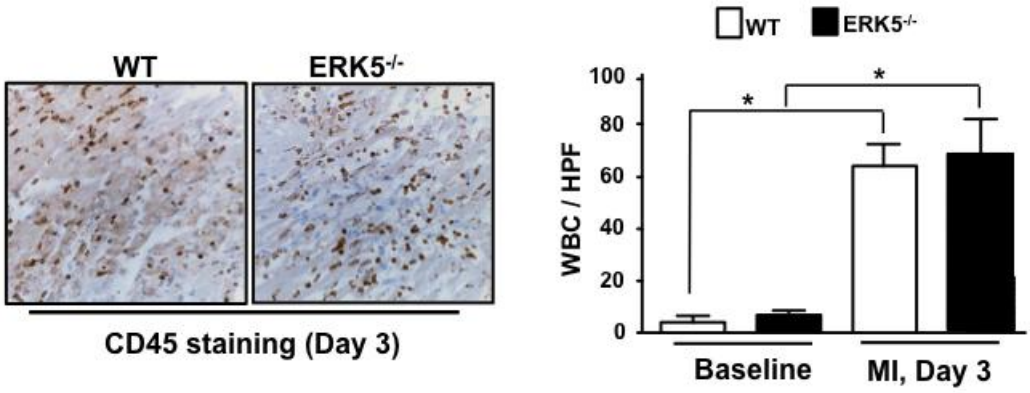


Figure S10. Myocardial white blood cells infiltrates. $CD45^+$ staining on day 3 following LAD coronary ligation in WT and platelet $ERK5^{-/-}$ mouse hearts. Data are shown as mean WBC count per high powered field (mean ± SEM, n=5; * $p < 0.05$ vs baseline, t-test for unequal variances).

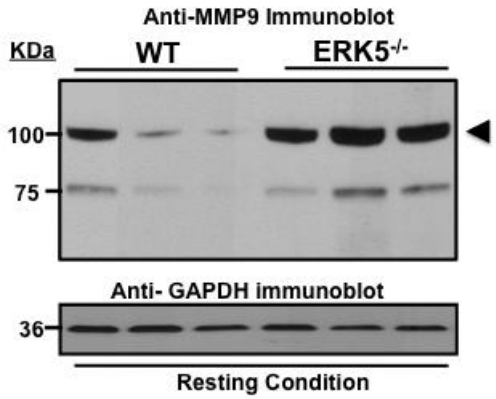
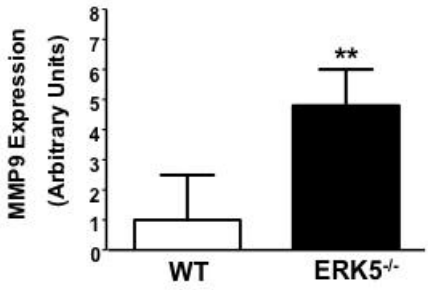


Figure S11. Baseline platelet MMP-9 expression in WT and ERK5$^{-/-}$ platelets. Isolated washed mouse platelet extracts were separated by SDS-PAGE then immunoblotted for MMP-9. Blots are representative of at least 4 different individual mice for each strain. The arrowhead indicates the expected molecular mass of MMP-9. MMP-9 content is expressed as mean MMP-9/GAPDH (mean ± SEM, n=3; ***P*<0.05 vs WT, t-test for equal variances).



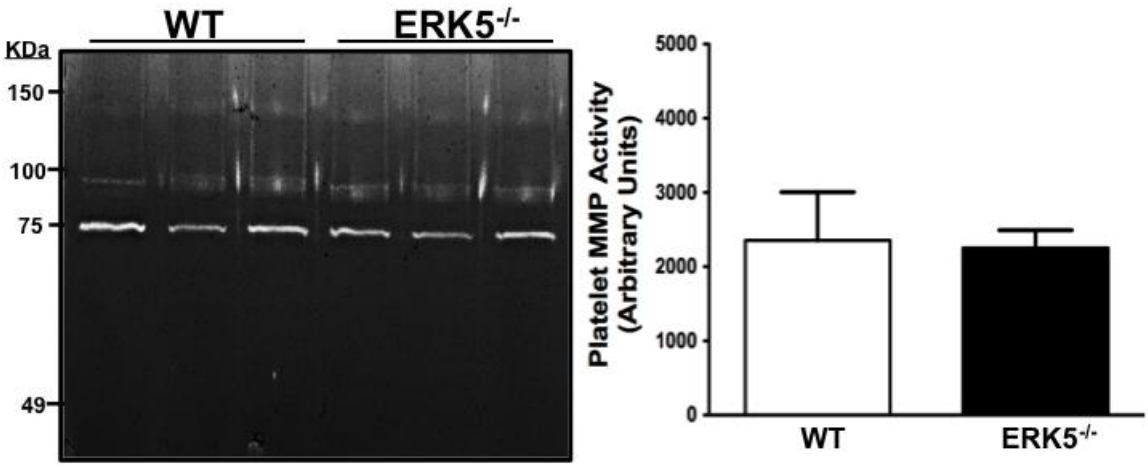


Figure S12. Baseline platelet MMP activity in WT and ERK5$^{-/-}$ mice. Washed mouse platelet extracts were protein-normalized and run in duplicate on non-reducing gels with a gelatin matrix. Gelatinase activity is shown as light bands on the final zymogram. Total MMP activity (all isoforms) was calculated for each lane (mean ± SEM, N=3, *$p < 0.05$ vs. WT).

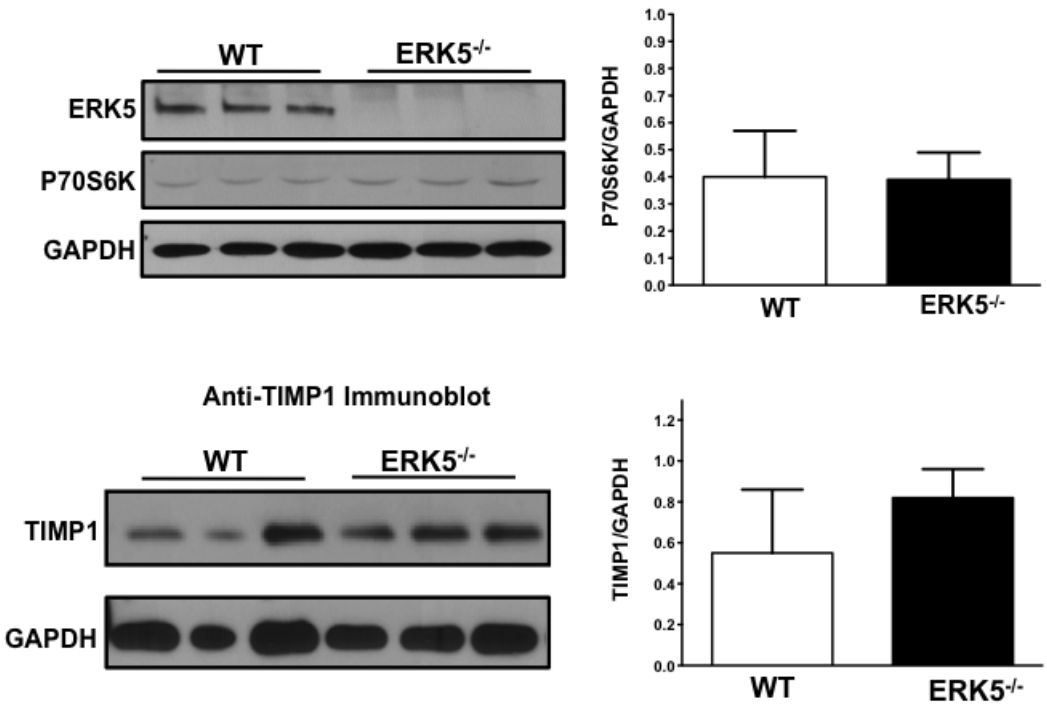


Figure S13. Platelet P70S6K and TIMP1 content is similar in WT and platelet ERK5$^{-/-}$ mice under resting conditions. Protein content is expressed as a ratio of P70S6K/GAPDH (± SEM, n=3-6 in each group, *P*=NS, t-test for equal variances).

Movie 1. Parasternal long axis view of a WT mouse heart on day 9 following MI.

Movie 2. Parasternal long axis view of a platelet ERK5$^{-/-}$ mouse heart on day 9 following MI.

Supplemental References

1. Cameron SJ, Abe J, Malik S, Che W, Yang J. Differential role of mek5alpha and mek5beta in bmk1/erk5 activation. *J Biol Chem*. 2004;279:1506-1512
2. Ramesh S, Morrell CN, Tarango C, Thomas GD, Yuhanna IS, Girardi G, Herz J, Urbanus RT, de Groot PG, Thorpe PE, Salmon JE, Shaul PW, Mineo C. Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing enos via beta2gpi and apoer2. *J Clin Invest*. 2011;121:120-131