Supplemental Methods and Figures

Materials

Suppliers for monoclonal antibodies were: PE-labeled JON/A antibody, anti-mouse GpIbα and GpVI (Emfret); PE-conjugated anti-P-selectin antibody, allophycocyanin (APC)-conjugated anti-mouse CD144 (VE-Cadherin) (Clone:eBioBV13), PE-conjugated anti-mouse CD31 (PECAM-1) (Clone:390), PE-conjugated anti-mouse CD309 (Flk-1) (Clone:Avas12a1), and labeled isotype specific control antibodies (eBioscience); monoclonal anti-ERp72 antibody (Cell Signaling Technology, #5033); Anti-αIIb (SZ22) was a generous gift from Dr. C. Ruan;[1] Anti-fibrin antibody hybridoma (clone 59D8) was a generous gift from Dr. H. Weiler.[2] Suppliers for polyclonal rabbit antibodies were: anti-PDI, anti-PLCγ2, anti-β3 (H-96), anti-ERp5 (goat polyclonal IgG) (Santa Cruz). Anti-CD41 F(ab)2 (BD Bioscience) was conjugated with Alexa 488 (Invitrogen). Additional materials were from: IRDye 800-conjugated goat anti-mouse IgG, IRDye 680-conjugated goat anti-rabbit IgG (LI-COR Bioscience); PVDF membrane (Millipore); 3,3′-dihexyloxacarbocyanine iodide (DiOC6) (Sigma, 318426), α-thrombin, collagen, ATP Standard and CHRONO-LUME (CHRONO-LOG), convulxin (Enzo). For studies on ATP secretion in human platelets purified α-thrombin from Dr. J.W. Fenton II was used.[3] Biotin-HPDP (Therma Scientific); 3-(N-Maleimidylpropionyl)biocytin (Molecular Probes).

Generation and characterization of tissue specific ERp72-deficient mice

The embryonic stem (ES) cells that were generated by EUCOMM (Clone ID: EPD0629-2, MGI Allele ID:104864) harbored a modified *pdia4* allele in which the second exon was flanked by loxP sites (a 34 base pair recognition sequence for Cre-recombinase) and the PGK-Neomycin (neo) drug resistance cassette flanked by FRT sites (base pair sequence recognized by Flp-recombinase). After confirmed by long range PCR, the ES cell clones were injected into murine blastocysts and transferred to pseudopregnant females. The chimeric offspring were mated with wild-type C57BL/6 mice, the germ-line transmission of the targeted allele was confirmed by PCR analysis of tail DNAs. The pairs of primer 1F: 5'-CCTCTCAGAGTATTACT CAAGGCTG-3' and primer 1R1: 5′-CCAACTGACCTTGGGCAAGAACAT-3′ yielded a 289 bp product for flox-neo (fln) allele and no product for wildtype (WT) allele; the pairs of primer 2F: 5′-CTGCTGTGTCACCTCACCAACTCT-3′ and primer 2R: 5'-AGAAGTGGGAGGAATTGAACAGTTC-3′ yielded a 300 bp product for WT allele and a 356 bp product for fln or flox allele. Once the targeting construct was successfully incorporated into the mouse germline, the *neo* cassette was removed by crossing with mice expressing Flp recombinase (deleter mice), the offspring was confirmed by PCR analysis of tail DNA using Primer 1F and Primer 1R2: 5′-ACCCATCACGTTCTCCTCTACAAC-3′, which' yielded a 584 bp product for Flp-excised flox allele (floxed), a 414 bp product for WT allele and no product for fln allele.

To generate tissue-specific ERp72-knockout mice and experimental control mice, ERp72$^{fl/fl}$ mice homozygous for the ERp72 floxed allele were mated with Tie2-Cre mice and Pf4-Cre mice, both of which were on a C57BL/6 background (Jackson Laboratory).[4] The null allele with the excision of the second exon was confirmed by PCR using the pairs of primer 3F: 5′-TACTCAAGGCTGGCTTTCTAA-3′ and primer 3R: 5′-CTGTTCAGAAACTATGATTACCA-3′, which' yielded a 221bp product for null allele.

The knockout efficiency of ERp72 at mRNA level was assessed by reverse transcription polymerase chain reaction (RT-PCR) as previously described,[5] with the control expression of other PDI members and β-actin. The primers used were as follows: ERp72, sense 5′-gctgttggtcctgctctt-3′, anti-sense 5′-cagccacaaagttatcaaag-3′; PDI, sense 5'-ctcgacaaagatggggttgt-3′, antisense 5′-gcaagaacagcaggatgtga-3′; ERp57, sense 5′-atcttgctaacaagtttgaagaca-3′, antisense 5′-catcatagtaagcggtgag taagtc-3′; ERp46, sense 5′-aaaggaattcccaggcttgt-3′, antisense 5′-ccgttgtgttctcccacttt-3'; ERp5, sense 5′-ggtgagctgcaccttctttc-3′, antisense 5′- gctgctttcttccattctgg-3′; Cre, sense 5′-cccatacagcacacctttg-3′, antisense 5′-tgcacagtcagcaggtt-3′; β-actin, sense 5′-gtgctatgttgctctagacttcg, antisense 5′-atgccacaggattccatacc.

The crosses between homozygous ERp72$^{fl/fl}$ mice and homozygous ERp72$^{fl/fl}$ expressing Tie2-Cre mice and Pf4-Cre mice yielded homozygous conditional ERp72$^{fl/fl}$ mice (Tie2-Cre/ERp72$^{fl/fl}$ and Pf4-Cre/ERp72$^{fl/fl}$) in the expected Mendelian

ratio. The mice did not have any obvious phenotypic abnormalities or developmental defects. The tissue distribution of Pf4-Cre and Tie2-Cre expression has previously been characterized.[4,6] We documented normal ERp72 expression in the liver and other non-vascular, non-hematopoetic tissues (not shown). All experiments with mice were performed in accordance with Temple University and Soochow University institutional guidelines and approval of the Animal Care Committees. Mice (8 to 12 weeks old) were anesthetized using 1% pentobarbital by intraperitoneal injection (100 mg/kg body weight).[7] Complete blood cells counts, white blood cell differential counts and red blood cell and platelet indices were measured using a Sysmex Coulter Counter (XT2000-iV).

β3 integrin-null mice

Previously described β3 integrin-null mice on a C57BL/6 background with matched C57BL/6 wild type control mice[8] were used in some experiments.

Isolation of endothelial cells from mice

Mouse endothelial cells were isolated from lungs as described previously[8,9] with minor modifications. Briefly, the mice were euthanized by exsanguination, followed by exposure of thoracic cavity. After the left atrium was cut, 10 ml cold PBS with 0.1% heparinized solution was injected via the right ventricle to completely flush blood cells from the lungs. Lungs were removed and minced into small pieces, followed by incubation for 60 minutes at 37°C with 5 ml 0.1% collagenase A (Roche Inc.). The digested tissue suspension was aspirated into to a 20-ml syringe with a 14-

gauge cannula, and clumps were triturated into a single-cell suspension. The single-cell suspension was filtered through a 70-μm strainer. The filtered cell suspension was centrifuged for 10 minutes at 300 *g*, and the cell pellet was washed once with buffer (0.5% BSA, 2 mM EDTA, and PBS, pH 7.2). The cell pellet was suspended with 90 μl binding buffer per 10^7 total cells, followed by the addition of 10 μl CD31 MicroBeads (130-097-418, Miltenyi Biotec) per 10^7 total cells. After incubation at 4°C for 15 minutes in the refrigerator, bead-bound ($CD31^+$) cells were collected by MACS magnetic separator and MS Column (130-042-2011; Miltenyi Biotec) according to the manufacturer's protocol. After the isolated cells were verified for expression of VE-cadherin, Flk1, and CD31 by flow cytometry, they were analyzed for ERp72, PDI and ERp57 expression by Western blotting.

RNA extraction, Reverse transcription (RT)-PCR, and PCR

Total RNA was extracted from mouse platelets by using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions.[8] Reverse transcription (RT) was performed with total RNA (10 ng) using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The PCR reactions were carried out using cDNA obtained from RT or Tail DNA and Dream Taq™ Green PCR Master Mix (Fermentas) according to the manufacturer's instructions.

Western blotting

Proteins from platelet lysates were separated by 8% SDS-PAGE and transferred onto PVDF Membrane (Millipore). The membranes were blocked with 1% BSA in PBS.

After extensive washing with TBS containing 0.1% Tween 20, the immunoblots were incubated with the primary antibodies for 2 hours. Antibody binding was detected by using IRDye 800-conjugated goat anti-mouse IgG (LI-COR Bioscience) or IRDye 680-conjugated goat anti-rabbit IgG (LI-COR Bioscience) and visualized with ODYSSEY infrared imaging system (LI-COR). The data are representative of at least three experiments.

Bleeding time analysis

Bleeding time assay was performed using a razor blade to transect the mouse-tail 3 mm from the tip, with the tail immersed in a 12-ml test tube containing PBS at 37°C.[7] Bleeding times were determined when the bleeding stopped for more than ten seconds. If the bleeding time was longer than 15 minutes, the assay was stopped and bleeding time was count as 15 minutes. The control and experimental groups were matched for age, sex and body weight.

Coagulation assays

The prothrombin time (PT) and the activated partial thromboplastin time (APTT) were measured using a START 4 Coagulation Analyzer (Diagnostica Stago) according to the manufacturer's instructions.[8] Mouse blood samples were collected through the inferior vena cava into 147 mM sodium citrate in a ratio of 9:1 and centrifuged at 3000 g for 10 minutes. Plasma was incubated at 37°C for two minutes. The PT and the APTT were measured using NEOPLASTINE® CI PLUS 10 and PTT Automate 5 (Diagnostica Stago), respectively.

Flow cytometry and aggregation and secretion studies of human platelets

Flow cytometry studies on human platelets were performed using washed platelets suspended into Tyrode's buffer as described.[8] Aggregation studies were performed using washed platelets prepared and suspended in modified Tyrode's buffer to which calcium (1 mM) was added as described.[8] The percent aggregation was calculated from the amplitude of the tracings at 5 minutes and normalized to the response of the untreated control within an individual experiment. Secretion of ATP was monitored in the Chronolog lumi-aggregometer as previously described.[8]

Flow cytometry and aggregation and secretion studies of mouse platelets

Flow cytometry studies on mouse platelets were performed using platelet-rich-plasma (PRP) prepared and diluted into Tyrode's buffer as described.[7] The PRP was prepared from blood obtained into acid-citrate dextrose solution through the inferior vena cava.[7] Aggregation studies were performed using washed platelets prepared as described.[10] Platelet concentration was measured using a Sysmex Coulter Counter, and counts were adjusted to 200,000/μL for aggregation studies. Secretion of ATP was monitored in the Chronolog lumi-aggregometer as previously described.[11]

$FeCl_3$-induced platelet accumulation and thrombosis of the mesenteric artery

To follow platelet accumulation in vivo, Alexa-488-conjugated anti-mouse CD41 F(ab)2 antibody was injected into mice through tail vein. The mesentery was exteriorized through a midline abdominal incision. A single arteriole was visualized

using a Leica DM 2000 fluorescent microscope and the diameter was measured. Vessel injury was generated by placing a 1 × 2 mm patch of No.1 Whatman filter paper soaked in 5% $FeCl_3$ above the exposed artery for 1 minute. The filter paper was removed and platelet accumulation observed and photographed every minute for 15 minutes. The fluorescent intensity (FI) of the platelet accumulation and thrombus formation was analyzed by Image J software. The FI divided by the area analyzed gave the relative FI for each injury and is indicated by FI/μm^2. The area analyzed was obtained from the length analyzed multiplied by the diameter of each injured vessel. The length of the thrombi was from the beginning of the upstream edge of the thrombi to 1 mm downstream of this site. Background fluorescent intensity of the uninjured vessels was negligible.

Intravital microscopy of laser-induced thrombosis of the cremaster muscle arterioles

Laser-induced injury of the mouse cremaster muscle arteriole was performed as previously described.[8,12-14] Alexa 647-labeled anti-fibrin antibody and Alexa 488-labeled anti-CD41 F(ab)2 fragments (BD Biosciences) were infused at 0.1 μg/g into the jugular vein followed by infusion of recombinant ERp72 protein with BSA as a control (200 μg/mouse). In the experiments using β3-null mice, intravenous administration of the cationic lipophilic fluorochrome 3, 3′-dihexylcarbocyanine iodide (DiOC6) (2.5 μL of a 100 μM solution/g of body weight)[15,16] was used to visualize platelets instead of anti-CD41 antibody, as the mice did not express CD41 antigen on platelet surface. After 5 minutes, arterioles (30 to 45 μm diameter) were injured using

a Laser Ablation system (Intelligent Imaging Innovations (I3)) through a Zeiss microscope (Axio Examiner D1) objective parfocal with the focal plane. The laser power was set to 55-65% and the laser fired at the vessel wall in 1 to 3 pulses until thrombi were induced. Approximately 10 thrombi were studied in a single mouse. Injuries in which puncture of the vessel occurred or injuries in which no thrombus formed were excluded. The average number of laser pulses/injury was equal for all conditions (data not shown). Data was captured using a CCD camera (Cool SnapTM HQ2)–using Slidebook 5.0 image acquisition and analysis software (I3). Data were collected for 5 minutes after vessel wall injury. Image analysis was performed using Slidebook Version 5.0 (I3). Data was obtained from a total of 30 thrombi per group for each experimental condition.

Determination of relative amounts of 3-(N-Maleimidylpropionyl)biocytin (MPB) label per αIIb and β3 protein

Relative amounts of MPB label per protein was determined by using the IRDye® 800CW (Green) Streptavidin (LI-COR) to detect the MPB and IRDye® 680RD (red) goat anti-Rabbit secondary antibody (LI-COR) to detect the primary rabbit antibody against β3 (Santa Cruz). The membrane was then stripped with NewBlot^{TM} IR Stripping Buffer (LI-COR) and re-probed using IRDye® 680RD (red) goat anti-mouse secondary antibody (LI-COR) to detect the primary mouse antibody against αIIb (SZ22). The intensity of each band is calculated using Image J program. The ratio of MPB to protein was compared to the untreated sample.

Labeling of thiols in αIIbβ3 with Biotin-HPDP

Biotin-HPDP labeling of platelet αIIbβ3 was performed by adding 100 μM biotin-HPDP to washed non-activated or thrombin (0.5-1 U/ml)-activated platelets in albumin free Tyrode's buffer for 10 min, after which 6M guanidine (pH 7.4) was added for 50 min. The samples were dialyzed against TBS (20 M Tris, 150 mM NaCl, pH 7.4) x 3 (each dialysis > 3 hours, including an overnight dialysis), centrifuged (16,000g for 20 min), and the supernatant loaded on a concanavalin A column and αIIbβ3 purified as described.[17,18]

Mass Spectrometry

αIIbβ3 was in-solution trypsin digested without DTT reduction. The resulting peptides were C_{18} desalted and enriched using avidin affinity cartridge based on manufacture's protocol (Sciex, Rebwood City, CA, Cat. 4326694). The enriched biotin HPDP modified peptides were analyzed by LC-MS/MS on Q Exactive mass spectrometer (QE-MS) coupled with Ultimate 3000 nano UHPLC system. In brief, the peptides were injected into a trap column (Acclaim PepMap 100, 75 x 2 cm, C18, 3μm, 100Å) and then separated by a C18 column (Acclaim PepMap RSLC, 75 x 50 cm, C_{18}, 2μm, 100Å). A 3 hours binary gradient with the solvent A (2% acetonitrile (ACN, 0.1% formic acid (FA)) and Solvent B (85% ACN 0.1% FA) was used for peptides separation. The eluted peptides were directly analyzed by QE-MS with the spray voltage of 2.15 KV, capillary temperature of 275 °C. The full scan MS spectra were acquired in a positive mode with AGC target of 3E + 6 and resolution of 140,000. The MS/MS spectra were generated by high-energy collision dissociation

with normalized collision energy of 27%. The MS/MS spectra were searched against Swiss-Prot human database using Mascot (V.2.3) search engines on Proteome Discoverer (V1.4) platform. The biotin-HPDP modification and oxidation of methionine were set as variable modification. The identified proteins and peptides were displayed using Scaffold software (V.4.6).

Statistics

Data were analyzed using the statistical software GraphPad Prism 5. For parametric comparison, the values were expressed as the mean ± SEM, one-way ANOVA (analysis of variance) followed by the Tukey's test for multiple groups and the two-tailed Student's t-test for 2 groups were used. For nonparametric comparison between two groups the area under the curve (AUC) of the laser injury experiments, Wilcoxon-Mann Whitney test was used.[14] For the non-parametric comparison between multiple groups the area under curve of median FI over 300 seconds was analyzed with a Kruskal-Wallis test.[19] A P value less than 0.05 was considered significant.

References

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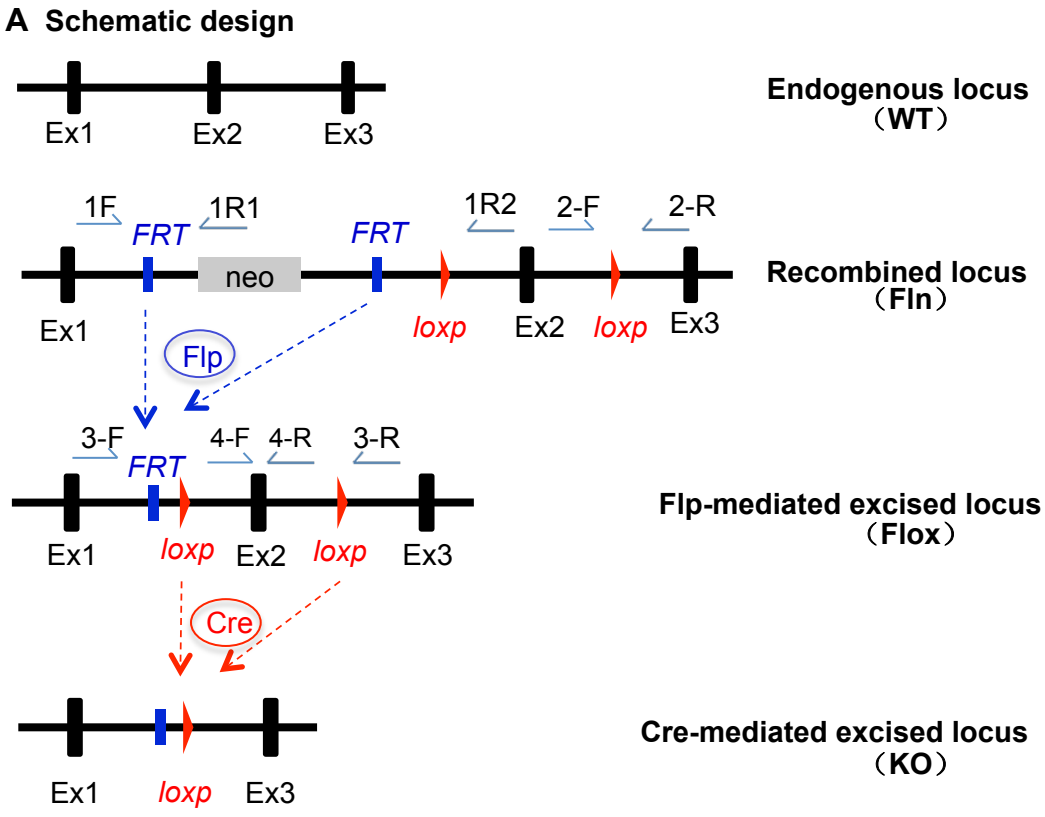
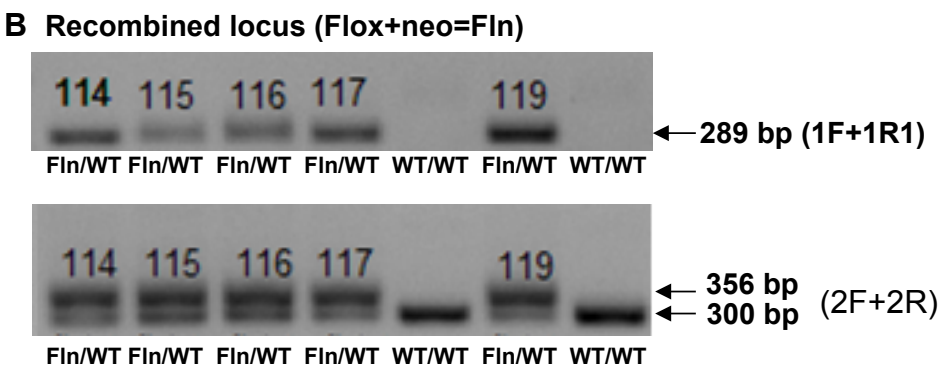
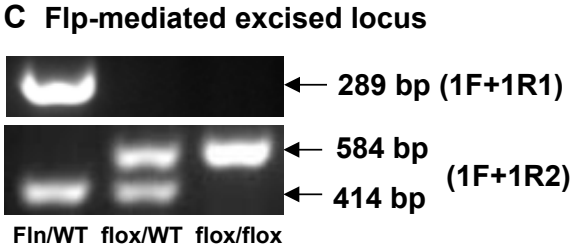
Supplemental Figures and Legends







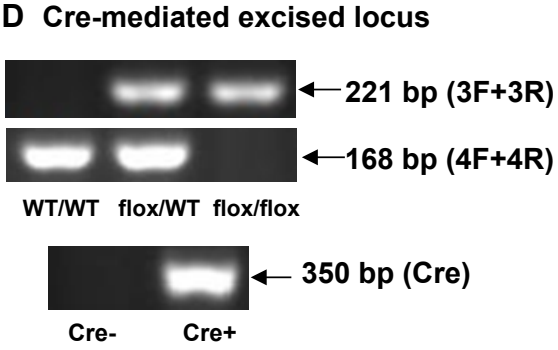


Figure S1. Generation of ERp72-conditional knockout mice. (A) Scheme of the targeting strategy for generation of the embryonic ES cells harboring a modified pdia4 allele, in which the second exon was flanked by loxP sites and a PGK-Neomycin (neo) drug resistance cassette flanked by FRT sites was inserted (recombined locus). (B) The genotyping indicates the mice (#114, #115, #116, #117, and #119) were offsprings with germ line transmission. The targeted *neo* cassette insertion was confirmed by PCR using a pair of primers 1F and 1R1, which yielded a 289 bp product (upper panel). The second loxp site was detected by PCR using a pair of primers 2F and 2R, which yielded a 356 bp product for floxed allele and a 300 bp product for WT allele (lower panel). (C) The targeted neo cassette was removed by Flp-mediated recombination of FRT sites, forming the Flp-mediated excised locus (floxed allele). Genotyping of targeted mouse tail DNA using primers 1F, 1R1 and 1R2 yielded a 289 bp product for fln allele, a 584 bp product for floxed allele and a 414 bp product for WT allele. (D) ERp72-floxed mice were mated with Tie2-Cre transgenic mice. Genotyping of the endothelial cells of the offspring with primers 3F and 3R yielded a 221 bp product for deletion of exon 2 and a 168 bp product for wild-type allele, respectively. Genotyping for Cre cDNA produced a 350 bp product.

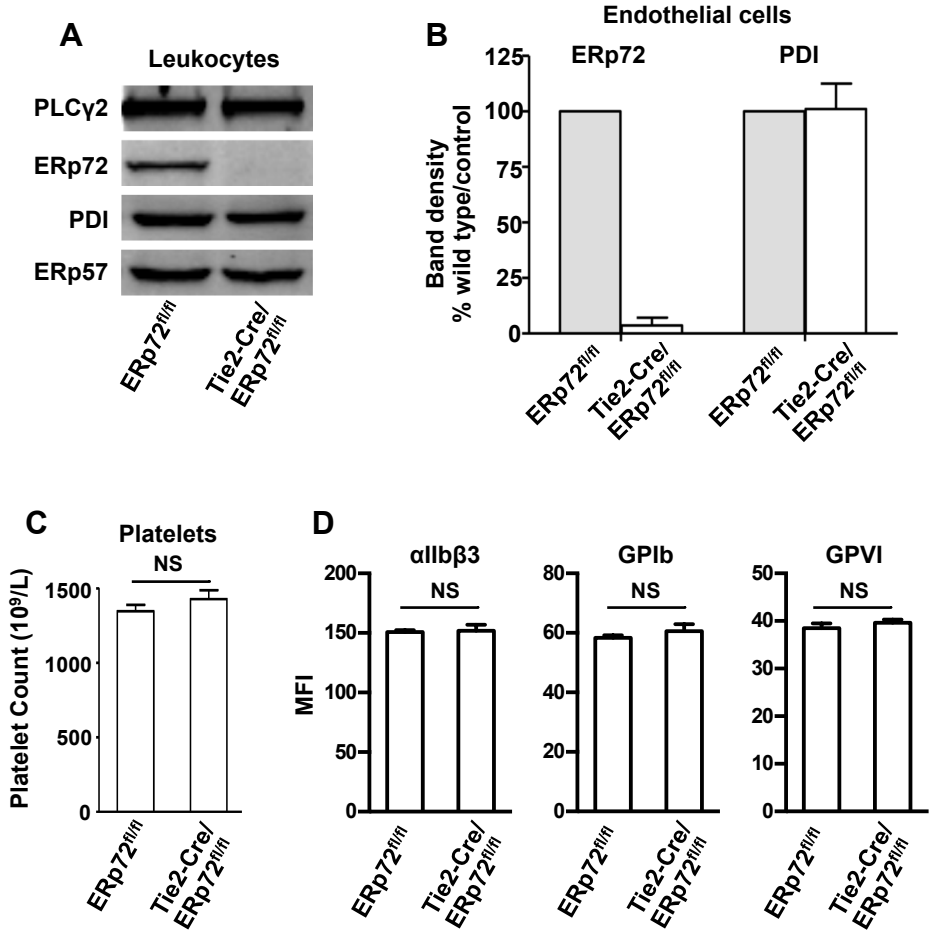


Figure S2. Characterization of Tie2-Cre/ERp72$^{fl/fl}$ mice. (A) Leukocytes from Tie2-Cre/ERp72$^{fl/fl}$ mice have decreased ERp72. Mononuclear cell were prepared as described (Zhou J, et al *J Clin Invest.* 2015;125(12):4391-4406) and analyzed by Western blot. (B) Normal PDI expression in ERp72-deficient endothelial cells; mean ± SE, n = 3, t-test. (C) Comparison of platelet counts between ERp72$^{fl/fl}$ and Tie2-Cre/ERp72$^{fl/fl}$ mice; mean ± SEM, *n* = 13, *t* test. NS = non significant. (D) Glycoprotein (αIIbβ3, GPIb or GPVI) expression on platelets from Tie2-Cre/ ERp72$^{fl/fl}$ and ERp72$^{fl/fl}$ littermate controls was analyzed by flow cytometry. Mean fluorescent intensity (MFI) is on the y axis; mean ± SE, n = 4, t-test.

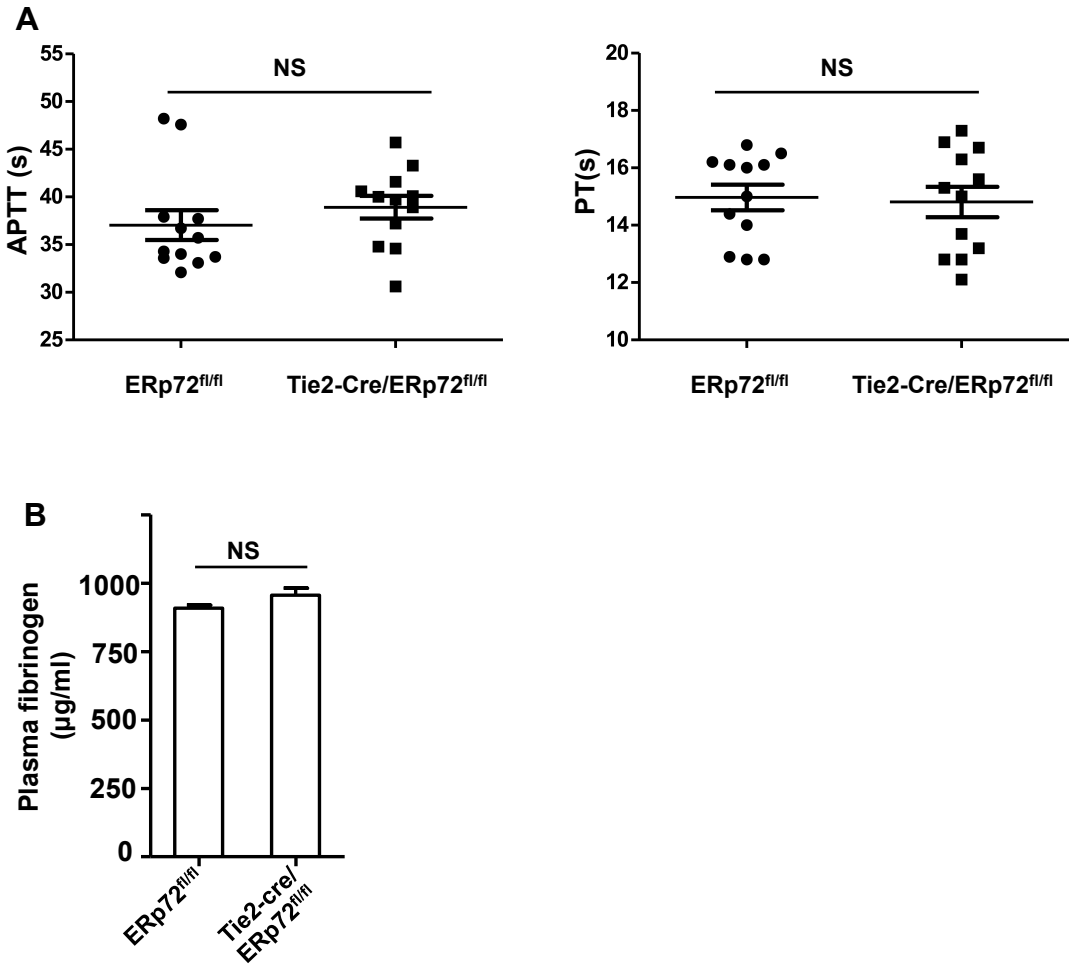


Figure S3. **Normal prothrombin times (PT), partial thromboplastin times (PTT) and fibrinogen levels in plasma of Tie2-Cre/ERp72$^{fl/fl}$ mice**. (A) The PT and PTT of plasma from Tie2-Cre/ERp72$^{fl/fl}$ and ERp72$^{fl/fl}$ littermate control mice was performed as in Methods. t-test, n = 12, for all samples. (B) Plasma from Tie2-Cre/ERp72$^{fl/fl}$ mice and their ERp72$^{fl/fl}$ littermate control mice was collected and prepared as described in Supplemental Methods. After dilution at 1:25,000, plasma was incubated with plates at 4℃ overnight. The levels of fibrinogen was measured by an ELISA kit (USCN Life Science, Catalogue number SEA193Mu); mean ± SEM, *n* = 4 per group, t test.

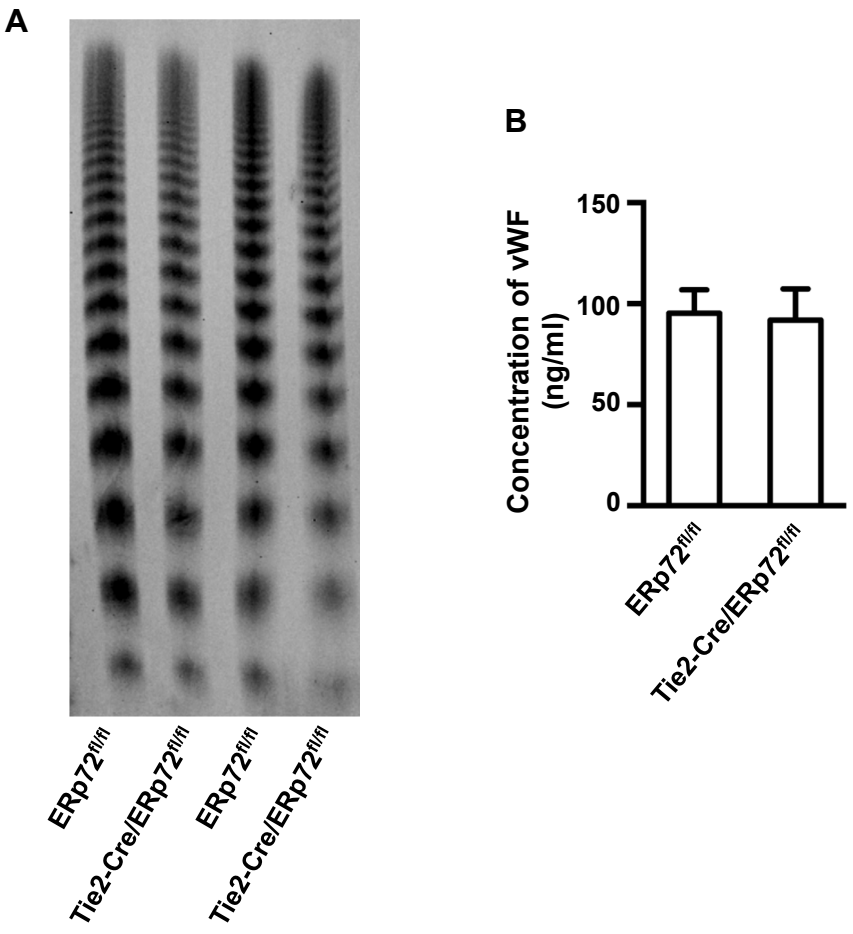


Figure S4. **Analysis of plasma von Willebrand factor in Tie2-Cre/ERp72$^{fl/fl}$ mice**. (A) Blot of vWF multimer analysis of plasma (1 µl) from Tie2-Cre/ERp72$^{fl/fl}$ and ERp72$^{fl/fl}$ littermate control mice. Multimer analysis was performed using 1 mm thick 1.5 % agarose gels. The protein was blotted onto a PVDF membrane and the blot developed using rabbit anti-human vWF-HRP antibody (Dako, P0226). (B) Plasma vWF concentration in ERp72$^{fl/fl}$ and Tie2-Cre/ERp72$^{fl/fl}$ mice was determined using an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., CEA833Mu); mean ± SEM, *n* = 4 per group, t test.

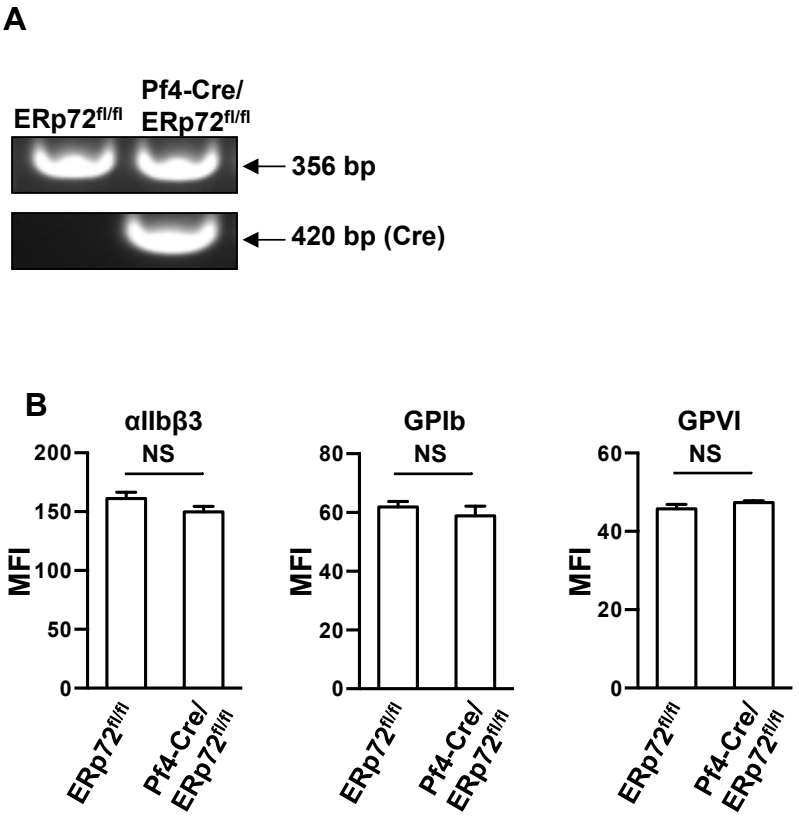


Figure S5. Characterization of Pf4-Cre/ERp72$^{fl/fl}$ mice. (A) Genotyping of Pf4-Cre/ERp72$^{fl/fl}$ mice. Homozygous ERp72$^{fl/fl}$ mice were mated with Pf4-Cre-positive homozygous ERp72$^{fl/fl}$ mice. Genotyping of tail DNA from the offspring yielded a 356 bp product for the second floxed site using primers 5′-CTGCTGTGTCACCTCACCAACTCT-3′ and 5′-AGAAGTGGGAGGAATTGAACAGTTC-3′, and a 420 bp product for Cre enzyme using primers 5′-CCCATACAGCACACCTTTTG-3′ and 5′-TGCACAGTCAGCAGGTT-3′. (B) Glycoprotein (αIIbβ3, GPIb or GPVI) expression on platelets from Pf4-Cre/ERp72$^{fl/fl}$ and ERp72$^{fl/fl}$ littermate controls was analyzed by flow cytometry; mean ± SE, n = 10, t-test.

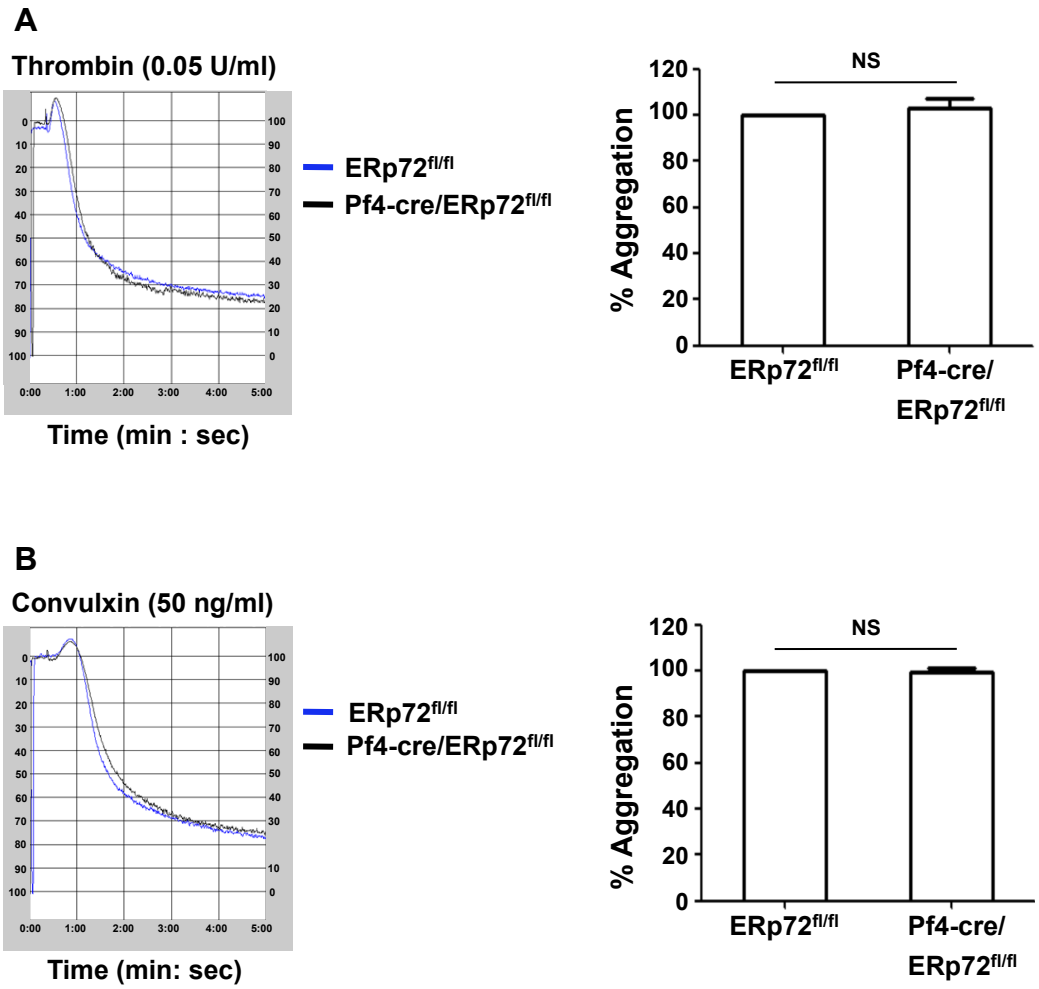


Figure S6. Decreased aggregation of ERp72-null platelets is overcome by higher concentrations of agonists. (A) thrombin-induced aggregation and (B) convulxin-induced aggregation; mean ± SEM, *n* = 3, t test.

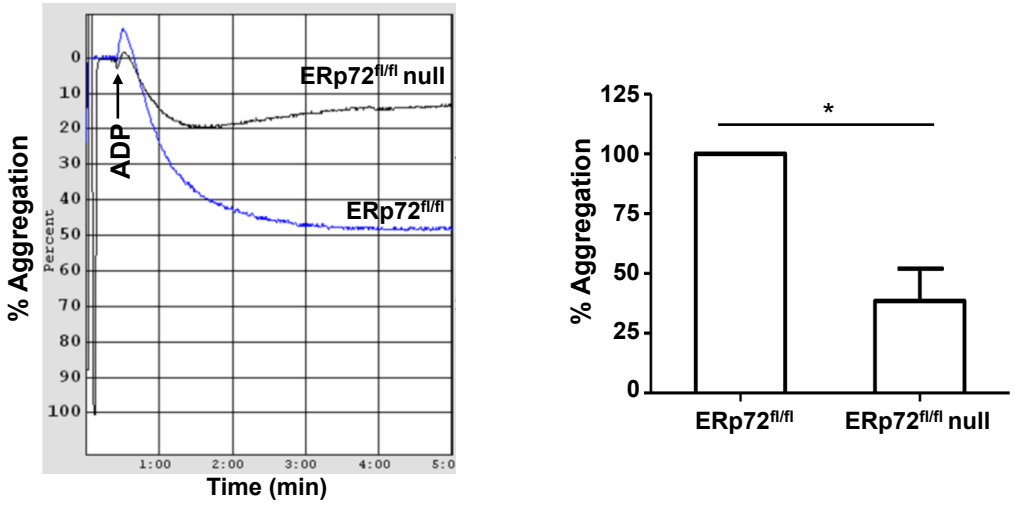


Figure S7. ERp72 is critical for ADP induced platelet aggregation. Representative aggregation tracings (left panels) and combined results (right) showing the defect in ERp72-deficient platelets using ADP (12 μM); mean ± SEM, n = 3, **P* < 0.05, t-test. Platelet-rich plasma was prepared and aggregation performed in the lumi-aggregometer.

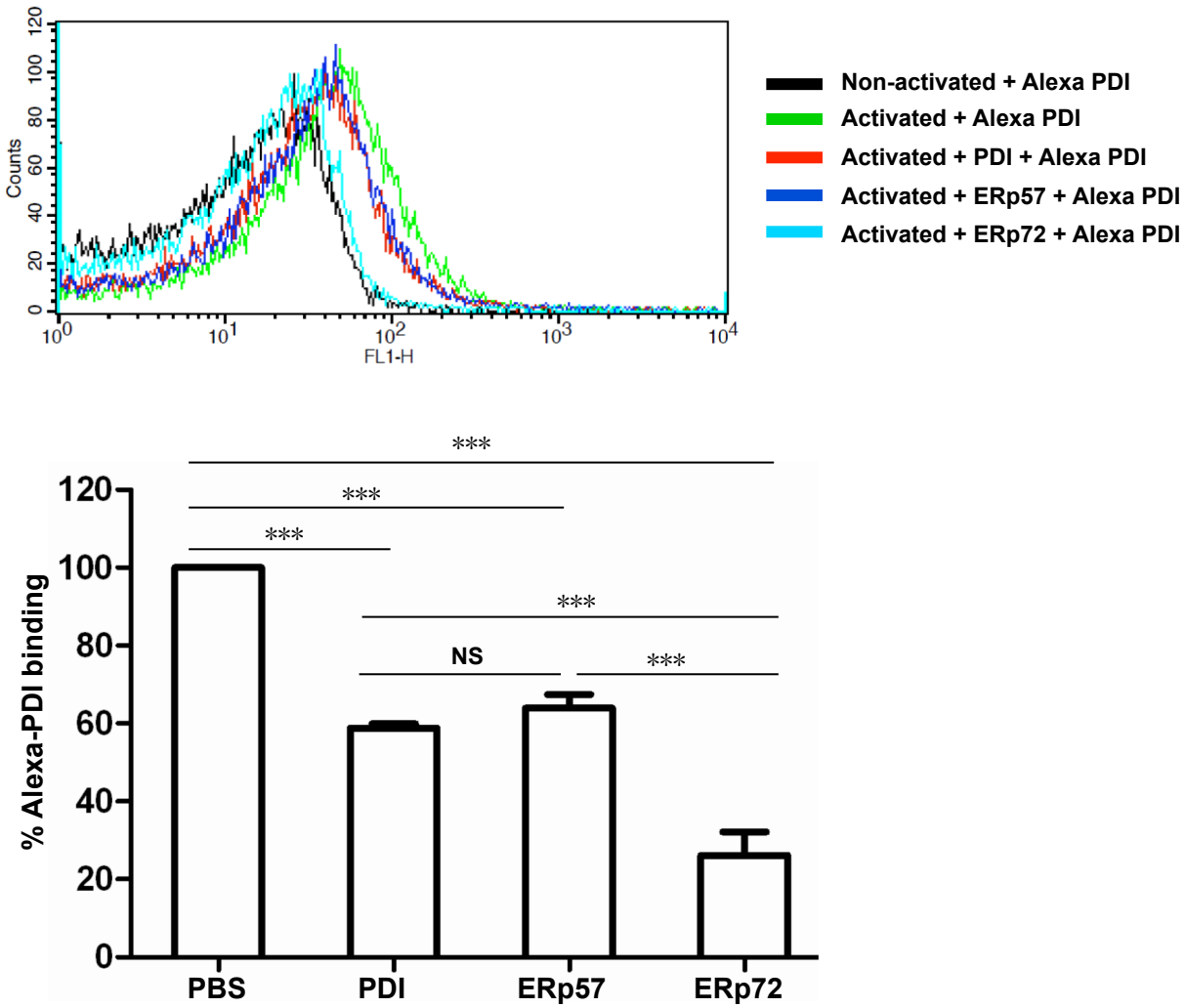


Figure S8. Binding of PDI, ERp57 and ERp72 to activated platelets. Human platelets were activated with thrombin (0.5 U/ml for 3 min), incubated with Alexa 488-labeled PDI (250 nM) for 10 min, and analyzed by flow cytometry. Samples were incubated with a 10 fold excess of unlabeled PDI, ERp57 or ERp72 for 3 min before addition of Alexa-488 labeled PDI and compared to samples incubated with PBS; mean ± SEM, n =3, *** $P < 0.001$, ANOVA.

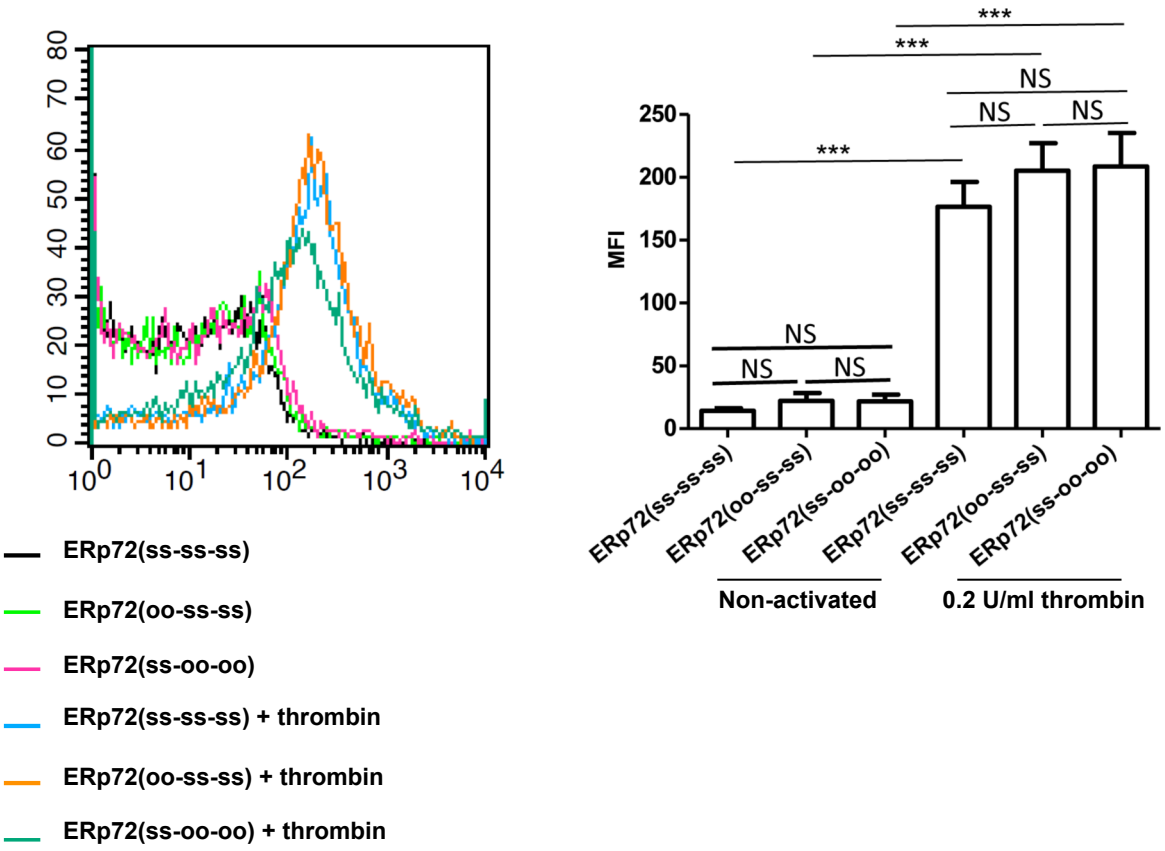


Figure S9. Similar binding of ERp72(ss-ss-ss), ERp72(oo-ss-ss) and ERp(ss-oo-oo) to the platelet surface. Representative histogram showing binding of Alexa labeled variants of ERp72 to non-activated and thrombin (0.2 U/ml)-activated platelets (left). Cumulative data (right); mean ± SEM, n = 3, *** $P <$ 0.001, ANOVA. Washed wild-type mouse platelets were activated and incubated with Alexa 488 ERp72 (250 nM) for 10 min and analyzed by flow cytometry.

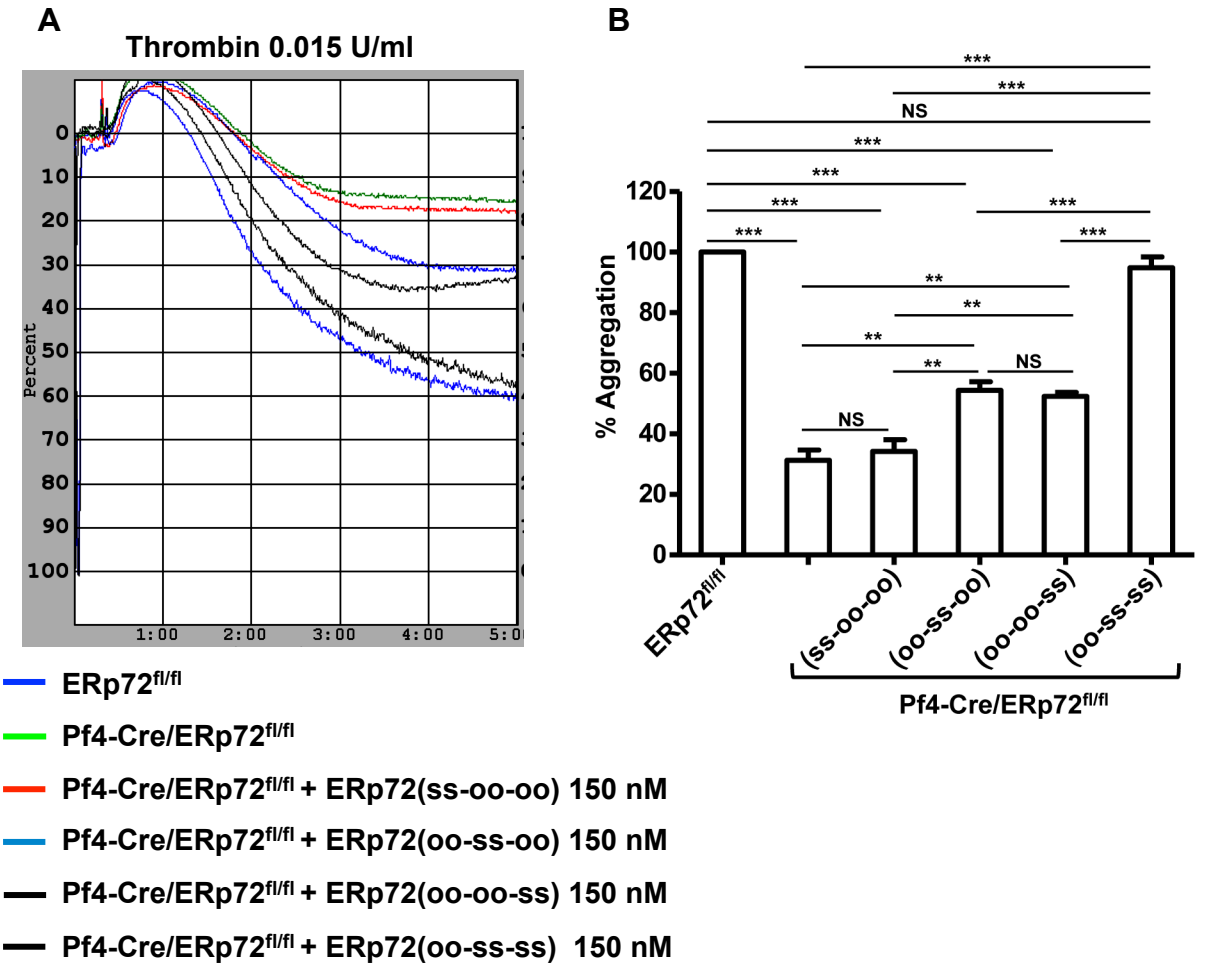


Figure S10. Effect of double active site mutants that contain only a single functional active site on aggregation of ERp72-null platelets. **(**A and B**)** Correction of the aggregation defect of ERp72-null platelets (2 × 10^8 platelets/ml) from Pf4-Cre/ERp72$^{fl/fl}$ mice compared with wild type (ERp72$^{fl/fl}$) littermate mice by mutant ERp72 proteins. The mutant proteins were added 5 minutes prior to the addition of thrombin (0.015 U/ml). Representative tracings (A) and cumulative data for aggregation (B); mean ± SEM, *n* = 3 for each sample, ***P* < 0.01, ****P* < 0.001, ANOVA.

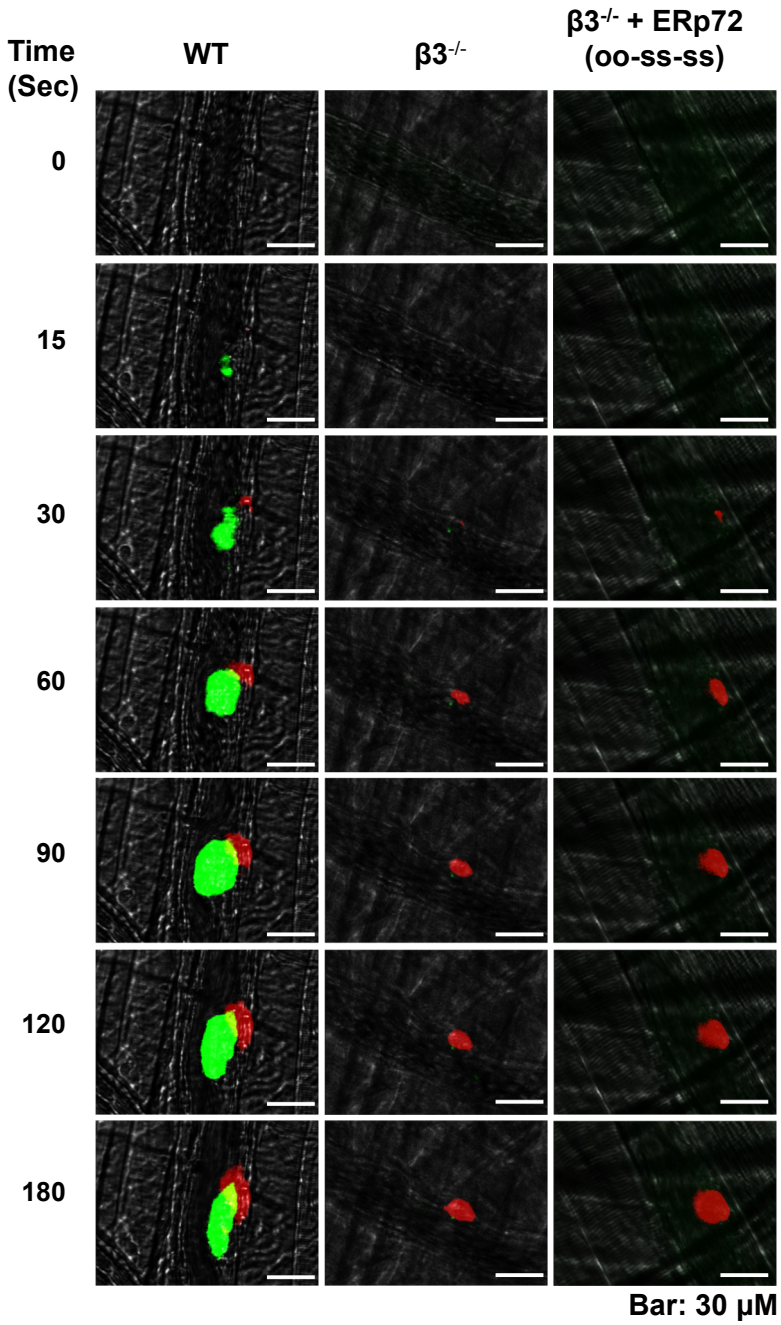


Figure S11. Recombinant ERp72(oo-ss-ss) protein directly enhances fibrin deposition. Images for Figure 7 B and C. Cremaster arteriole injury was induced in wild-type mice after they received intravenous injections of anti-fibrin antibodies conjugated to Alexa Fluor 647, and 3′-dihexyloxacarbocyanine iodide (DIOC6) (2.5 μL of a 100 μM solution/g of body weight). Representative images for platelet accumulation visualized by these two methods (green) and fibrin formation (red).

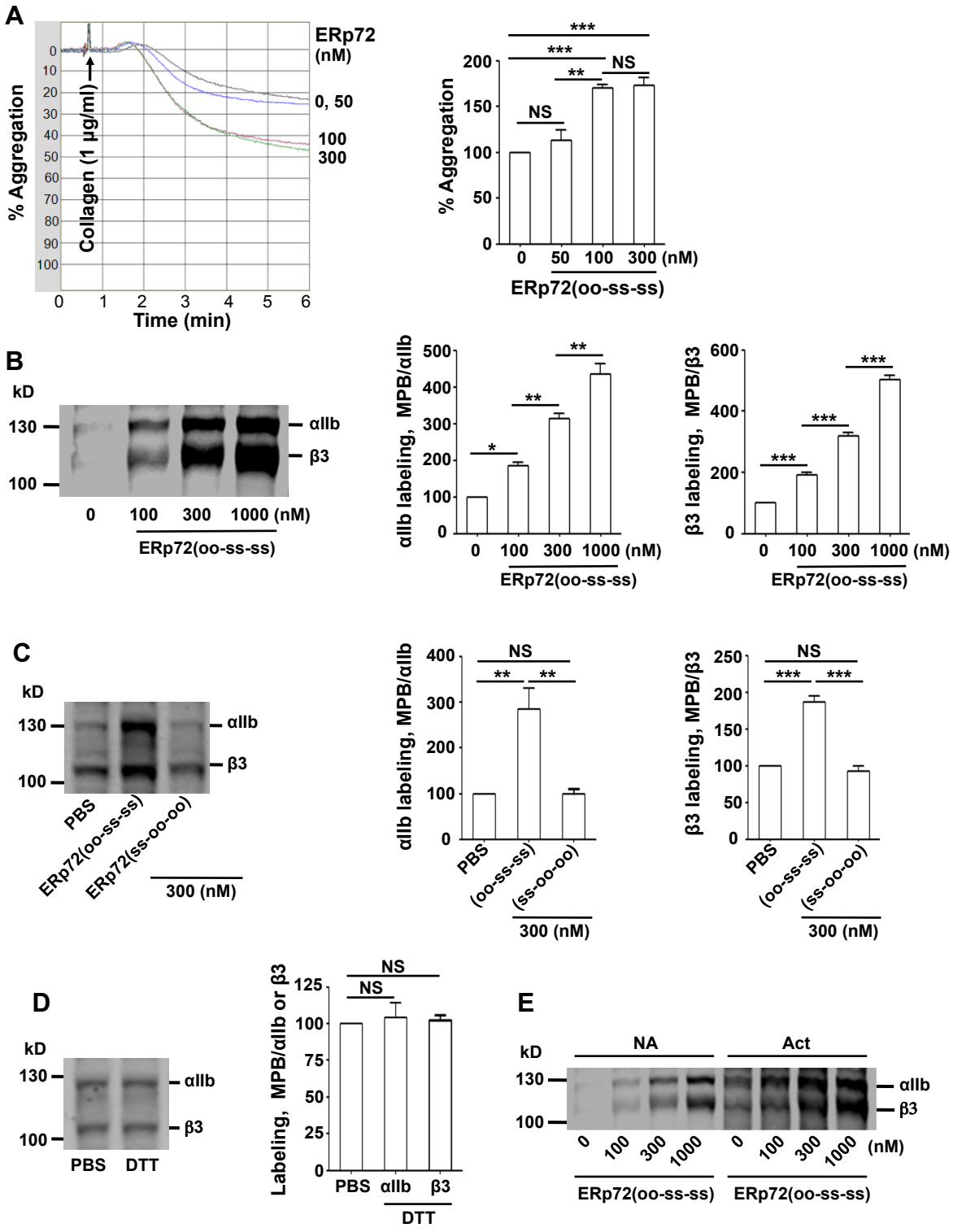
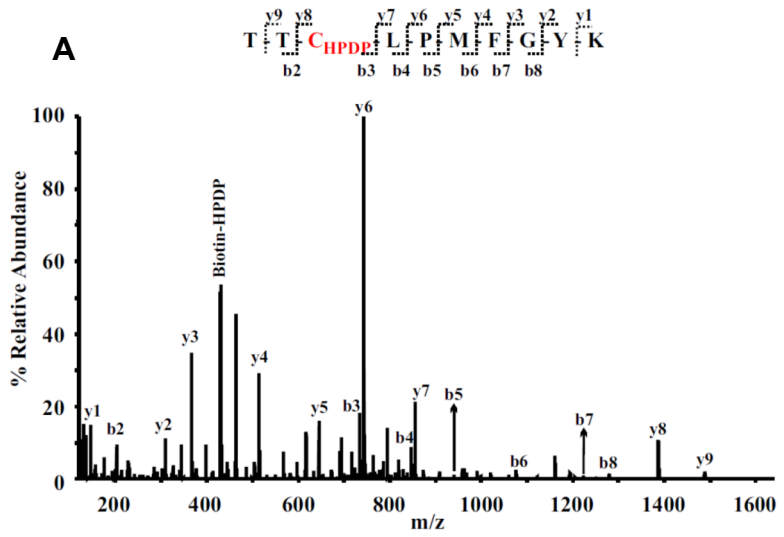

A
% Aggregation
Collagen (1 µg/ml)
Time (min)
ERp72 (nM)
0, 50
100
300
% Aggregation
ERp72(oo-ss-ss)
NS
B
kD
130
100
αIIb
β3
0 100 300 1000 (nM)
ERp72(oo-ss-ss)
αIIb labeling, MPB/αIIb
β3 labeling, MPB/β3
C
PBS
ERp72(oo-ss-ss)
ERp72(ss-oo-oo)
300 (nM)
D
PBS DTT
Labeling, MPB/αIIb or β3
PBS αIIb β3
DTT
E
NA
Act
0 100 300 1000 (nM)

Figure S12. ERp72 potentiates platelet aggregation and generates thiols in αIIb and β3. (A) Effect of adding wild-type ERp72 (50-300 nM) on collagen-induced platelet aggregation (2 x 10^8 platelets/ml). Effect of (B) ERp72(oo-ss-ss), (C) ERp72(ss-oo-oo), or (D) DTT (2.5 μM) on MPB labeling of thiols in αIIb and β3. In (B-D) the left panel shows a representative MPB blot and the right panel(s) cumulative data of the ratio of MPB labeling to αIIb or β3 protein; mean ± SEM, $n = 4$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA. (E) Effect of adding ERp72(oo-ss-ss) and platelet activation with thrombin (1 U/ml) on MPB labeling of αIIb and β3. Labeling was performed by adding MPB (100 uM) to washed non-activated platelets.



B. Cysteine residues labeled by biotin-HPDP in αIIbβ3

(numbering does not include the 31 amino acid signal peptide in αIIb or the 26 amino acid signal peptide in β3)

αIIb

non-activated platelets

Cys56, Cys65, Cys130, Cys146, Cys473, Cys545, Cys687

activated platelets

Cys56, Cys65, Cys130, Cys146, Cys473, Cys484, Cys490, Cys545, Cys687

β3

non-activated platelets

Cys49, Cys184, Cys495, Cys631, Cys687

activated platelets

Cys49, Cys184, Cys495, Cys631, Cys687

Additional cysteine residues found labeled with biotin-HPDP in a single sample.

αIIb: non-activated Cys107; activated, Cys826.

β3: non-activated, Cys273; activated, Cys232, Cys635, Cys663.

Figure S13. (A) Representative MS/MS spectrum of a doubly-charged ion (m/z 794.87) corresponding to the peptide sequence of 182-TTCLPMFGYK-191 with a biotin-HPDP modification at Cys184 in the β3 subunit. The observed y- and b-ion series confirmed the peptide sequence, and a biotin-HPDP modified Cys (+428.2 amu) found between b^2 and b^3 as well as between y^7 and y^8 ions in the spectrum confirmed the modification of Cys184. (B) Table of all biotin-HPDP labeled cysteine residues found in 3 separate experiments.

Valid	...	Sequence	Prob	Masc...	Masc...	Masc...	NTT	Modifications	Observed	Actual Mass	Charge	Delta ...	Delta ...	Rete...	Intensity	TIC	Start	Stop	# Ot...	Other Prot...	Spectrum ID
☑	✓	(R)TLGPSQEETGGVFLCPWR(A)	100%	59.3	25.0	59.3	2	Biotin-HPDP (+428)	802.38	2,404.13	3	-0.0054	-2.2	11400		3.035E7	73	90	0		Essex_enrichmen...
☑	✓	(R)TLGPSQEETGGVFLCPWR(A)	100%	101.6	25.0	101.6	2	Biotin-HPDP (+428)	1,203.07	2,404.13	2	-0.010	-4.2	11400		2.376E7	73	90	0		Essex_enrichmen...
☑	✓	(R)AEGGQCPSLLFDLR(D)	100%	32.0	25.0	32.0	2	Biotin-HPDP (+428)	645.31	1,932.92	3	-0.0058	-3.0	11300		2.505E7	91	104	0		Essex_enrichmen...
☑	✓	(K)TPVGSCFLAQPESGR(R)	100%	55.2	25.0	55.2	2	Biotin-HPDP (+428)	988.97	1,975.93	2	-0.0027	-1.3	9840		1.034E8	156	170	0		Essex_enrichmen...
☑	✓	(K)TPVGSCFLAQPESGR(R)	100%	46.6	25.0	46.6	2	Biotin-HPDP (+428)	988.97	1,975.93	2	-0.0024	-1.2	9890		1.444E7	156	170	0		Essex_enrichmen...
☑	✓	(K)TPVGSCFLAQPESGR(R)	100%	43.4	25.0	43.4	2	Biotin-HPDP (+428)	659.65	1,975.93	3	-0.0030	-1.5	9830		2.52E7	156	170	0		Essex_enrichmen...
☑	✓	(K)TPVGSCFLAQPESGR(R)	100%	40.7	25.0	40.7	2	Biotin-HPDP (+428)	988.97	1,975.93	2	-0.0030	-1.5	9830		5.232E7	156	170	0		Essex_enrichmen...
☑	✓	(K)TPVGSCFLAQPESGR(R)	100%	29.5	25.0	29.5	2	Biotin-HPDP (+428)	659.65	1,975.93	3	-0.0024	-1.2	9890		3247000	156	170	0		Essex_enrichmen...
☑	✓	(R)RAEYSPCR(G)	99%	23.4	25.0	23.4	2	Biotin-HPDP (+428)	705.33	1,408.64	2	-0.0033	-2.4	5680		1189000	171	178	0		Essex_enrichmen...
☑	✓	(R)RAEYSPCR(G)	99%	19.1	25.0	19.1	2	Biotin-HPDP (+428)	705.33	1,408.64	2	-0.0028	-2.0	5630		5400000	171	178	0		Essex_enrichmen...
☑	✓	(K)SCVLPQTK(T)	99%	26.6	25.0	26.6	2	Biotin-HPDP (+428)	652.33	1,302.65	2	-0.0015	-1.2	7710		2.856E7	503	510	0		Essex_enrichmen...
☑	✓	(K)HSPICHTTMAFLR(D)	100%	47.0	25.0	47.0	2	Biotin-HPDP (+428)	971.47	1,940.92	2	-0.0074	-3.8	7770		2650000	572	584	0		Essex_enrichmen...
☑	✓	(K)HSPICHTTMAFLR(D)	100%	33.0	25.0	33.0	2	Biotin-HPDP (+42...	653.31	1,956.92	3	0.00031	0.16	6780		7515000	572	584	0		Essex_enrichmen...
☑	✓	(K)HSPICHTTMAFLR(D)	100%	30.7	25.0	30.7	2	Biotin-HPDP (+428)	647.98	1,940.92	3	-0.0089	-4.6	7810		7027000	572	584	0		Essex_enrichmen...
☑	✓	(K)HSPICHTTMAFLR(D)	100%	27.3	25.0	27.3	2	Biotin-HPDP (+428)	647.98	1,940.93	3	0.0011	0.59	7760		9500000	572	584	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	50.7	25.0	37.0	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0042	-2.6	10300		2.832E7	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	39.5	25.0	28.5	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0020	-1.2	10400		6117000	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	40.3	25.0	24.1	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0036	-2.2	10400		1.375E7	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	39.9	25.0	23.2	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0046	-2.8	10300		8.828E7	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	28.8	25.0	28.8	2	Biotin-HPDP (+42...	823.91	1,645.80	2	-0.0053	-3.2	9480		8911000	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	27.5	25.0	27.5	2	Biotin-HPDP (+42...	823.91	1,645.80	2	-0.0040	-2.4	9790		4384000	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	31.9	25.0	16.7	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0027	-1.7	10300		2.053E7	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	100%	27.8	25.0	27.8	2	Biotin-HPDP (+42...	823.91	1,645.80	2	-0.0061	-3.7	10300		1.567E7	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	96%	16.9	25.0	16.9	2	Biotin-HPDP (+428)	815.91	1,629.81	2	-0.0054	-3.3	10500		3235000	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	95%	15.3	25.0	15.3	2	Biotin-HPDP (+42...	823.91	1,645.80	2	-0.0023	-1.4	9740		5485000	715	725	0		Essex_enrichmen...
☑	✓	(R)VVLCELGNPMK(K)	95%	18.7	25.0	18.7	2	Biotin-HPDP (+42...	823.91	1,645.81	2	0.000040	0.024	9630		6546000	715	725	0		Essex_enrichmen...

Table. Representative % probability of identification of biotin-HPDP containing trypic fragments in the αIIb subunit prepared from non-activated platelets.

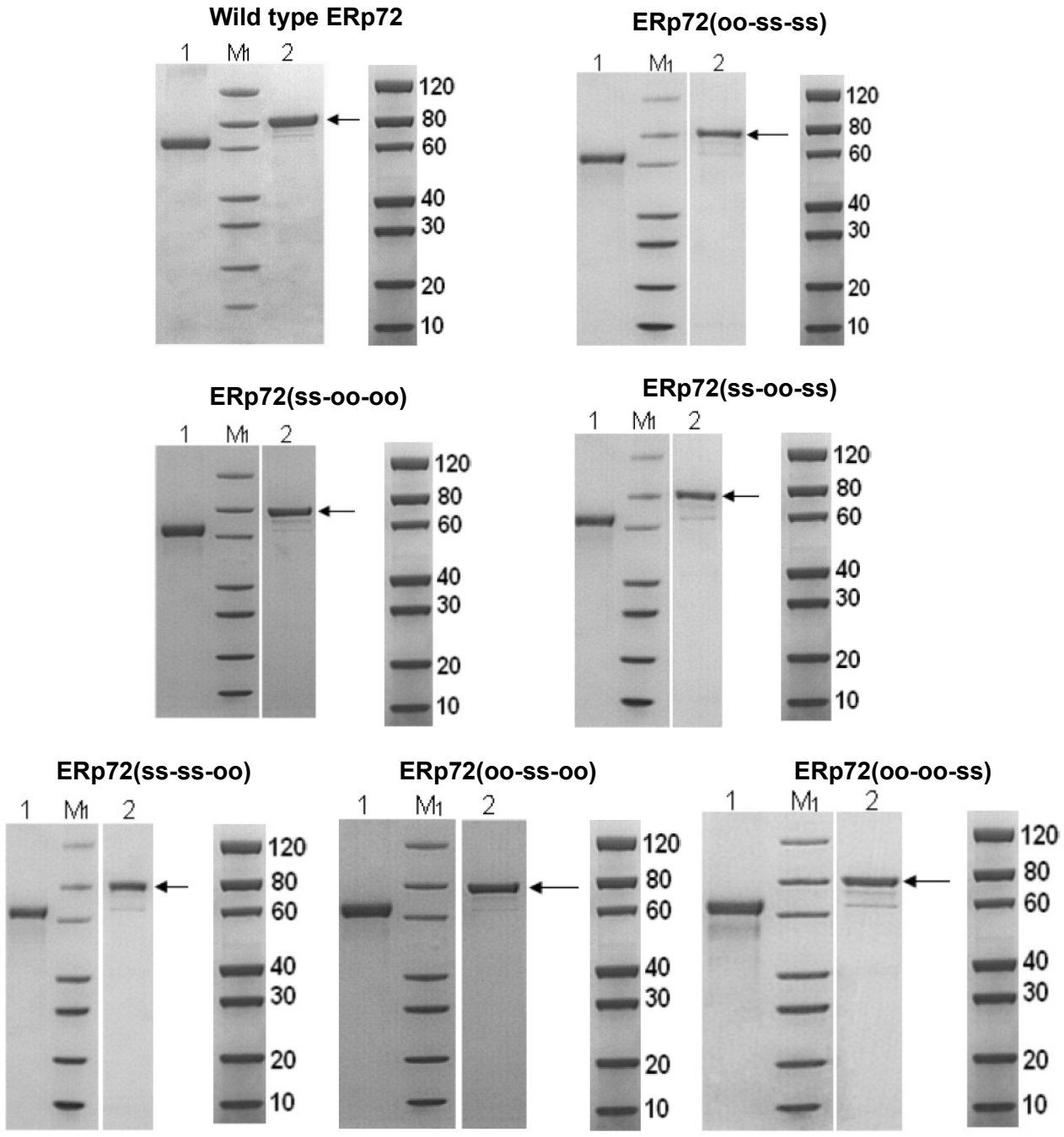


Figure S14. Purity of ERp72 proteins on SDS gel electrophoresis. SDS-PAGE analysis of purified recombinant ERp72 proteins. Each lane 1 contains BSA (2 μg); Each lane 2 contains the ERp72 variant (1.65-2 μg/lane). The other lanes have the molecular weight numbers.