Supplemental Methods

Materials. Thiazole orange, epinephrine, apyrase, aspirin, ADP, D-(+)-Galactose and A23187 were from Sigma-Aldrich (St. Louis, MO). U46619 was from CalBiochem (San Diego, CA), and PAR4 activating peptide from Bachem (Torrance, CA). Goat anti-RGS10, goat anti-human integrin α_{IIb} (c-20), and donkey anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-RGS18 and Alexa Fluor 488-labeled rat anti-TLT-1 were from Novus Biologicals (Centennial, CO). Rabbit anti-β-actin was from Cell Signaling Technology (Danvers, MA), and donkey anti-rabbit IgG-HRP from GE Healthcare (Chicago, IL). Alexa Fluor 568- and 647-labeled anti-CD41 $F(ab')_2$ fragments (Clone MWReg30), Alexa Fluor 647- and FITC-labeled anti-P-selectin (clone RB40.34), and fluorescent BUV395 Annexin V were purchased from BD Bioscience (San Jose, CA). PE-labeled anti-$\alpha_{IIb}\beta_3$ integrin (clone JON/A) was purchased from Emfret Analytics (Eibelstadt, Germany). α2-3,6,8 Neuraminidase and ApoI-HF were purchased from New England Biolabs (Ipswich, MA). FITC-labeled Ricinus Communis Agglutinin I (RCA-I), peroxidase-conjugated anti-goat secondary, and DAB Peroxidase Substrate Kit were purchased from Vector Laboratories (Burlingame, CA). Pacific-blue anti-mouse lineage cocktail, APC/Cyanine7 anti-mouse CD117 (c-kit), PerCP/Cy5.5 anti-mouse Ly-6A/E (Sca-1), and PE/Dazzle™ 594 anti-mouse CD150 (SLAM), APC anti-mouse CD42d, and FITC anti-mouse CD41 antibodies were purchased from BioLegend (San Diego, CA). Alexa Fluor 647-labeled anti-fibrin antibody (clone 59D8) was a generous gift from Dr. Hartmut Weiler (Blood Research Institute, WI) and Dr. Rodney Camire (Children's Hospital of Philadelphia, PA). Alexa Fluor 488-labeled Annexin V was a generous gift from Dr. Sriram Krishnaswamy (Children's Hospital of Philadelphia, PA). Vybrant™ DyeCycle™ Violet Stain was purchased from Thermo Fisher Scientific (Waltham, MA).

Mice. Cas9 mRNA was generated from pMJ920-Cas9 plasmid using mMESSAGE mMACHINE T7 Ultra Transcription Kit according to the manufacturer's instructions (Life Technologies, AM1345). The quality of the Cas9 mRNA was determined by analyzing Cas9 mRNA pre- and post-polyadenylation with a 2100 Bioanalyzer. gRNAs were designed for *Rgs10* and *Rgs18* genes by following the protocol described in Ran et al.[1] T7 promoter was added to the gRNA templates by PCR amplification. The PCR product was purified and then used as a template for in vitro transcription according to the manufacturer's specifications (MEGAshortscript T7 kit, Life Technologies). The gRNAs were then purified using the MEGAclear kit (Life Technologies). gRNA quality was verified on agarose gel. Zygotes from C57BL/6 mice were injected with Cas9 mRNA (100 ng/μl) and gRNAs (50 ng/μl). Embryos were then transferred to pseudo-pregnant C57BL/6 females. After birth, 10-day-old mice were tail-snipped and genomic DNA was extracted for

genotyping and sequencing. The lone founder mouse (*Rgs10*$^{-/-}$*Rgs18*$^{+/-}$) was backcrossed to genetically identical parental WT C57BL/6 mice. Successive breeding with mice from the same colony was performed as necessary to generate *Rgs10*$^{+/+}$*Rgs18*$^{+/+}$, *Rgs10*$^{-/-}$*Rgs18*$^{+/+}$, *Rgs10*$^{+/+}$*Rgs18*$^{-/-}$, and *Rgs10*$^{-/-}$*Rgs18*$^{-/-}$ mice. Age- and sex-matched WT, single and double knockouts for experimental use were generated from homozygous parents for each genotype.

Genotyping. Mice were genotyped for *Rgs10* using a three primer PCR-based strategy. Forward Primer: 5'-GTGGATAACAGTCCAGCTTCTC-3', Reverse Primer 1: 5'-CCAGAGCCCATCTCACATTTA-3', Reverse Primer 2: 5'- GTTCCTCAGCCTTCGTCAAT-3'. PCR was performed with the following conditions: Denaturation at 95°C for 5 minutes; 35 cycles of (95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute); extension at 72°C for 7 minutes. Mice were genotyped for *Rgs18* by PCR and endonuclease digestion strategy. Forward Primer: 5'-TGTGTAAATGTGTGGATCCTTGT-3', Reverse Primer: 5'-ACTTTCAATCCATAATCATACGCTGTATTCTG-3'. PCR was performed with the following conditions: Denaturation at 95°C for 2 minutes; 35 cycles of (95°C for 30 seconds, 59°C for 45 seconds, and 72°C for 45 seconds); extension at 72°C for 5 minutes. Samples were then incubated with ApoI-HF according to manufacturer protocol. Additionally, to routinely verify PCR genotyping results, samples were processed with ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Santa Clara, CA) according to manufacturer protocol prior to addition of Forward Primer for Sanger sequencing through the Genomics Analysis Core at the University of Pennsylvania.

Preparation of diluted whole blood. Whole blood was isolated via the retro-orbital plexus from isoflurane-anesthetized mice using heparinized micro-hematocrit capillary tubes. Blood was diluted 1:20 with HEPES-Tyrode's Buffer (HTB; 137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/liter BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM, NaH_2PO_4, pH 7.4) and stored at 37°C prior to analysis.

Vascular injury: platelet and fibrin accumulation. Alexa Fluor 568–labeled anti-CD41 $F(ab')_2$ fragments, Alexa Fluor 488–labeled anti–P-selectin, and Alexa Fluor 647-labeled anti-fibrin were administered via a catheter in the jugular vein. Arterioles 30-50 µm in diameter were studied. Penetrating vascular injuries were produced with a pulsed nitrogen dye laser fired through the microscope objective. Thrombus formation was observed for 3 min at 1.9 frames per second and analyzed using SlideBook 6 software (Intelligent Imaging Innovations, Denver, CO). Transient occlusions were defined as hemostatic thrombi that completely filled the blood vessel, but either

did not fully block blood flow or blocked it temporarily. Stable occlusions were defined as fully occluded vessels that did not recover flow by the end of the observation period

Bone marrow megakaryocyte immunohistochemistry. Femurs were harvested from mice and fixed for at least 48 hours in 10% neutral buffer formalin. Decalcification, paraffin embedding, sectioning and slide mounting was performed by the Comparative Pathology Core at the University of Pennsylvania School of Veterinary Medicine. Immunohistochemistry to stain for α_{IIb} was performed as previously described.[2] Briefly, slides were incubated with goat anti-human integrin α_{IIb} followed by peroxidase-conjugated anti-goat secondary, stained with DAB Peroxidase Substrate Kit, and counterstained with hematoxylin. Imaging was performed using a 20X objective on a Nikon Eclipse E600 microscope and quantification of megakaryocytes performed blinded by counting large, positively stained brown cells with multi-lobed nuclei.

Megakaryocyte progenitor analysis. Bone marrow was isolated from femurs and tibias by flushing with PBS + 1X Penicillin-Streptomycin (Gibco, Waltham, MA) and counted using an automated trypan blue cell counter. To quantitate numbers of healthy, immunophenotypic bone marrow megakaryocyte progenitors (MegPs), cells were stained with the following fluorescently labeled antibodies: Pacific blue-anti-mouse lineage cocktail (Lin), PerCP/Cy5.5 anti-Sca-1, APC/Cy7 anti-c-Kit, PE anti-CD16/CD32, PE/Dazzle 594 anti-CD150, FITC anti-CD41, and fluorescent BUV395 Annexin V (to exclude apoptotic cells) and analyzed by flow cytometry. Healthy MegPs were defined as Annexin V$^{(-)}$Lin$^{(-)}$Sca-1$^{(-)}$c-Kit$^{(+)}$CD16/32$^{(-)}$CD150$^{(+)}$CD41$^{(+)}$ cells.

Cultured megakaryocyte analysis. Unfractionated bone marrow cells isolated as outlined above were seeded at 5 x 10^6 cells per well in a 6-well plate in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS (HyClone, Chicago, IL), 100 U/mL Pen/Strep (Gibco, Waltham, MA), 2 mM Glutamine (Gibco, Waltham, MA), and 50 ng/mL mouse thrombopoietin (TPO; R&D Systems, Minneapolis, MN). Cells were cultured for 5 days and media was replenished on Day 3 of culture. On Day 5, terminal megakaryocytes (Megs) and Meg ploidy were quantitated by staining with APC anti-mouse CD42d, FITC anti-mouse CD41, and Vybrant DyeCycle followed by flow cytometric analysis. Megs were defined as CD42d$^{(+)}$CD41$^{(+)}$ cells and ploidy was determined by measuring distinct histogram peaks in DyeCycle within the CD42d$^{(+)}$CD41$^{(+)}$ subpopulation. Following enrichment via a 3%/1.5% BSA gradient, cells were seeded into 24-well plates coated with fibronectin (Thermo Fisher, Waltham, MA) and containing 10% FBS, 100 U/mL Pen/Strep, and 10 ng/mL TPO. After 48 hours, cells extending at least one proplatelet protrusion were counted and imaged using a phase contrast inverted microscope at 200X total magnification

attached to an 18 mega pixel digital camera (OMAX, Kent, WA). Three wells were examined per condition and at least 100 cells quantitated per well.

Platelet depletion and recovery. Twenty-four hours prior to depletion, mouse whole blood was acquired from the retro-orbital plexus and counted with a Procyte Hematological Analyzer (Idexx Laboratories, Westbrook, ME) to establish base-line counts. To assess the rate of platelet production, mice were injected via the retro-orbital plexus with 0.2 μg/g bodyweight of platelet-depleting rat anti-GPIbα antibody (Emfret Analytics, Eibelstadt, Germany) multiplied by the percent of platelets relative to Wild type (WT) baseline (to account for differences in base-line platelet counts between genotypes). Twenty minutes post-injection, platelets counts were <5% of baseline for each genotype. Every 24 hours for five days, whole blood was obtained from the retro-orbital plexus and analyzed for platelet counts as they recovered.

Desialylation and phosphatidylserine exposure. Diluted whole blood was prepared as outlined in "Preparation of diluted whole blood". For desialylation studies, diluted blood was incubated with vehicle, 200 mM galactose (competitive inhibitor for RCA-I binding) or 10 U/mL α2-3,6,8 neuraminidase (sialidase) as indicated for 15 minutes at 37°C, prior to a 15-minute incubation with FITC-labelled RCA-I to measure desialylation and CD41 for gating. RCA-I lectin binding increases upon reduced sialylation. For WT vs DKO studies, samples were only incubated with RCA-I and Alexa Fluor-labelled CD41 prior to flow cytometric analysis. For phosphatidylserine exposure studies, diluted blood was incubated with Alexa Fluor 488-labelled Annexin V and 10 μM A23187 (calcium ionophore) or vehicle for 5 minutes prior to flow cytometric analysis.

Bone marrow chimeras. Four days prior to irradiation, mice begun treatment ad libitum per os with Sulfamethoxazole and Trimethoprim (Aurobindo Pharma, Hyderabad, India) to prevent infections. Mice were then irradiated with two doses of 550 rad each spread 2 hours apart. Following irradiation, donors were retro-orbitally injected with 2×10^7 unfractionated bone marrow cells in sterile DMEM isolated from recipients as outlined above. After six weeks, reconstitution of bone marrow was complete and platelet counts for mice were determined as described.

Mouse lung immunofluorescence. Mice were injected with DyLight488-labelled anti-mouse GPIbβ (Emfret Analytics, Eibelstadt, Germany) 24 hours prior to experiment. Three minutes prior to euthanasia, mice were either injected with either saline as a vehicle control or 18 μg/mL collagen and 150 μg/mL epinephrine to induce systemic thrombosis as a positive control. Following euthanasia, lung was harvested, fixed for 24 hours in 4% neutral buffered formalin, cryoprotected with sucrose, and then frozen in optimal cutting temperature (OCT) compound (Sakura Finetek,

Torrance, CA). After sectioning, samples were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs, Burlingame, CA). Images were acquired from at least three fields per sample using a Nikon Eclipse TE2000-U at 20X magnification equipped with blue (DAPI) and green (FITC) filters and analyzed with Slidebook6 software.

References

1. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. 2013;8:2281.
2. Capitano M, Zhao L, Cooper S, et al. Phosphatidylinositol transfer proteins regulate megakaryocyte TGF-β1 secretion and hematopoiesis in mice. *Blood*. 2018;132(10):1027-1038-1038.

Supplemental Figure Legends

Supplemental Figure 1. Characterization of mice. (A) Initial weight gains of female (left) and male (right) WT, RGS18$^{-/-}$, RGS10$^{-/-}$, and RGS10$^{-/-}$18$^{-/-}$ mice. At least 4 measurements were collected per genotype per day, mean ± SEM. (B) Blood counts, hematocrit and hemoglobin of 8-week-old WT, RGS18$^{-/-}$, RGS10$^{-/-}$, and RGS10$^{-/-}$18$^{-/-}$ mice. At least 9 measurements were collected per genotype. NS indicates P > 0.05, mean ± SEM.

Supplemental Figure 2. *In vitro* platelet activation pairwise comparisons. Statistical comparisons to WT controls for flow cytometric analysis of (A, C, E) P-selectin expression and (B, D, F) integrin $\alpha_{IIb}\beta_3$ activation of platelets from matched WT, RGS18$^{-/-}$, RGS10$^{-/-}$, and RGS10$^{-/-}$18$^{-/-}$ mice. Platelets were stimulated with increasing doses of: (A, B) PAR4 activating peptide (PAR4P, AYPGKF), (C, D) ADP and (E, F) TxA_2 analogue (U46619) and gated by FSC/SSC and CD41 positivity. At least 4 measurements were collected per genotype per condition. NS indicates P > 0.05, mean ± SEM.

Supplemental Figure 3. Maximum ADP and U46619 P-selectin and Jon/A fold-change. Fold-change in binding relative to WT controls of (A, C) anti-P-selectin and (B, D) Jon/A antibodies in response to maximally stimulating concentrations of (A, B) ADP (5 µM) and (C, D) TxA_2 analogue, U46619 (2.5 µM). At least 4 measurements were collected per genotype per condition. NS indicates P > 0.05, mean ± SEM.

Supplemental Figure 4. *In vitro* analysis of megakaryocytes. (A) Percentage of megakaryocyte progenitors (MegPs) in the lineage cocktail(-), Sca-1(-), c-Kit(+), CD16/32(-) [$L^-S^-K^+C^-$] subpopulation of bone marrow cells. MegPs were defined as CD150(+) and CD41(+) cells within the aforementioned subpopulation. (B) Number of megakaryocytes (Megs) per unit input of MegP after culturing for 5 days in the presence of thrombopoietin (TPO). Megs were defined as CD42d(+) and CD41(+) cells. (C) Percentage of TPO-cultured CD42d(+)/CD41(+) Megs that are diploid (2N), tetraploid (4N) or greater than or equal to octoploid (≥8N) as assessed via DNA dye. (D) Percentage of proplatelet-forming cells after BSA-gradient enrichment and culture on fibronectin-coated plates. Cells were defined as proplatelet-forming if they possessed visible membrane extensions or protrusions. N = 4. All results shown as mean ± SEM.

Supplemental Figure 5. Exploring mechanisms of platelet clearance. Flow cytometric analysis of: (A, B) P-selectin on (A) resting or (B) epinephrine-stimulated platelets from WT, RGS18$^{-/-}$, RGS10$^{-/-}$, and RGS10$^{-/-}$18$^{-/-}$ mice; (C) RCA-I:FITC binding to untreated, galactose-

blocked, or sialidase-treated WT platelets (N = 3); (D) RCA-I:FITC binding to WT and RGS10$^{-/-}$18$^{-/-}$ platelets (N = 6); and (E) Annexin-V:488 binding to resting or A23187 (calcium ionophore) treated WT and RGS10$^{-/-}$18$^{-/-}$ platelets (N = 3). (F) Platelet counts of lethally irradiated WT mice reconstituted with WT or RGS10$^{-/-}$18$^{-/-}$ bone marrow as compared to global RGS10$^{-/-}$18$^{-/-}$ mice (N = 4). All results shown as mean ± SEM. NS = not significant. ** $p \leq 0.01$.

Supplemental Figure 6. *Ex vivo* effects of aspirin and prasugrel administered *in vivo*. (A) TLT-1 exposure on the platelet surface in response to increasing doses of PAR4P. (B, C) Jon/A binding in response to ADP. All mice were given either aspirin plus prasugrel or vehicle (0.5% methylcellulose) daily by oral gavage for a total of 5 days prior to assessment. N = 4, mean ± SEM.

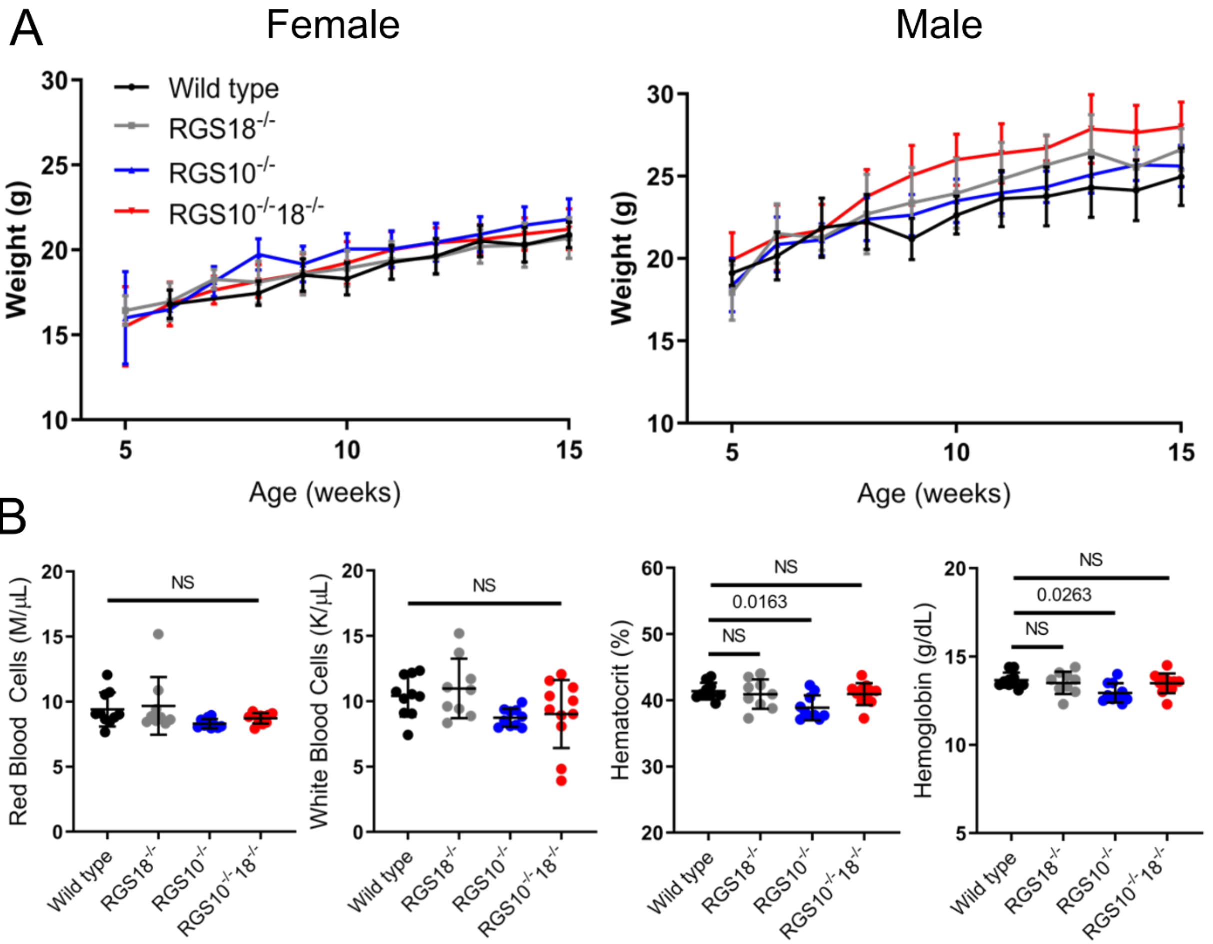
Supplemental Figure 7. Immunofluorescence analysis of pulmonary thrombosis. Mice were injected with DyLight488-labelled anti-GPIbβ antibody 24 hours prior to harvesting lung, fixation, sectioning and immunofluorescent staining for DAPI. Three minutes prior to euthanizing, mice were either injected with (A, B) vehicle control (saline) or (C) 18 µg/mL collagen and 150 µg/mL epinephrine to induce systemic thrombosis as a positive control. Images were acquired from at least three fields per sample using a Nikon Eclipse TE2000-U equipped with a blue (DAPI) and green (FITC) filters and analyzed with Slidebook6 software.

Supplemental Video Legends

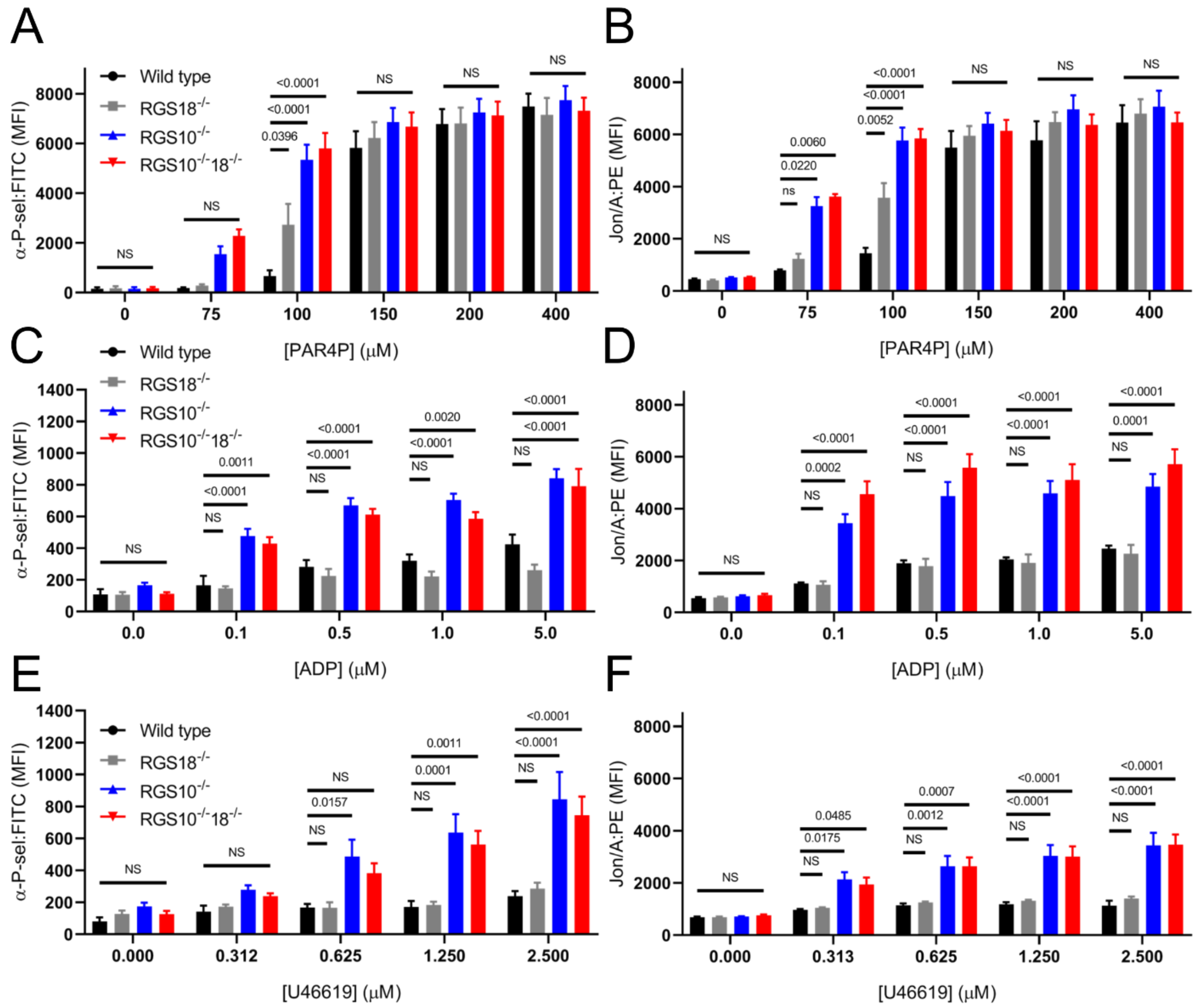
Supplemental Video 1. Wild type vs. RGS10$^{-/-}$18$^{-/-}$ hemostatic response to injury. Representative timelapse videos of platelet accumulation and activation and fibrin deposition following laser injury in wild type (left) and RGS10$^{-/-}$18$^{-/-}$ (right) mouse cremaster arterioles. Both videos are composites of 3 fluorescence channels overlaid on brightfield image: platelets (CD41) are labeled red, P-selectin (P-sel) is green, and fibrin is blue. Overlay of CD41 and fibrin appears magenta, CD41 and P-selectin appears yellow, fibrin and P-selectin appears cyan, and overlay of all three channels appears white. Images were captured at 2 composite frames per second and playback is at 20 frames per second. Timestamp is hh.mm:ss.000.

Supplemental Video 2. Transient vs. Stable Occlusions during RGS10$^{-/-}$18$^{-/-}$ hemostatic response to injury. Representative timelapse videos of a transient (left) and stable (right) occlusion in RGS10$^{-/-}$18$^{-/-}$ mouse cremaster arterioles. Both videos are composites of 3 fluorescence channels overlaid on brightfield image: platelets (CD41) are labeled red, P-selectin (P-sel) is green, and fibrin is blue. Overlay of CD41 and fibrin appears magenta, CD41 and P-selectin appears yellow, fibrin and P-selectin appears cyan, and overlay of all three channels appears white. Images were captured at 2 composite frames per second and playback is at 20 frames per second. Timestamp is hh.mm:ss.000.

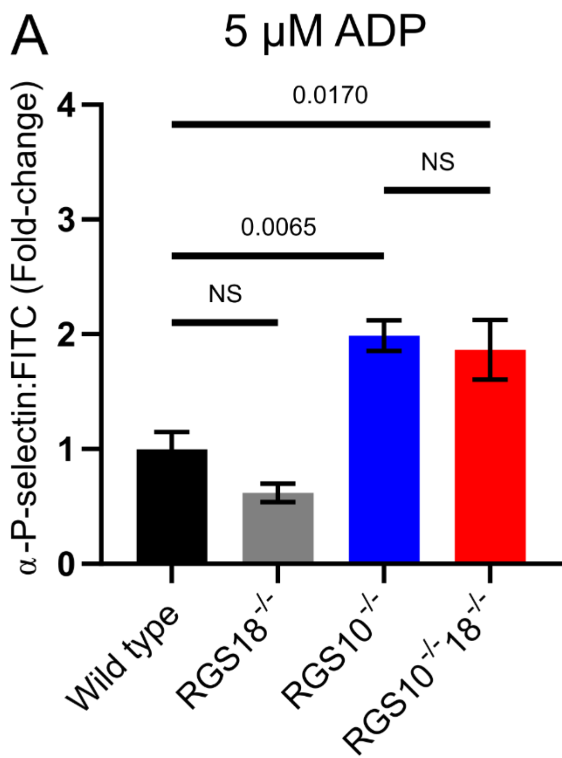
Supplemental Figure 1

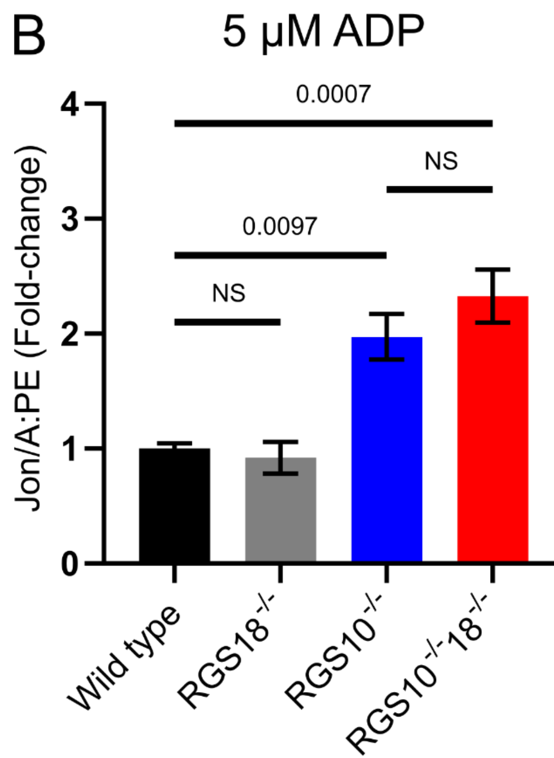


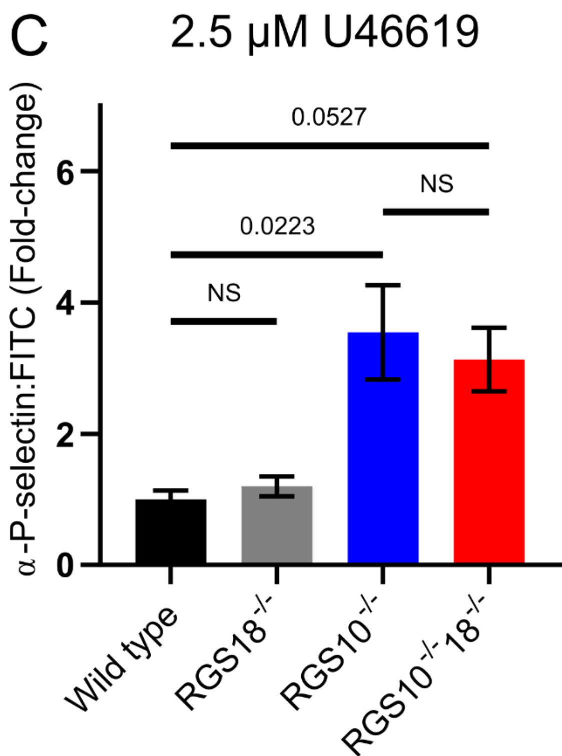
Supplemental Figure 2

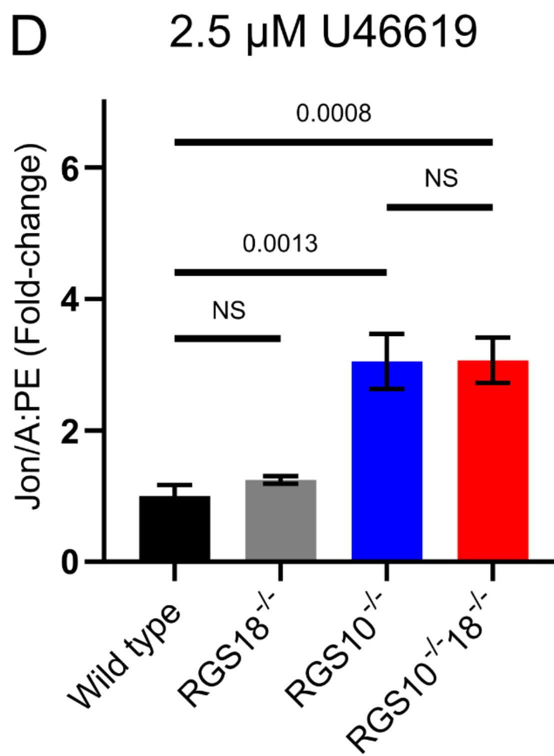


Supplemental Figure 3









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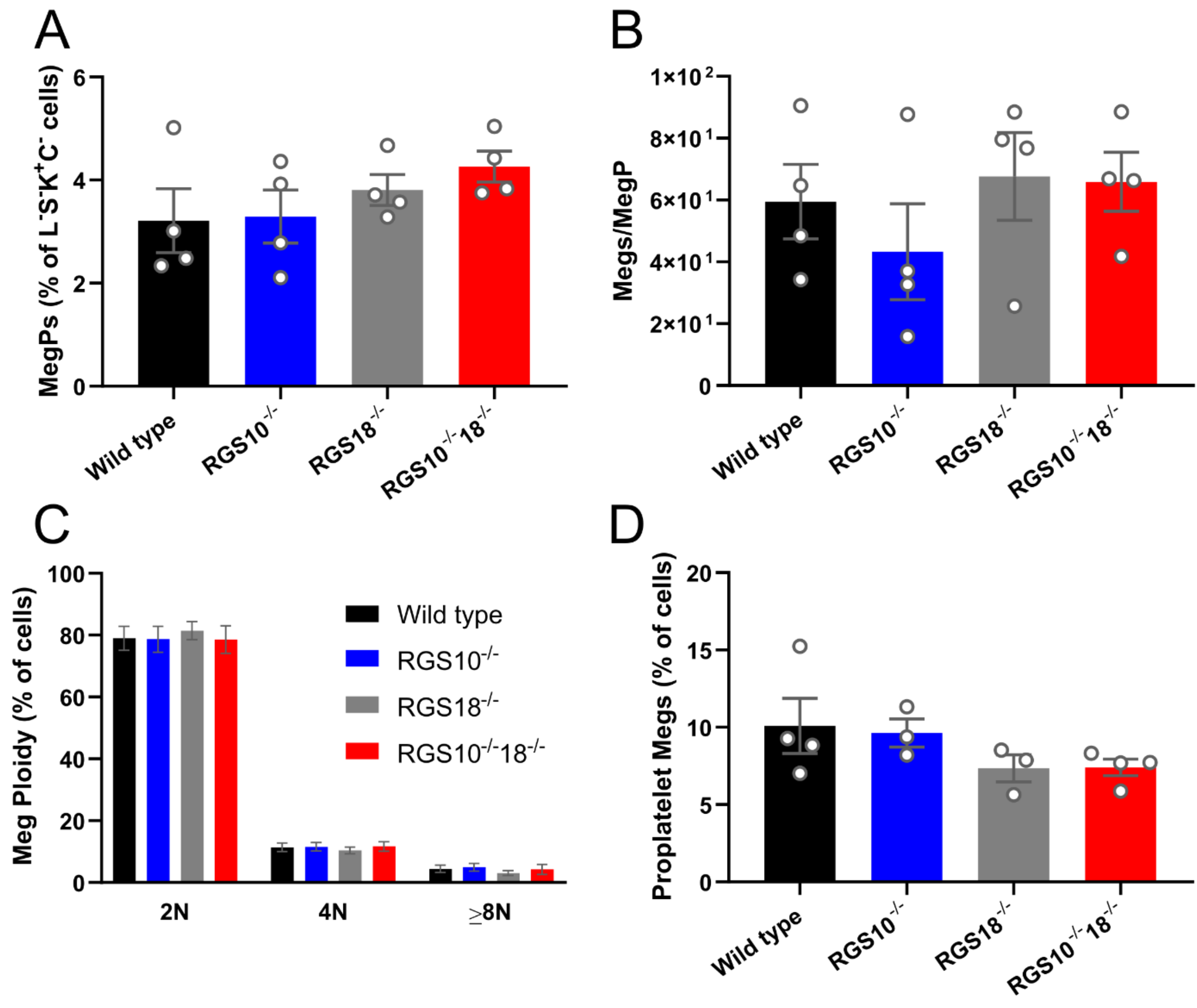
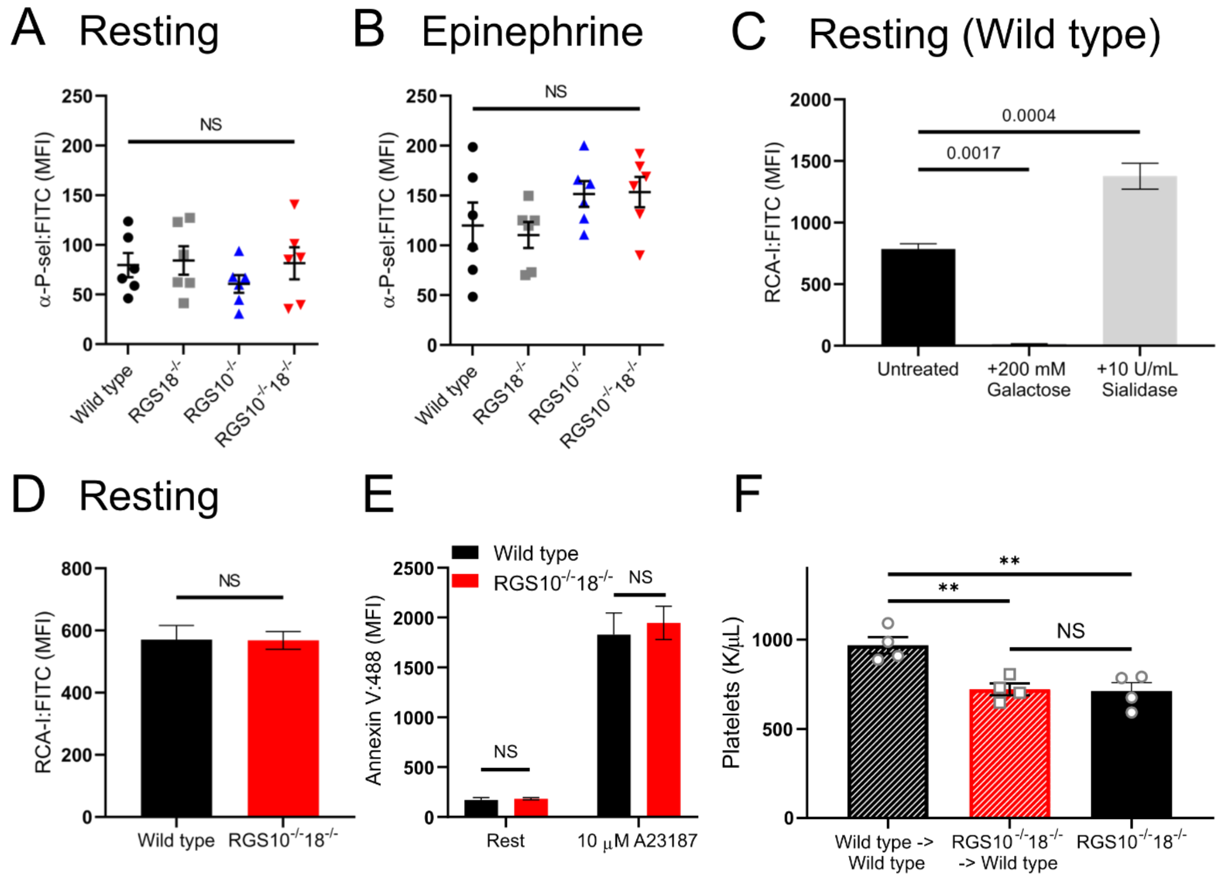
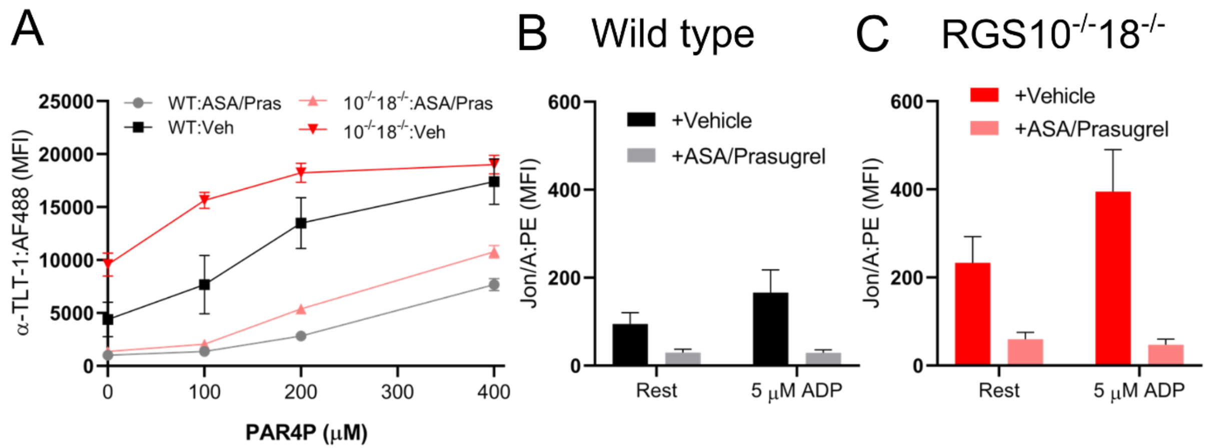


Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7

DAPI GPIbβ DAPI + GPIbβ

A Wild type

100 μM

B RGS10$^{-/-}$18$^{-/-}$

100 μM

C Wild type + Coll-Epi

100 μM

D