Supplemental Table 1

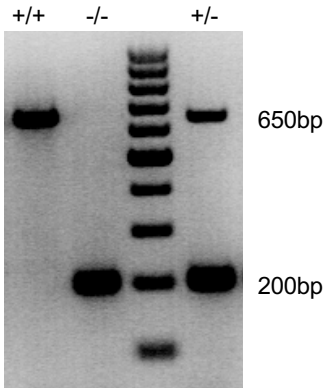
Mutant line	**GRK6-/-**	
Number of Mice	82 (WT 18, +/- 44, -/- 20)	
Expected number percentage	~20	25%
Actual	20	25%

Supplemental Table 2. Hematological parameters

		WT		GRK6$^{-/-}$		
Parameter	Units	Mean	SD	Mean	SD	P-value
Platelets	10^3 Cells/μL	832	63	734	57	0.03
Mean Platelet Volume	10^{-15} Liters	5.6	0.23	4.77	0.65	0.46
Red Blood Cells	10^6 Cells/μL	8.01	0.47	8.31	0.27	0.70
White Blood Cells	10^3 Cells/μL	8.14	3.88	10.68	2.84	0.20

Supplemental Figure 1
Xi, et al.

A.



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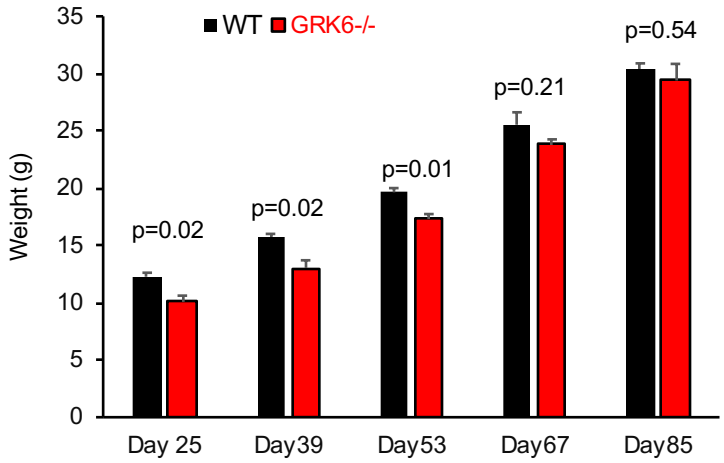
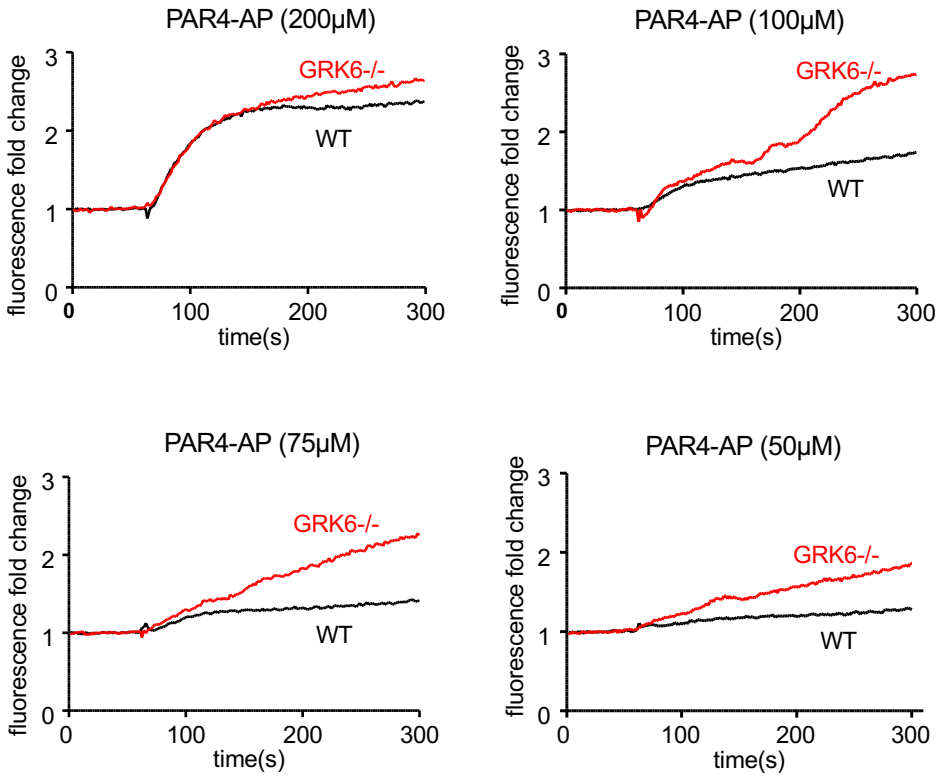
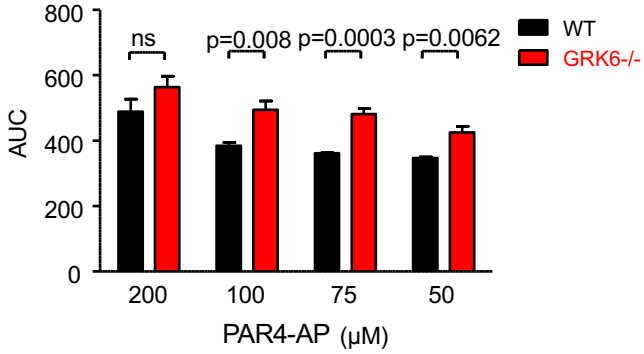


Supplemental Figure 2
Xi, et al.

A.





Supplemental Figure 2
Xi, et al.

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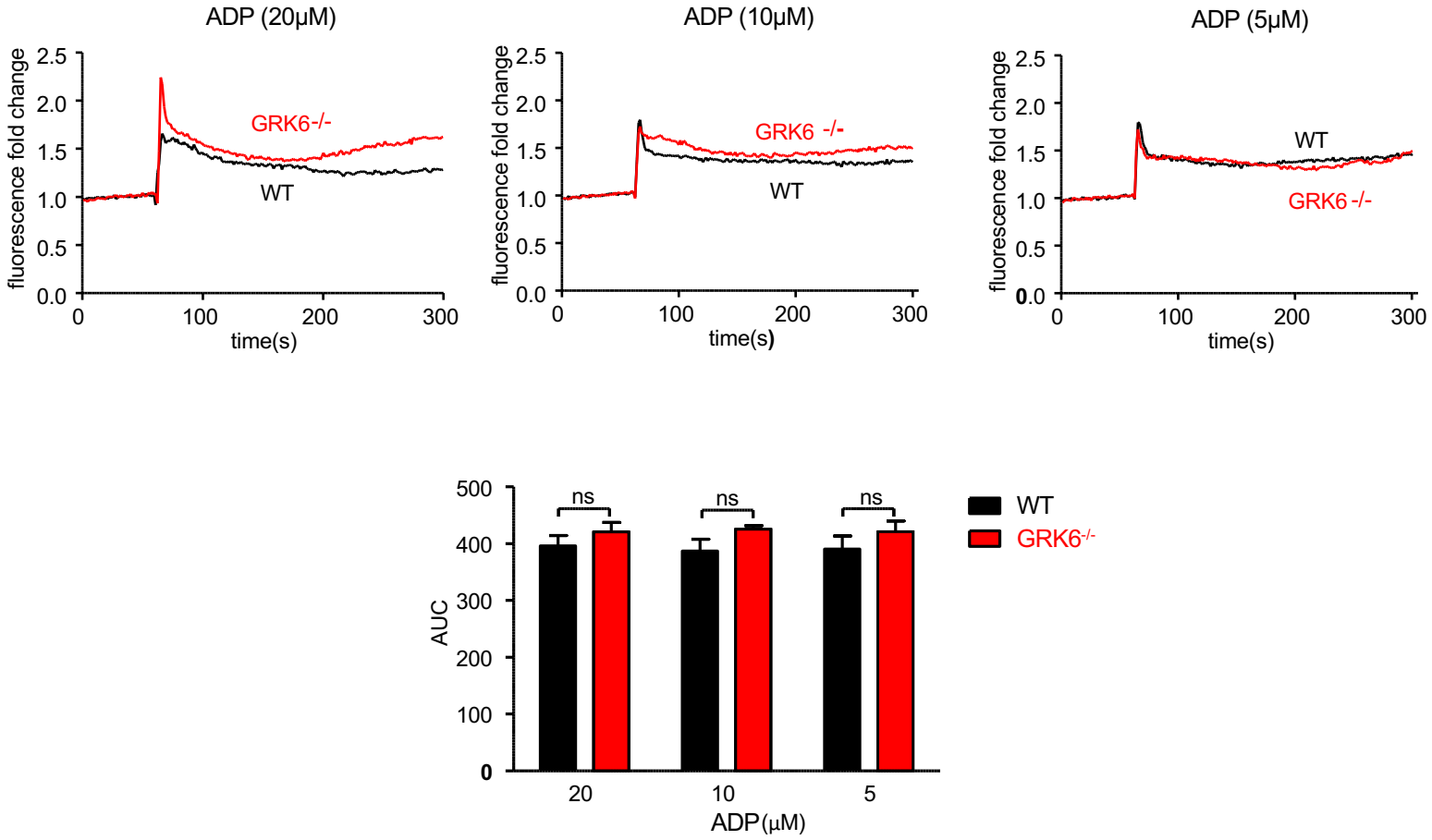
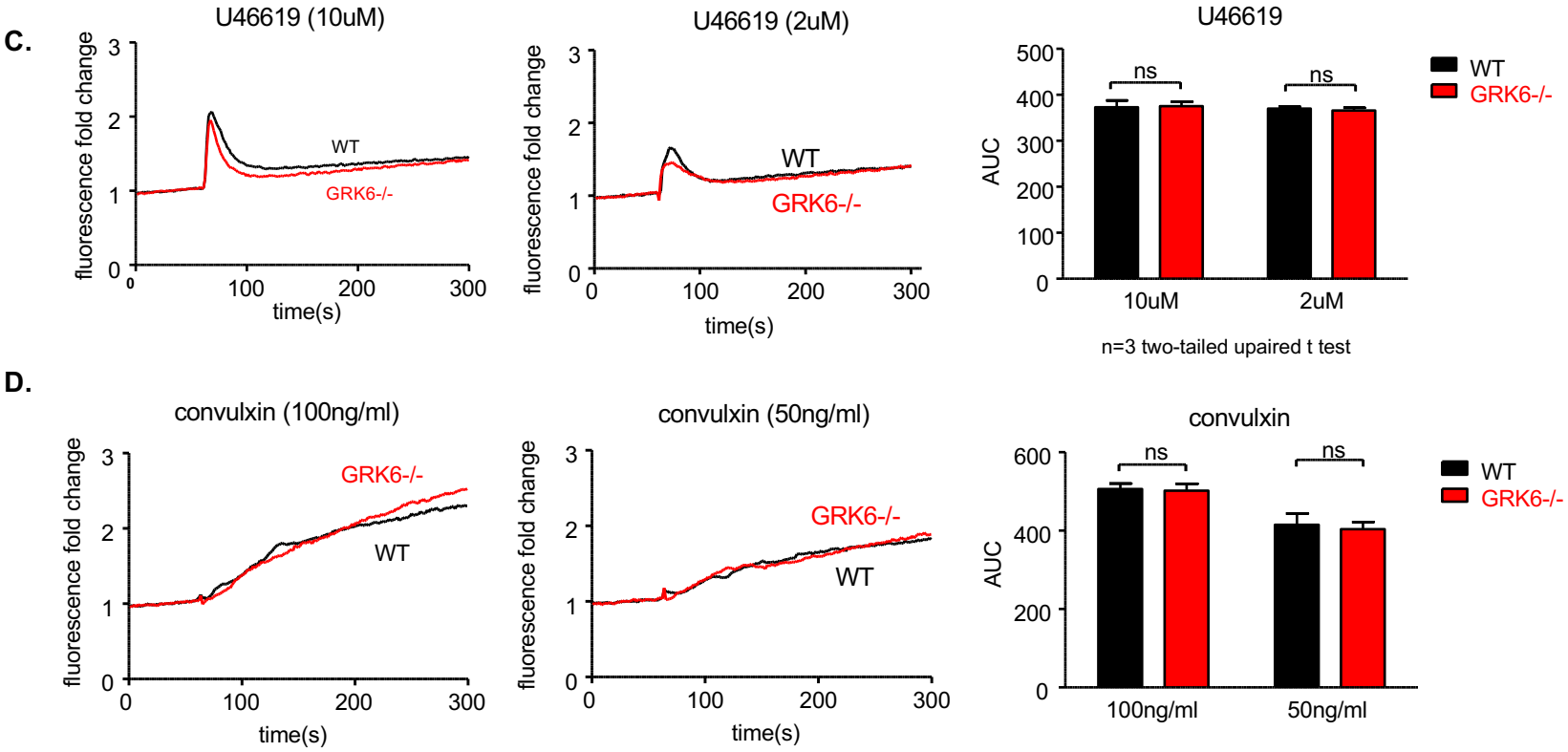
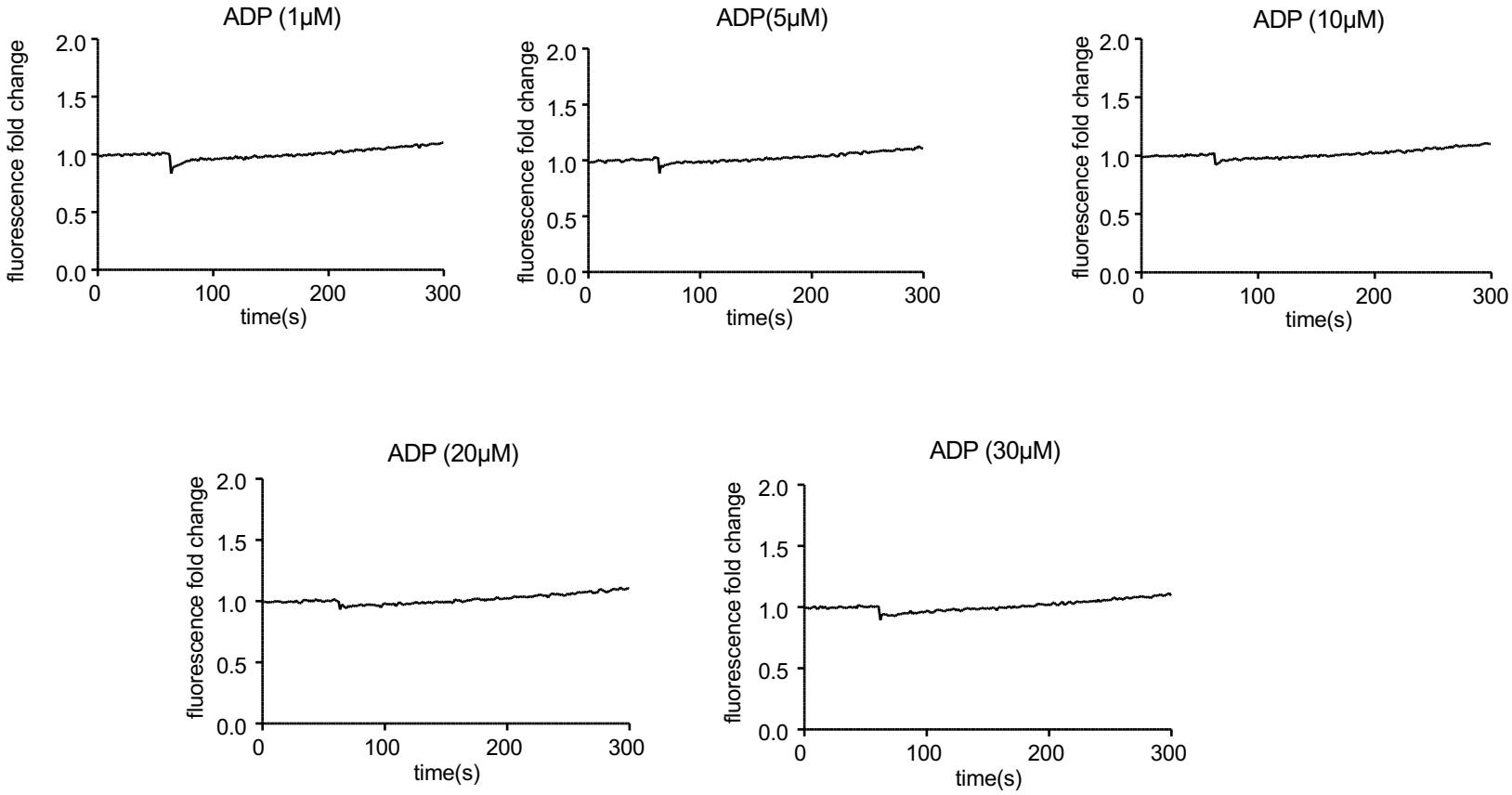


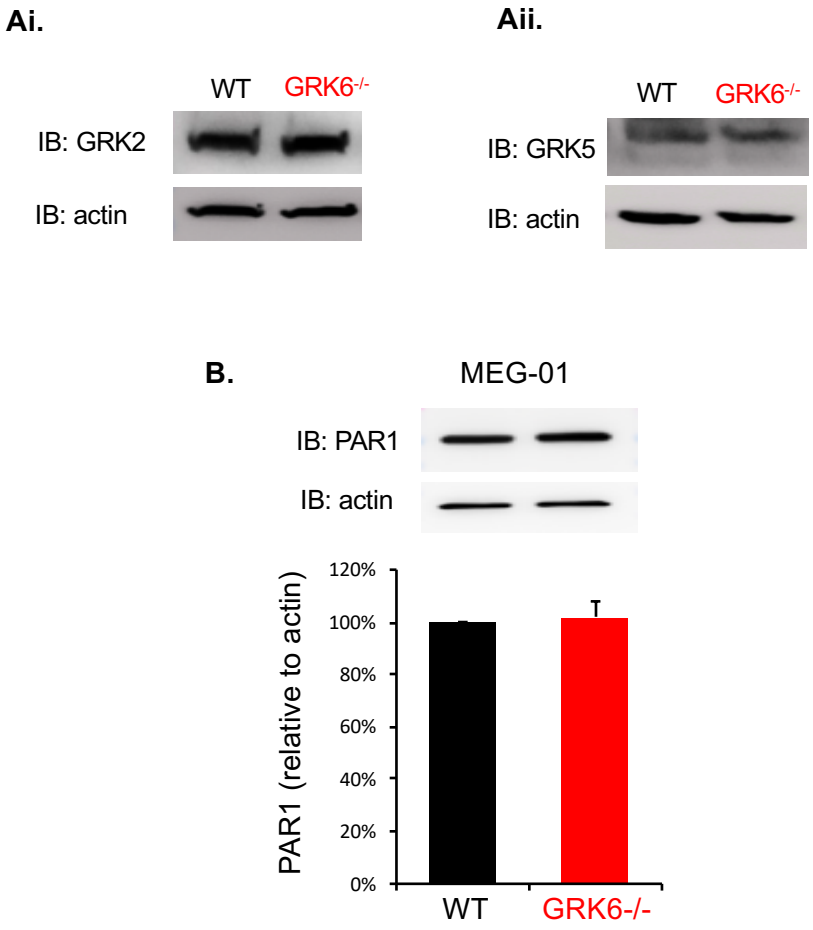
Supplemental Figure 2
Xi, et al.



Supplemental Figure 3
Xi, et al.

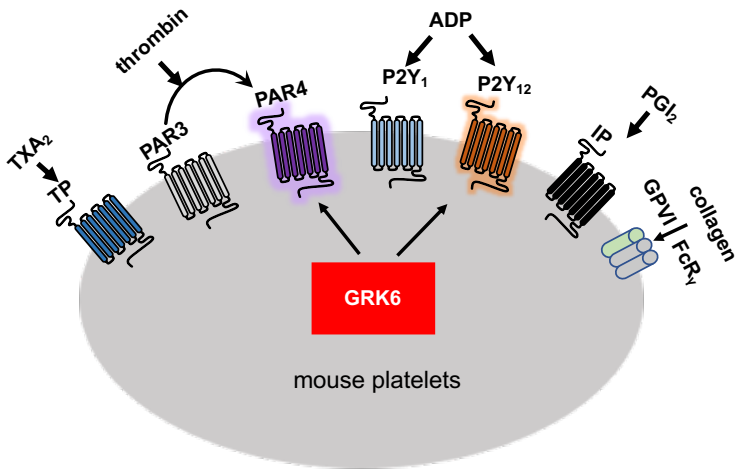


Supplemental Figure 4
Xi, et al.



Supplemental Figure 5
Xi, et al.

A.



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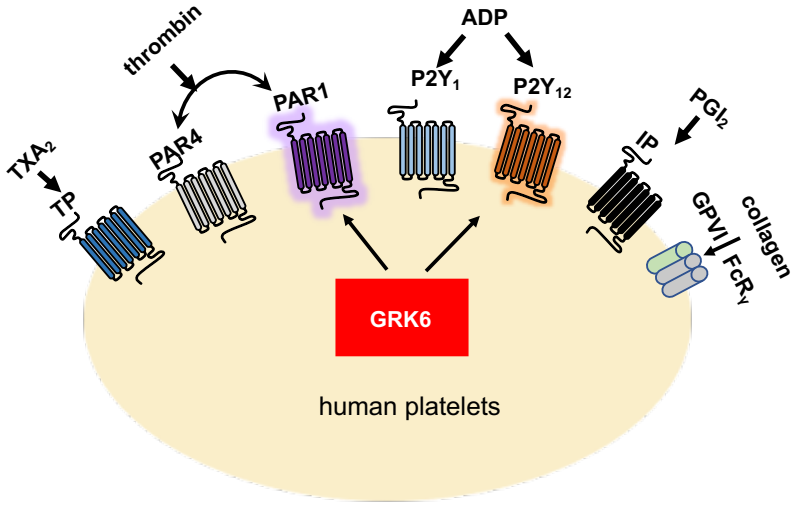
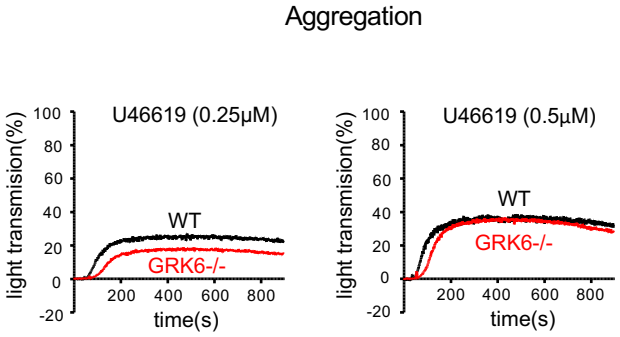


Supplemental Figure 6
Xi, et al.



Supplemental Table 1. **Mendelian ratio of offspring from +/G188S hemizygous breeding.**

Supplemental Table 2. Hematological parameters of GRK6$^{-/-}$ and wild-type (WT) littermate controls. Hematological parameters were measured with a Hemavet 950 (Drew Scientific Group) multi-species hematology system. Data are represented as mean ± standard deviation for 5 WT and 5 GRK6$^{-/-}$ mice.

Supplemental Figure 1. **Characterization of GRK6$^{-/-}$ mice**. (**A**) Genotyping. Mice were genotyped using a strategy that we developed. Primers pairs were chosen both external and internal to the excised region of the GRK6 gene, to differentiate between wild type and GRK6-knockout mice. The following primers were used in PCR reaction: Forward primer: 5'-GCTGTGTGGCACTGAGTTGA -3'; Reverse primer: 5'-GTCTGGAATTGTTCCAGGGAAGG -3'; Internal Forward primer: '5-CCTGCCAGTGAGTTCTAGGTC -3'; Internal Reverse primer: '5-CACCCACAGACATCTGCC -3'. We performed PCR with the following conditions: denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute); extension at 72°C for 7 minutes. Bands of the expected size were observed for the WT allele (650 bp) and the mutant allele (200 bp) by 1.5% agarose gel. (**B**) Mouse weights were recorded once a week beginning at Day 25. Weights are represented as mean ± SEM for 4 WT and 4 GRK6$^{-/-}$ mice.

Supplemental Figure 2: **The impact of deleting GRK6 on Ca^{2+} release in GRK6$^{-/-}$ platelets.** Platelets were loaded with fluo-4 AM, the cells were stimulated with PAR4-AP (A), ADP (B), U46619 (C), or CVX (D) and changes of Ca^{2+} were recorded using a FlexStation 3 system. The bar graph summarizes 3-4 experiments, respectively (mean ± SEM).

Supplemental Figure 3. The impact of deleting GRK6 on Ca^{2+} release in MEG-01 cells. MEG-01 cells were loaded with fluo-4 AM, the cells were stimulated with ADP at indicated concentrations (N=3). Changes of Ca^{2+} were recorded using a FlexStation 3 system.

Supplemental Figure 4. (**A**) Deletion of GRK6 in MEG-01 cells does not affect GRK2 (Ai) or GRK5 (Aii) protein expression. N=2. (**B**) Deletion of GRK6 does not affect PAR1 protein expression. N=3.

Supplemental Figure 5. A model of GRK6 regulation in GPCR dependent signaling (A) in mouse or (B) in human platelets.

Supplemental Figure 6. **Aggregation in platelets from GRK6$^{-/-}$ mice.** Platelets aggregation in response to TxA_2 mimetic (U46619) at the concentrations indicated. N=2.

Supplemental Methods

Materials. Apyrase, aspirin and ADP were from Sigma-Aldrich (St. Louis, MO), thrombin was from Haematologic Technologies, Inc. (Essex Junction, VT), U46619 from CalBiochem (San Diego, CA), collagen from Chrono-log (Havertown, PA). Fura-2/AM were from Molecular Probes (Eugene, OR). PAR4 agonist peptide, AYPGKF, were from Bachem (Torrance, CA). Anti-phosphoserine antibody and fluo-4 AM was purchased from thermo fisher scientific (Waltham, MA). Prostacyclin (PGI_2) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-PAR1 was from Novus Biologicals (Centennial, CO). Anti-PAR1 (ATAP2) and anti-GRK6 were from Santa Cruz (Santa Cruz, CA) and Abcam (Cambridge, MA) and donkey anti-goat IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GRK5/6 was purchased from EMD Millipore (Burlington, MA), Anti-GRK5 was from Novus Biologicals (Centennial, CO), anti-GRK2 and Rabbit anti-actin (13E5) was purchased from Cell Signaling Technology (Danvers, MA). Donkey anti-rabbit IgG-HRP was purchased from GE Healthcare UK Limited. Anti-Jon/A-PE antibody was purchased from Emfret Analytics (Wuerzburg, Germany). Alexa Fluor 568-labeled anti-CD41 $F(ab')_2$ fragments (Clone MWReg30) and Alexa Fluor 647-labeled anti-P-selectin (clone RB40.34) were purchased from BD Bioscience (San Jose, CA). Alexa-647 labeled anti-fibrin antibody (clone 59D8) was a generous gift from Dr. Hartmut Weiler (University of Wisconsin) and Dr. Rodney Camire (Children Hospital of Philadelphia).

Mice. Generation of GRK6 knockout mice using CRISPR-Cas9 genome-editing system was performed as described by Henao-Mejia et al.[1] Briefly, 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 postpolyadenylation with a 2100 Bioanalyzer. gRNAs were designed by following the protocol described in Ran et al.[2] 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 founder mice were each bred to the F1 generation using C57BL/6 mice for further analysis. All mouse protocols and procedures were

approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania or Thomas Jefferson University.

Intracellular calcium was measured as described[3]. Isolated platelets were suspended in Tyrode's buffer without Ca^{2+} and loaded with fura-2 AM (10 μM) in the presence of Pluronic F-127 (2 mg/ml) for 15 minutes at 37 °C. The platelets were then washed and resuspended in Tyrode's buffer with no extracellular Ca^{2+} and 0.5μM EGTA. Changes in fura-2 AM fluorescence were detected with a JASCO FP-8300 spectrofluorometer, exciting at 340 and 380 nm, and measuring emission at 510 nm. For fluo-4 AM calcium release, $1x10^6$ of MEG-01 or $1.6x10^7$ fluo-4 AM stained mouse platelets were loaded in each well of a 96-well reading plate and agonists were added by the Flexstation 3 multi-mode microplate reader (Molecular Devices).

Isolation of human platelets. Blood was obtained from healthy donors using protocols approved by Thomas Jefferson University IRB. Written informed consent of all donors was obtained prior to blood collection. Blood was anticoagulated 1:5 with ACD (65 mM Na_3 citrate, 70 mM citric acid, 100 mM dextrose, pH 4.4) and centrifuged at 129xg for 20 min to obtain platelet rich plasma (PRP). Washed platelets were prepared by sedimentation at 341xg for 10 min. Platelets were washed with HEN buffer (150 mM NaCl, 1 mM Na_2EDTA, 10 mM HEPES, pH 6.5) containing 1 μM PGI_2 and resuspended in modified Tyrode's buffer (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).

Gel filtration of mouse platelets. Heparinized whole blood was collected from the retro-orbital plexus from isoflurane-anesthetized mice and centrifuged at 280xg for 5 min. Platelet rich plasma (PRP) was carefully removed from the supernatant and supplemented with 1 μM PGE1. PRP was loaded onto a 2 mL column of Sepharose 2B and eluted with Tyrode's buffer containing 1 mg/ml bovine serum albumin. The most turbid fractions collected were counted and adjusted with Tyrode's buffer if necessary.

Co-immunoprecipitation experiments in platelets. Washed human platelets and/or MEG-01 cells were lysed with ice-cold 5x Nonidet P-40 buffer (1% NP-40 in 50 mM Tris, 150 mM NaCl with protease inhibitor). For PAR1 phosphorylation experiments, 1μM okadaic acid was added to the lysis buffer to inhibit protein phosphatase activity. After centrifugation at 16,000xg for 20 min at 4 °C, supernatants were precleared with protein A agarose (for rabbit antibodies) or protein G agarose (for goat and mouse antibodies) for

an hour and incubated overnight at 4 °C with an immunoprecipitating antibody or normal rabbit, goat or mouse IgG. Protein/antibody complexes were isolated with protein A- or G-agarose for 2 h at 4 °C. After 5 washes with lysis buffer, the beads were boiled in SDS sample buffer.

Immunoblotting. Platelets were lysed in NP-40 (50 mM Tris, 150 mM NaCl, 2mM EDTA, 1mM EGTA, 1% NP40, pH 7.4) or Triton X-100 lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4) in the presence of protease inhibitors. MEG-01 cells were lysed with RIPA buffer supplemented with protease inhibitors. The lysates were boiled in sample buffer before sulfate-polyacrylamide gel electrophoresis (SDS PAGE) analysis. Binding of the primary antibodies was detected using HRP-conjugated secondary antibodies and the ECL-system (Amersham Biosciences). Individual bands were quantified by densitometry and analyzed using ImageJ software (NIH).

Flow cytometric analysis of platelet activation. Platelet activation was detected as previously described.[4] Briefly, heparinized whole mouse blood was diluted 1:40 in Tyrode's buffer. The blood was incubated with agonists in presence of saturating amounts of fluorophore-conjugated mAbs for 15 min at room temperature and analyzed on a BD LSRII (BD Biosciences, San Jose, CA). The platelet population was gated based on FSC/SSC and CD41 positivity. Platelets were stimulated with PAR4 agonist peptide (PAR4-AP), ADP, TxA_2 mimetic (U46619), or convulxin (CVX) at the concentrations indicated.

Platelet aggregation. Heparinized whole mouse blood isolated via the retro-orbital plexus from isoflurane-anesthetized mice was centrifuged at 1800 rpm for 5 minutes. Platelet-rich plasma (PRP) was collected by spinning at 800 rpm for 5 minutes. Platelets was then collected by spinning at 2800 rpm for 5 minutes and resuspended 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). Platelet counts (Beckman-Coulter Z1) were adjusted to $2.5x10^8$/ml. Aggregation was observed in a dual-channel Chrono-log lumi-aggregometer.

Cells culture and transfection

Meg01 cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia, United States) and cultured according to protocol. The AIO-GFP(Cas9) plasmid was transfected into Meg01 using Nucleofector II (Lonza, Basel, Switzerland) as

previously described[5]. Twenty-four hours following transfection, individual GFP-positive cells were sorted. Positive clones were identified by sequencing and western blots.

cAMP formation. Except for the basal cAMP determinations, washed platelets ($2.5x10^8$ /ml) were incubated for 30 min at 37°C with 500 μM IBMX to inhibit cAMP phosphodiesterase activity. Platelets were stimulated with 15 μM PGI_2 and ADP for 10 min as indicated. The reaction was stopped with the addition of 1 volume of 10% ice cold TCA after which the samples were mixed by vortexing, lysed by rapid freezing and thawing, and then spun to remove precipitates. cAMP was measured using the Biotrak EIA system from GE Healthcare. Samples used to measure basal cAMP levels were not incubated with IBMX, PGI_2 or ADP.

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3. Ma P, Gupta S, Sampietro S, et al. RGS10 shapes the hemostatic response to injury through its differential effects on intracellular signaling by platelet agonists. *Blood Adv*. 2018;2(16):2145-2155.
4. Ramanathan G, Gupta S, Thielmann I, et al. Defective diacylglycerol-induced Ca2+entry but normal agonist-induced activation responses in TRPC6-deficient mouse platelets. *J Thromb Haemost*. 2012;10(3):419-429.
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