Supplemental Material

Arf6 controls platelet spreading and clot retraction via integrin $\alpha_{IIb}\beta_3$ trafficking

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Supplemental Materials and Methods

Antibodies: The mouse antibodies used were: anti-Rac1, anti-Cdc42 and anti-Rab4 (BD Biosciences, San Jose, CA); anti-RhoA (Cytoskeleton, Denver, CO); anti-phospho-tyrosine (4G10 clone platinum, Millipore, Germany); anti-P-selectin and anti-fibronectin (Abcam, Cambridge, MA); and anti-β-actin (Sigma, St. Louis, MO). The rabbit antibodies used were: anti-Rab4 and anti-Rab11 (ThermoFisher Scientific, Grand Island, NY); anti-phospho-myosin light chain (pMLC) and anti-integrin β_3 (Cell Signaling, Danvers, MA); anti-Arf1/3, anti-Sema3F, anti-VMAT2 (vesicular monoamine transporter 2) and anti-vWF (von Willebrand factor) (Sigma, St. Louis, MO); anti-fibrinogen (Dako, Denmark); anti-vitronectin (Molecular Innovation, Novi, MI); anti-PF4 (Platelet Factor 4; R&D Systems, Pittsburgh, PA) and anti-GIT1 (Santa Cruz, Dallas, TX). FITC-anti-CD41/61 and PE-Jon/A antibodies were from Emfret Analytics (Germany). Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse antibodies were from ThermoFisher Scientific (Grand Island, NY). The rabbit anti-syntaxin 11, anti-VAMP8 (vesicle associated membrane protein 8/endobrevin), anti-SNAP23 (synaptosomal-associated protein 23), anti-VAMP3 (vesicle associated membrane protein 3/cellubrevin), anti–RabGDI (Rab GDP dissociation inhibitor), and anti-Arf6 (ADP-ribosylation factor 6) antibodies were as described [1-3].

Reagents: Apyrase, human fibrinogen (Fg), poly-D-lysine and hirudin were from Sigma (St. Louis, MO). Human fibronectin was from Millipore (Billerica, MA). Thrombin, collagen, ADP, and CHRONO-LUME reagent were from CHRONO-LOG (Havertown, PA). U46619 and prostaglandin I_2 (PGI_2) were from Cayman (Ann Arbor, MI). Fibronectin-free Fg was from Enzyme Research Laboratory (South Bend, IN). Glutaraldehyde (70%) was from Electron Microscopy Sciences (Hatfield, PA). Bovine Serum Albumin (BSA) was from ThermoFisher Scientific (Grand Island, NY). Myristoylated Arf1 peptide and Arf6 peptide were from AnaSpec (Fremont, CA). FITC-Fg was from Molecular Innovations (Novi, MI). Other reagents were of laboratory grade.

Whole Blood Counting: Blood was harvested from WT and KO littermates *via* heart puncture into 0.38% sodium citrate (final) with 0.2 U/mL apyrase and 10 ng/mL PGI_2. Whole blood counts were performed using a Hemavet (Erba Diagnostics, Inc.). Statistical analyses of both blood counts and platelet size were performed using SigmaPlot 12.0 (Systat Software Inc.).

Preparation of Washed Mouse/Human Platelets: Mouse blood was harvested *via* cardiac puncture into 0.38% sodium citrate (final). Human blood was collected using Acid Citrate Dextrose (ACD) tubes (BD, Franklin Lakes, NJ) from donors with informed consent. Whole blood was diluted (1:1 v/v) in

HEPES Tyrode buffer (pH 6.5, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)/KOH, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4) with 0.2 U/mL apyrase and 10 ng/mL PGI_2. Platelets were isolated as described [1,4], suspended in HEPES Tyrode buffer (pH 7.4) with 1 mM $CaCl_2$ and concentrations were measured using Z2 Counter (Beckman Coulter, Inc., Miami, FL).

Platelet Aggregometry and ATP Release: Aggregation of washed platelets (2.5 x 10^8/mL) was measured under stirring conditions at 37°C and ATP release was monitored using CHRONO-LUME reagent as described [1].

Rac1 and RhoA GTPase Pull-down Assay: Rac1-GTP and RhoA-GTP pull-down assays were performed as described [4]. Washed platelets (5 x 10^8/mL) were stimulated with 0.1 U/mL thrombin for indicated times. Reactions were stopped with 2 x ice-cold GTPase-pulldown buffer (20 mM HEPES, pH 7.4, 128 mM NaCl, 3 mM $MgCl_2$, 2% Triton X-100, 0.2% SDS, 1% deoxycholic acid, 20% glycerol, 2 x EDTA-free protease inhibitor cocktail). The lysates were cleared by centrifugation at 15,000 x g for 5 min. An aliquot of the lysates was used as input. The remaining lysates was incubated with glutathione-agarose beads bound to a glutathione-S-transferase-(GST)-fusion protein containing mouse $PAK3_{RBD(65-137)}$ [5] for Rac1-GTP or mouse $Rhotekin_{RBD(7-89)}$ [6] for RhoA. The bead-bound complexes were washed with HEPES-wash buffer (20 mM HEPES, pH 7.4, 128 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10% glycerol) and the proteins were eluted with 2 x SDS sample buffer.

Western Blotting and Quantification: Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to immobilon-P polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA), and incubated with the primary antibodies then with an appropriate alkaline phosphatase conjugated-secondary antibodies. Membranes were developed with ECF substrate and scanned using a TyphoonTM imager (GE Healthcare). The images were quantified using ImageQuantTL software.

Electron Microscopy: Washed resting platelets (4 x 10^8/mL) from WT and KO mice were fixed with 0.1% glutaraldehyde in White's Saline and prepared for electron microscopy as described [1]. Sample grids were imaged on an Hitachi H-7000 transmission electron microscope.

Static Platelet Adhesion: Washed platelets (5 x 10^8/mL), labeled with 7 µM calcein AM (BD Bioscience) in the presence of 0.2% of pluronic F-127 (Invitrogen), were seeded onto 96-well, opaque plates which were pre-coated with either 50 µg/mL human Fg or 5% bovine serum albumin (BSA) in buffer [25 mM

tris(hydroxymethyl)aminomethane (Tris), 137 mM NaCl, pH 7.4]. After incubation for 30 min at 37°C, the wells were washed twice with HEPES Tyrode Buffer (pH 7.4) and adherent platelets were measured by fluorescence using a SpectraMax plate reader (Molecular Devices; excitation/emission/cutoff at 485/538/515 nm, respectively). Standard curves were generated using fixed numbers of platelets from each genotype.

Platelet Spreading: Nunc Lab-Tek II chamber slides or nitric acid-washed cover slips were pre-coated with human Fg (50 μg/mL, Enzyme Research Laboratory), fibronectin (50 μg/mL, Millipore), denatured BSA (1%, ThermoFisher) and poly-D-lysine (Sigma) in PBS overnight at 4°C. Platelets (2 x 10^7/mL) in HEPES Tyrode buffer (pH 7.4) with 1 mM $CaCl_2$ were seeded on top of the surfaces at 37°C for increasing times. Unbound platelets were removed and the adherent platelets were fixed with 4% paraformaldehyde. The platelets were viewed using differential interference contrast (DIC) microscopy on a Nikon Eclipse E600 microscope (Nikon, Melville, NY). Images were taken using an AxioCam MR camera (Zeiss, Germany), processed with Zen 2011 (blue edition, Zeiss) and quantified with Image J software (V1.47, NIH).

Tail Bleeding and $FeCl_3$-induced Carotid Artery Injury Assays: Tail-bleeding times were measured in WT and KO littermates at 4-6 weeks age as described [1]. For the ferric chloride injury model, age-matched (8-12 weeks of age) WT and KO mice were used as described [1]. Statistical analysis of the data was performed using the Logrank test.

Study Approval: All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

References:

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2. Ren Q, Barber HK, Crawford GL, et al. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. *Mol Biol Cell*. 2007;18(1):24-33.

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4. Choi W, Karim ZA, Whiteheart SW. Arf6 plays an early role in platelet activation by collagen and convulxin. *Blood*. 2006;107(8):3145-3152.

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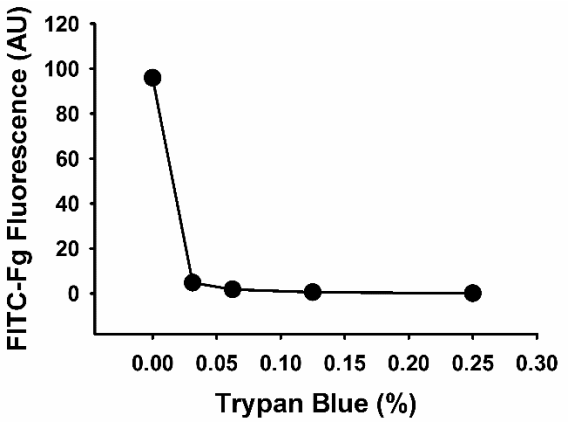
6. Ren XD, Schwartz MA. Determination of GTP loading on Rho. *Methods Enzymol*. 2000;325:264-272.

Supplemental Table 1:

Comparison of whole blood count between WT and KO

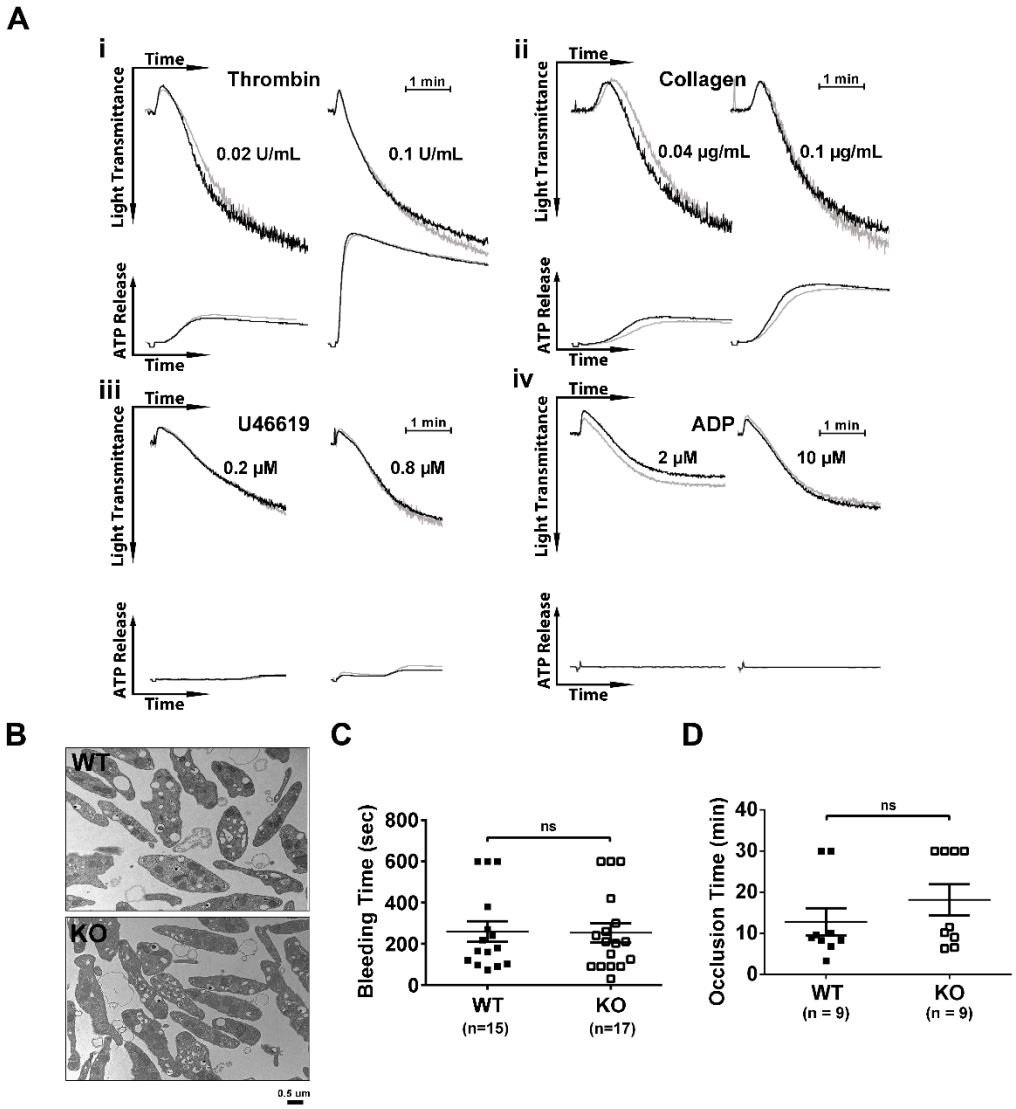
	WT (n=26)	KO (n=26)	*P*
RBC (M/μL)	10.63 ± 2.32	9.59 ± 2.36	.061
WBC (K/μL)	8.19 ± 1.90	7.54 ± 1.24	.117
Platelet (K/μL)	978.7 ± 416.3	893.9 ± 305.8	.602
Platelet Size (fL)	4.25 ± 0.45	4.03 ± 0.37	.021

Supplemental Figure 1



Supplemental Figure 1: Trypan blue is a potent quencher of FITC-Fg fluorescence. Equal amounts of FITC-Fg (0.05 mg/mL) were incubated with trypan blue at different concentrations. The FITC-Fg signal was measured using SpectraMax M5 (Molecular Devices) with Ex/Em/Cutoff at 485/538/515 nm. Quantification was graphed using Sigmaplot 12.0. Plot shown is representative of two independent experiments with duplicates.

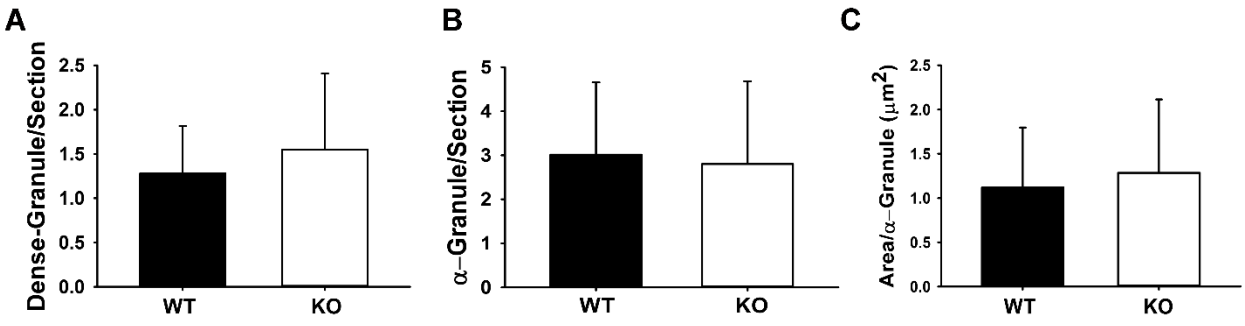
Supplemental Figure 2



Supplemental Figure 2: Arf6 deletion in mouse platelets did not affect platelet aggregation, morphology, or hemostasis. (A) Arf6 KO platelets aggregated similarly as WT in response to various agonists at different concentrations. Washed platelets from WT (black) and KO (grey) mice were freshly prepared and adjusted to 2.5 X 10^8/mL in HEPES Tyrode buffer. Thrombin (i), Collagen (ii), U46619 (iii), and ADP (iv) were used to stimulate platelet aggregation in stirring condition at 37°C in an aggregometer. The concentrations of agonists were as indicated. ATP release was monitored using CHRONO-LUME reagent (CHRONO-LOG) while platelet aggregation was measured. Data shown are representative of at least three independent experiments. Data was organized using Adobe Photoshop CS5. **(B)** Arf6 KO platelets had normal morphology. Washed platelets from WT and KO mice were

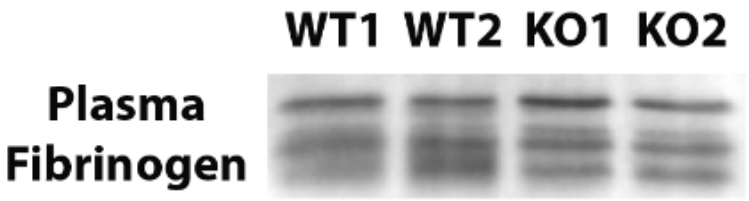
prepared in HEPES Tyrode buffer (pH 7.4) and adjusted to 4 X 10^8/mL. Resting platelets were subjected to electron microscope analysis (see Methods). Images were taken using an Hitachi H-7000 transmission electron microscope. The scale is indicated. **(C)** Arf6 KO mice did not have significant defects in thrombosis formation and hemostasis. (I) Tail bleeding assay. WT and KO mice (4-6 weeks of age) were anaesthetized by intraperitoneal injection with ketamine (75 mg/kg). The tail tip was transected at 3 mm and immediately immersed in 37°C saline. The time from transection to bleeding cessation was recorded. Mice were monitored for an additional minute for re-bleeding event. The recordings were terminated at 10 min. The data were analyzed using Logrank test. (II) $FeCl_3$-induced carotid artery injury model. WT and KO mice (8-12 weeks of age) were used. Application of fresh 4% $FeCl_3$ on left carotid artery for 3 min was used to induce an injury. The time from removal of $FeCl_3$ to blood flow cessation was recorded. Mice were monitored for an additional minute for unstable thrombus formation. The recordings were terminated at 30 min. Mice were euthanatized at the end of experiments. The data were analyzed by Logrank test.

Supplemental Figure 3



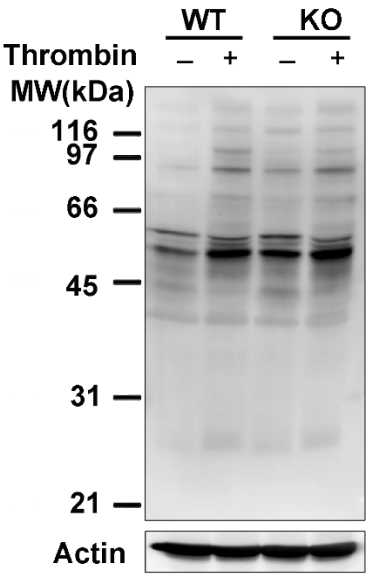
Supplemental Figure 3: Arf6 KO platelets did not have α granule biogenesis defects. Dense granule counting **(A)**, α granule counting **(B)** and area measurements **(C)** were performed using EM images and Image J software (NIH). Over 65 sections were quantified in each group. **(A)** The number of dense granule per section was quantified. **(B)** The number of α granule per section was quantified. **(C)** Area per α granule was quantified.

Supplemental Figure 4



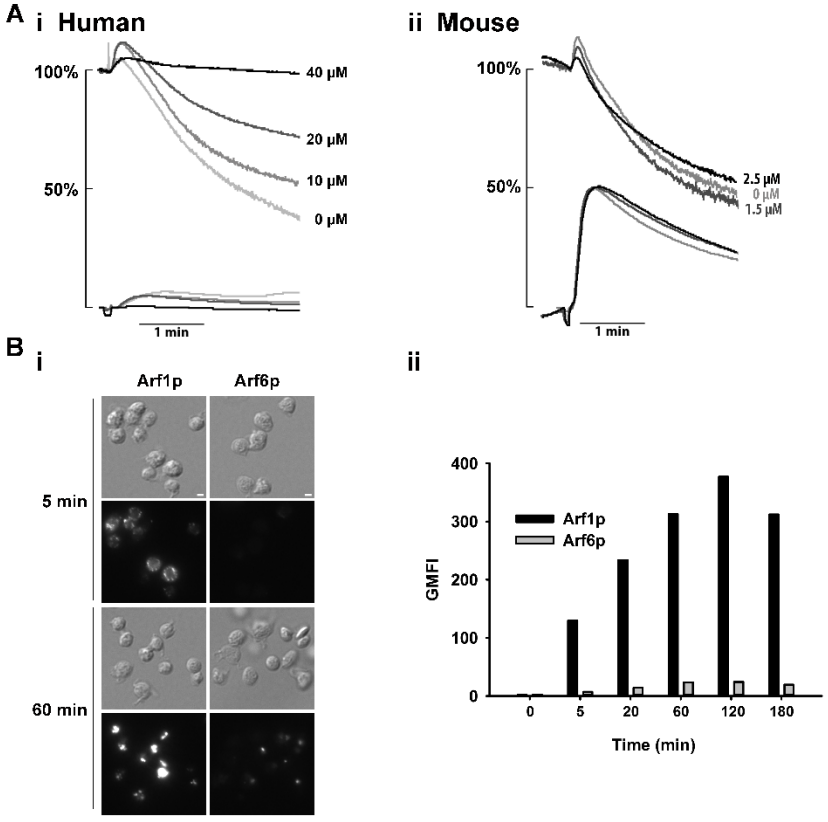
Supplemental Figure 4: Plasma Fg levels were comparable between WT and Arf6 KO mice. Platelet poor plasma (PPP) from individual mice were centrifuged at 16,000 x g for 3 min and the supernatants were harvested. It was then diluted 1:2,000 with HEPES Tyrode buffer (pH 7.4), denatured with SDS-PAGE sample buffer and subjected to western blotting. Fg was detected with anti-Fg antibody from Dako (Glostrup, Denmark).

Supplemental Figure 5



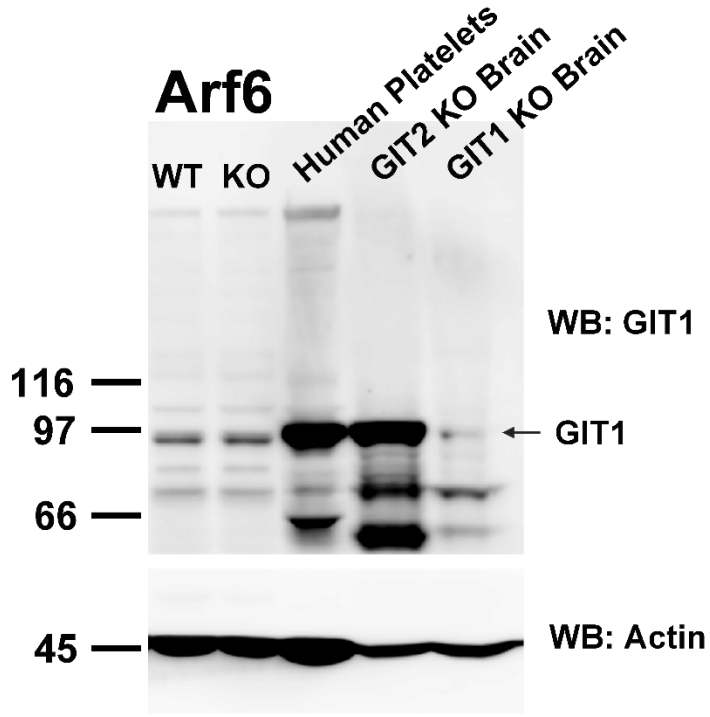
Supplemental Figure 5: Arf6 KO platelets did not show any defect in global tyrosine phosphorylation. Washed platelets from WT and KO mice were prepared at 4 X 10^8/mL in HEPES Tyrode buffer (pH 7.4) and kept resting or stimulated with thrombin (0.1 U/mL) for 5 min. The reactions were stopped by addition of SDS-PAGE sample buffer containing both protease inhibitor and phosphatase inhibitor cocktails. The lysates were subjected to western blotting for the global tyrosine-phosphorylation profile. β-actin was used as a loading control. Data are representative of at least two independent experiments.

Supplemental Figure 6



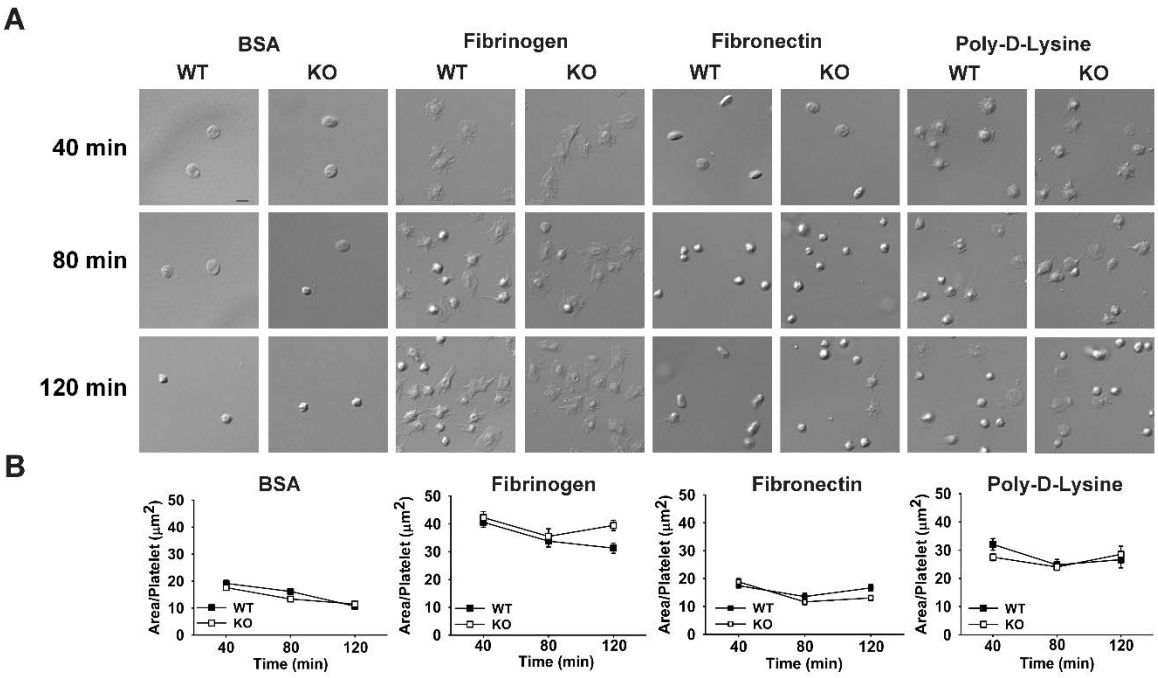
Supplemental Figures 6: myr-Arf6 peptide did not affect mouse platelet aggregation but inhibited FITC-Fg uptake by washed human platelets. (**A)** The effect of myr-Arf6 peptide on human and mouse platelet aggregation. Washed human or mouse platelets were prepared and adjusted to 2.5 X 10^8/mL in HEPES Tyrode buffer (pH 7.4). The platelets were pretreated with indicated dose of myr-Arf6 peptide for 2 min and then stimulated with thrombin (0.1 U/mL). Platelet aggregation was recorded. **(Ai)** Human platelet aggregation was inhibited by myr-Arf6 peptide pretreatment in a dose-dependent manner. **(Aii)** Mouse platelet aggregation was not inhibited by myr-Arf6 peptide pretreatment. **(B)** myr-Arf6 peptide inhibited the uptake of FITC-Fg by human platelets. Washed platelets (5 X 10^8/mL) were pretreated with myr-Arf1 (Arf1p) or myr-Arf6 (Arf6p) peptides for 5 min at RT, and then incubated with 0.05 mg/mL FITC-Fg at 37°C. At the indicated times, platelets were fixed with 2% paraformaldehyde at RT. **(Bi)** Platelets at 5 min and 60 min were imaged in the presence of 0.04% trypan blue. **(Bii)** Flow cytometry analysis was used to determine the fluorescent levels inside the platelets in the presence of 0.1% trypan blue.

Supplemental Figure 7



Supplemental Figure 7: The expression levels of GIT1 protein is much lower in mouse platelets than in human platelets. WT, Arf6 KO, and human platelets (1 X 10^7/lane) was loaded. Brain extracts (40 µg) from GIT1 KO and GIT2 KO (generous gift from Dr. Richard T. Premont) were included. GIT1 protein was probed for by western blotting. β-Actin was used as a loading control. The result is representative of at least two independent experiments.

Supplemental Figure 8



Supplemental Figure S8: Arf6 KO platelets had no defect in spreading on fibronectin-, poly-D-lysine- or BSA-coated surfaces. Washed platelet (WT and KO, 2 X 10^7/mL) in HEPES Tyrode buffer containing 1 mM Ca^{2+} were incubated on coverslips coated with BSA (1% in PBS), Fg (50 µg/mL), fibronectin (50 µg/mL) and poly-D-lysine (1 mg/mL) for the indicated times. Unbound platelets were removed and adherent platelets were fixed with 4% paraformaldehyde. DIC images were acquired as in Methods and platelet areas were measured using Image J.