Supplemental Materials and Methods

Reagents and antibodies

Low–molecular weight enoxaparin sodium (Lovenox) was purchased from Sanofi-Aventis, and heparin-coated capillaries were purchased from VWR. BSA, ADP, anti-β-actin antibody and red blood cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO). Anti-GPIbα (clone Xia.G5), anti-GPIX (clone Xia.B4), anti-GPVI (clone JAQ1), anti-integrin α2 (clone Sam.G4) and JON/A-PE were purchased from Emfret Analytics (Eibelstadt, Germany); recombinant mouse podoplanin/Fc chimera protein (clone 25463; R&D Systems); anti-$\alpha_{IIb}\beta_3$, and anti–P-selectin (BD Biosciences). Alexa Fluor 647-labeled anti-CD31 (clone MEC13.3) was purchased from BioLegend (San Diego, CA, USA). FITC-labeled CLEC-2 antibody was provided by Bernhard Nieswandt (University Clinic of Wuerzburg and Rudolf Virchow Center, Wuerzburg, Germany). Convulxin was provided by K.J. Clemetson (Theodor Kocher Institute, Bern, Switzerland), and Par4-activating peptide (Par4p; GL Biochem Inc., Boston, MA).

Immunoblot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 4-20% gradient gels (Bio-Rad, Hercules, CA) then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) using standard immunoblotting techniques. p34 (rabbit polyclonal anti-p34, Millipore, Billerica, MA), Arp2 (anti-Arp2, ECM Biosciences, Versailles, KY), Arp3 (anti-Arp3, clone FMS338, Sigma-Aldrich, St. Louis, MO) and β-actin (anti-β-actin clone AC-15, Sigma-Aldrich, St. Louis, MO)

proteins were detected using IRDye 680–conjugated goat anti-mouse, IRDye 680-conjugated donkey anti-goat or IRDye 800–conjugated goat anti-rabbit secondary antibodies, where appropriate, and visualized with the Odyssey Infrared Imaging System (Li-Cor Biosystems, Lincoln, NE).

Platelet aggregation

Platelet rich plasma was isolated from heparinized blood and adjusted to a concentration of 2.5 x 10^8/mL in Tyrode's Buffer containing 0.35% BSA and 1 mM $CaCl_2$. Aggregation was performed at 37^{o}C under stirring conditions using a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

Scanning Electron Microscopy (SEM)

Washed platelets were plated onto 100 μg/ml fibrinogen coated coverslips for 10 minutes before activation with agonists for 30 minutes. Cells were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.05% $CaCl_2$ for 30 minutes at room temperature, followed by subsequent treatment with 2% tannic acid for 10 minutes and 1% osmium tetroxide in water for 10 minutes. The coverslips were dehydrated with ethanol and dried in a Samdri-795 critical point dryer using carbon dioxide as the transitional solvent (Tousimis Research Corporation, Rockville, MD). Coverslips were mounted on aluminum planchets with double-sided carbon adhesive and coated with 10 nm of gold-palladium alloy (60Au:40Pd, Hummer X Sputter Coater, Anatech USA, Union City, CA). Images were

taken using a Zeiss Supra 25 FESEM operating at 5 kV, working distance of 5 mm, and 10 μm aperture (Carl Zeiss SMT Inc., Peabody, MA).

Transmission Electron Microscopy (TEM)

Sample processing was performed by the Harvard Medical School Electron Microscopy facility as described[1]. For preparation of cryosections, platelets were fixed with 1.25% formaldehyde, 2.5% gluteraldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 hours and then washed in 0.2 M sodium cacodylate buffer and pellets frozen in liquid nitrogen. Frozen samples were sectioned at −120°C, and the sections were transferred to formvar-carbon coated copper grids. Contrasting/embedding of the grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops, leaving a thin coat of methyl cellulose. The grids were examined in a Tecnai G2 Spirit BioTWIN transmission electron microscope (Hillsboro, OR) at 18,500x magnification at an accelerating voltage of 80 kV. Images were recorded with an AMT 2k CCD camera.

Differentiation of mouse megakaryocytes

Proplatelet formation was studied in mouse bone marrow–derived megakaryocytes. Cells were homogenized by pipetting, followed by passage through a 100-μm filter. The cell population was resuspended in 10% fetal bovine serum–supplemented DMEM with 2 mM l-glutamine, 50 U/mL penicillin/streptomycin, and fibroblast-conditioned media containing thrombopoietin and cultured for 4 days (37°C and 5%

CO_2). Mature megakaryocytes were layered over a BSA gradient and resuspended in culture media before seeding onto an immobilized fibrinogen matrix. A minimum of 100 megakaryocytes were analyzed to determine the percentage cells undergoing the process of proplatelet formation. For examination of spreading, cultured mouse megakaryocytes were seeded on collagen-coated plates (100 µg/mL type 1 collagen) for 3 hours before fixation in 3.7% formaldehyde, permeablization in ice-cold acetone and staining with Alexa fluor 647-conjugated phalloidin and Hoechst 33258 (Roche, Basel, Switzerland).

Dense granule secretion

Washed platelets were incubated for 30 minutes at 37°C with ^{3}H-serotonin (5-[1, 2-3H(N)]-hydroxytryptamine creatinine sulfate) (Perkin-Elmer, Waltham, MA) (6 µCi [0.222 MBq]/ml). After one washing step, platelets were re-suspended (3 x 10^8 platelets/ml) in modified Tyrode's Buffer containing 1 µM imipramine (Sigma-Aldrich, St. Louis, MO) and 1 mM $CaCl_2$. Platelets were stimulated with Par4p for 10 minutes and fixed with 0.1 M EDTA / 3.7% formaldehyde solution. The samples were then centrifuged for 5 minutes at 700 g and the supernatants were used for scintillation counting of secreted ^{3}H-serotonin. Total or 100% dense granule secretion was defined as the ^{3}H-serotonin in samples permeablized with 0.5% Triton X-100.

Peripheral blood cell counts

Whole blood was collected from the retro-orbital plexus into EDTA-containing tubes. Complete blood counts were determined using a Hemavet 950FS automated hematology analyzer (Drew Scientific, Waterbury, CT).

αIIbβ3 activation and α-granule secretion

Platelets were washed and diluted to 2.5 x 10^7/mL with Tyrode's buffer containing 0.35% BSA and 1 mM $CaCl_2$. Cells were then stimulated with Par4p, Cvx, or ADP for 10 minutes in the presence of 2 μg/mL PE-conjugated JON/A antibody (JON/A-PE)(Emfret Analytics, detects activated $\alpha_{IIb}\beta_3$) and Alexa Fluor 488-conjugated α–P-selectin antibody (2 μg/mL; BD Biosciences, Ann Arbor). Diluted samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI).

Quantification of platelet surface receptor expression

Diluted whole blood (Tyrode's buffer + BSA) samples were stained for 10 minutes with 5 μg/mL fluorophore-conjugated antibodies to the indicated platelet surface receptors and immediately analyzed by flow cytometry. Antibodies for GPIX, GPIbα, GPVI, integrin α2, and IgG controls were purchased from Emfret Analytics. FITC-labeled CLEC-2 antibody was provided by Bernhard Nieswandt (University Clinic of Wuerzburg and Rudolf Virchow Center, Wuerzburg, Germany).

Lectin Binding Assay

Washed platelets were resuspended in Tyrode's buffer – BSA and normalized to 3x10^8/ml. Platelets (3x10^6) were incubated in 25 μg/ml of FITC labeled Ricin

communis agglutinin I (RCA-FITC) for 20 minutes before dilution in PBS and analysis by flow cytometry. Aliquots of platelets were treated with recombinant α2-3,6,8 neuraminidase (New England BioLabs, MA, USA) for 10 minutes according to the manufacturer's instructions, before staining with RCA-FITC.

Reticulated Platelets

Heparinized blood from mice was incubated with 50 ng/ml of thiazole orange (Sigma-Aldrich, St. Louis, MO) diluted in PBS + 2 mM EDTA, for 30 minutes at room temperature followed by labeling with Alexa Fluor 647-conjugated anti-GPIX antibody (2 μg/ml). Samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI).

Histology

Femurs and spleens were isolated from euthanized mice and fixed in 3.7% formaldehyde for 24 hours before dehydration in 70% ethanol for 24 hours. Tissues were paraffin embedded, sectioned (4 μm) and stained with hematoxylin and eosin using standard methods.

Clot retraction

Washed platelets were resuspended in citrated human platelet-poor plasma at 4×10^8 platelets/ml. Coagulation was triggered by addition of thrombin (0.1 U/ml) in the presence of 10 mM $CaCl_2$. Clots were allowed to retract at 37°C for 2 hours and clot area was quantified using Image J image analysis software.

Tail vein bleeding time assay

Mice (12 weeks of age) were anaesthetized with a mixture of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively)(Med-Vet International, Mettawa, IL, USA). The tail was transected 2 mm from the tip and immediately immersed in warm PBS. Bleeding time was measured from the cutting of the tail until the cessation of blood loss. The PBS-blood mixture was mixed 1:1 with red blood cell lysis buffer and quantitated against known blood volumes at an absorbance of 490 nm.

Ferric chloride-induced thrombosis

The right common carotid artery was isolated in age and sex matched 12-16 week old mice anaesthetized with a ketamine/xylazine solution (100 mg/kg and 10 mg/kg, respectively). A 20% ferric chloride solution was applied with a 1x2 mm piece of filter paper for 60 seconds. Blood flow velocity was measured with a 0.5 mm Doppler flow probe connected to a TS420 Transonic flowmeter (Transonic, Ithaca, NY), and time to occlusion was recorded when blood velocity reached 25% of baseline velocity. Flow was monitored for 30 minutes.

Reverse passive Arthus reaction.

The rpA reaction was triggered by intradermal injection of anti-BSA antibodies anti-BSA (MP Biomedical, Santa Ana, CA; 60 μg diluted in PBS) followed by retro-orbital injection of Tyrode's buffer or washed platelets resuspended in Tyrode's buffer containing BSA, to reach a BSA concentration of 150 μg/g body weight. 4

hours later, mice were sacrificed and bleeding was visualized on the internal surface of the dermis.

1. Machlus KR, Wu SK, Stumpo DJ, et al. Synthesis and dephosphorylation of MARCKS in the late stages of megakaryocyte maturation drive proplatelet formation. *Blood*. 2016;127(11):1468–1480.

Table S1

	Control	*Arpc2*$^{fl/fl}$
Red Blood Cells, (10^9/ml)	9.74 ± 0.57	10.11 ± 0.47
White Blood Cells, (10^6/ml)	6.85 ± 0.46	8.49 ± 1.24
Neutrophils, (10^6/ml)	1.35 ± 0.25	1.91 ± 0.32
Lymphocytes, (10^6/ml)	5.01 ± 0.551	6.11 ± 0.87
Monocytes, (10^6/ml)	0.29 ± 0.02	0.32 ± 0.05
Eosinophils, (10^6/ml)	0.06 ± 0.03	0.14 ± 0.08
Basophils, (10^6/ml)	0.006 ± 0.004	0.016 ± 0.007

Table S1. **Normal blood cell counts in *Arpc2*$^{fl/fl}$*PF4-Cre* mice** (n=5).

Figure S1

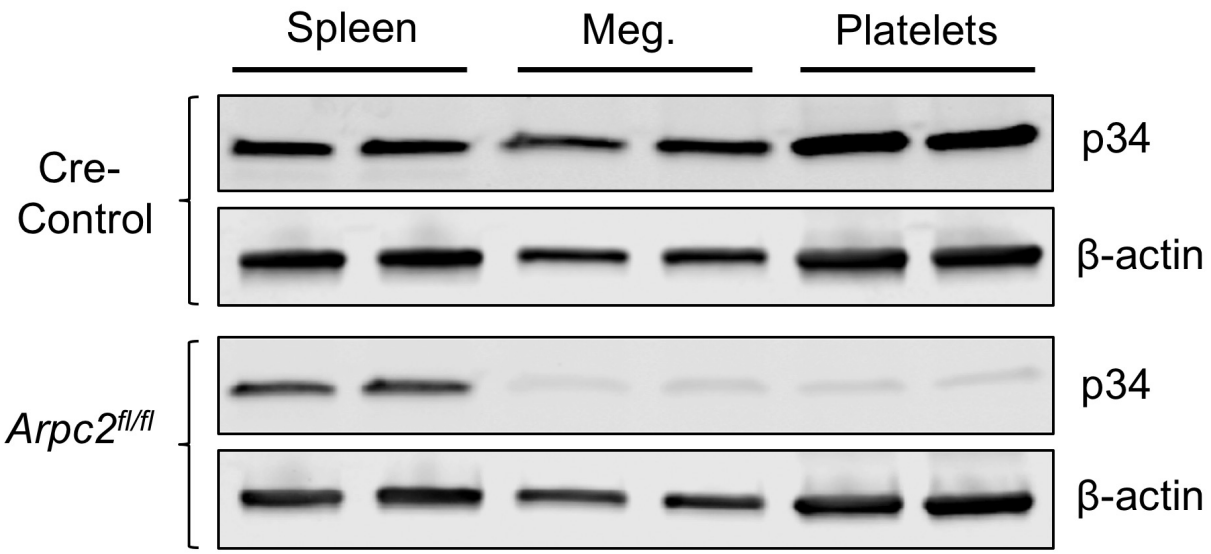

Figure S1. **Expression of p34 in spleen, megakaryocytes and platelets of *Arpc2$^{fl/fl}$PF4-Cre* mice.** Western Blot analysis of lysates from spleen, platelets and cultured megakaryocytes obtained from Cre- Control and *Arpc2$^{fl/fl}$PF4-Cre mice*.

Figure S2

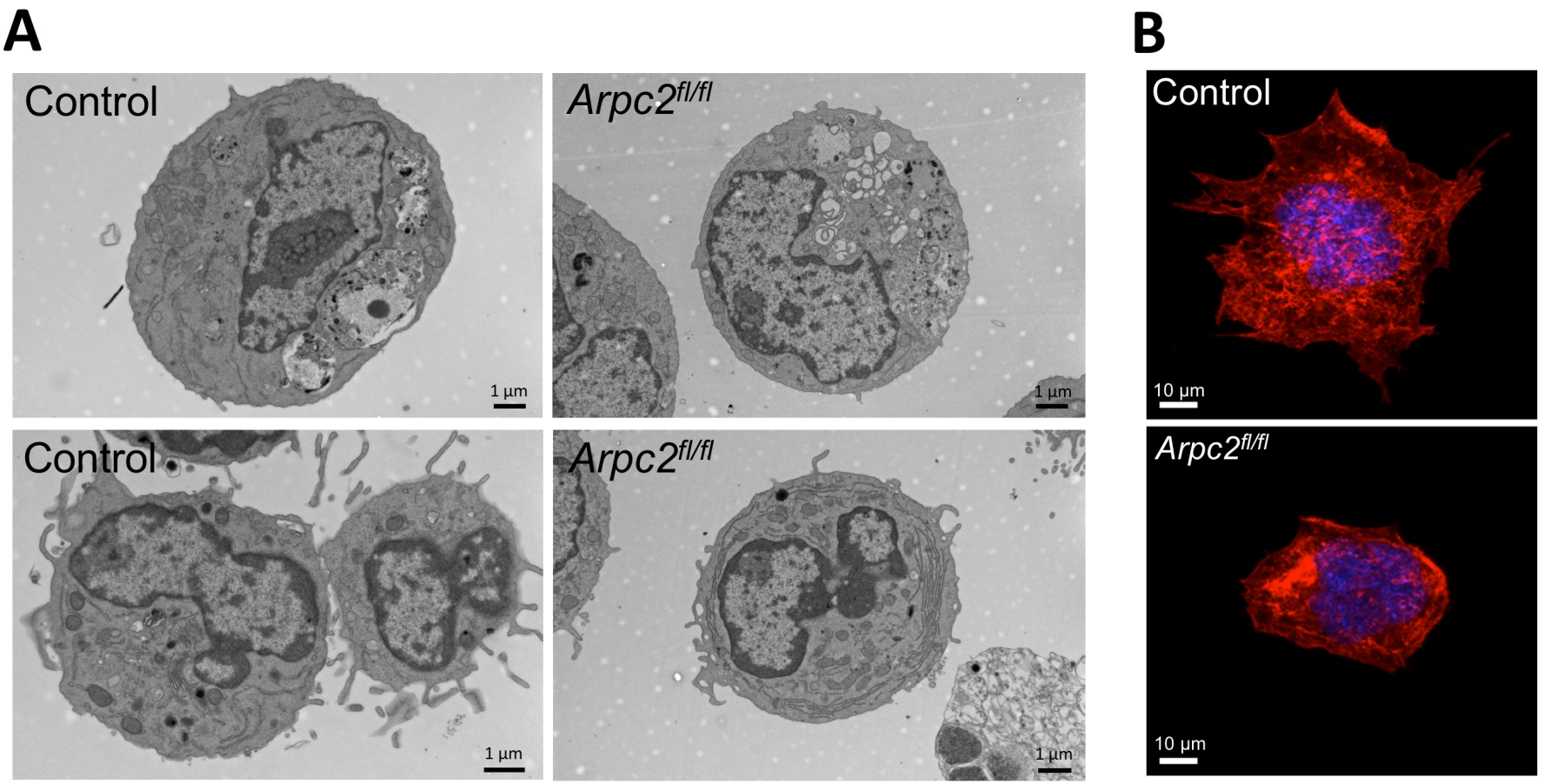

Figure S2. **Normal morphology and ultrastructure, but impaired spreading in megakaryocytes from *Arpc2$^{fl/fl}$PF4-Cre mice*.** (A) TEM micrographs of megakaryocytes isolated from bone marrow of the indicated mice. (B) Representative micrographs of cultured bone marrow-derived megakaryocytes stained for F-actin (red) and DNA (blue). Note the defect in spreading observed for *Arpc2$^{fl/fl}$PF4-Cre* megakaryocyte on collagen-coated surface.

Figure S3

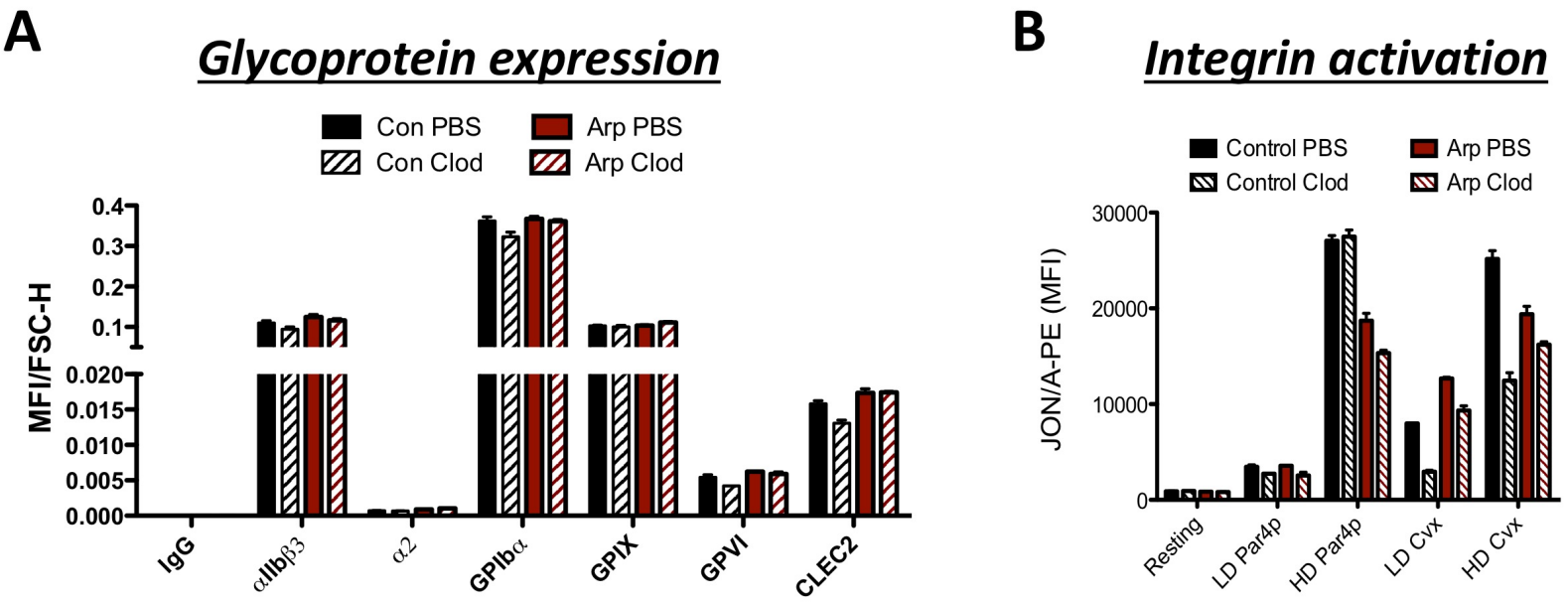

Figure S3. **Clodronate treatment does not significantly impact glycoprotein expression or integrin activation on *Arpc2*$^{fl/fl}$*PF4-Cre* platelets.** Flow cytometric quantification of (A) key adhesion and agonist platelet surface receptors (n=5) and (B) αIIbβ3 integrin activation in washed platelets isolated from Control or *Arpc2*$^{fl/fl}$*PF4-Cre* mice (n=3), 120 hours after treatment with PBS-liposomes or clodronate-liposomes.

Figure S4

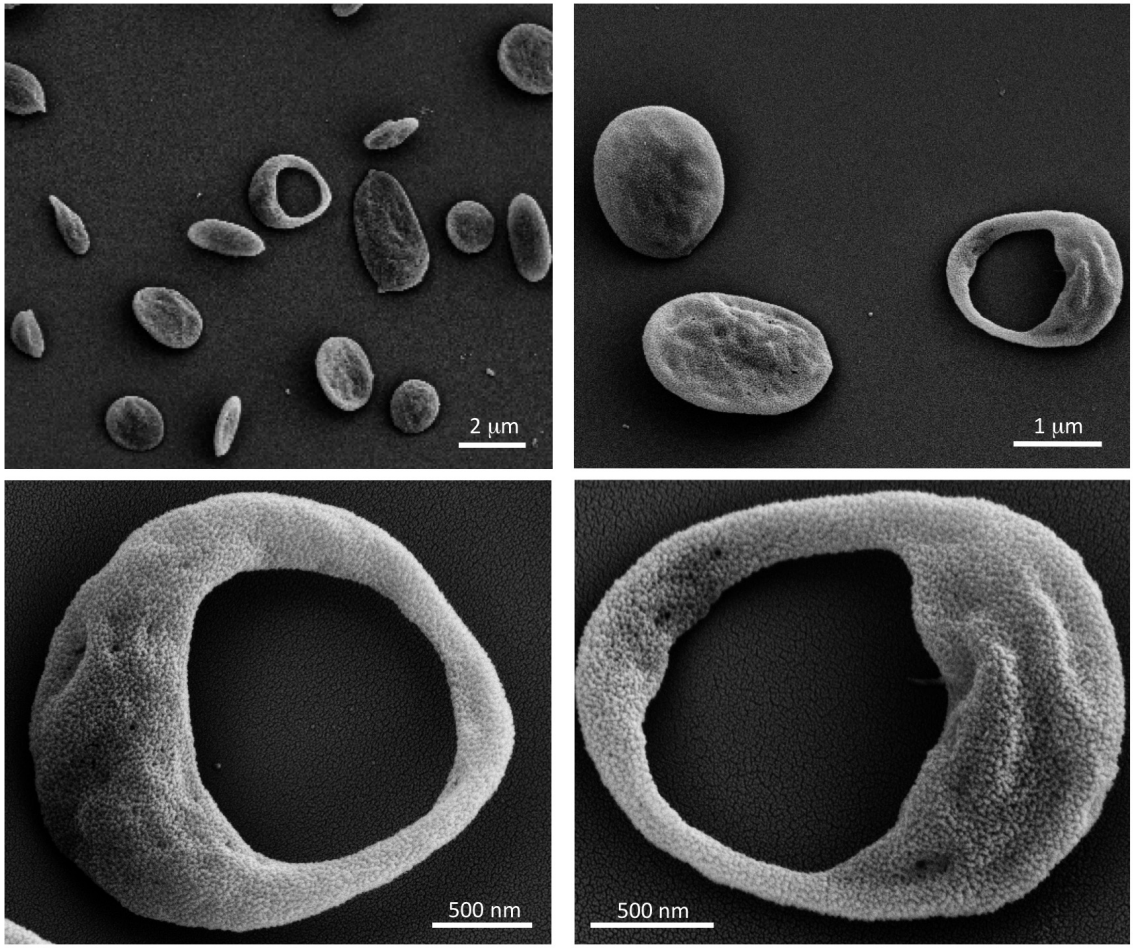

Figure S4. ***Arpc2$^{fl/fl}$PF4-Cre*** **platelets exhibit doughnut-like morphological defects.** Representative SEM images of resting platelets isolated from *Arpc2$^{fl/fl}$PF4-Cre* mice indicating potential for aberrant membrane structure.

Figure S5

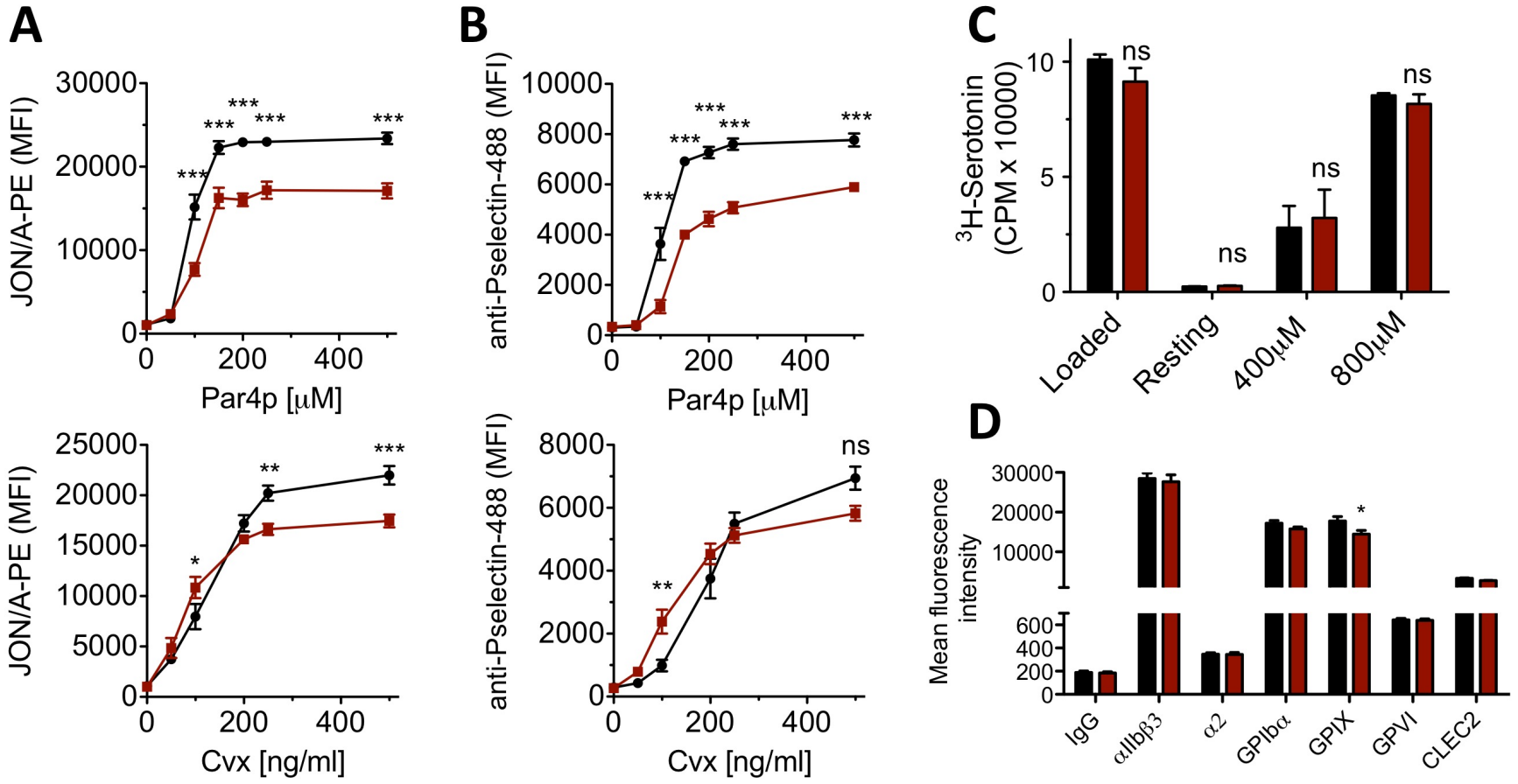

Figure S5. **Loss of Arp2/3 results in a minor impairment of platelet function.** (A,B) Flow cytometric quantification of αIIbβ3 activation (A) and P-selectin surface expression (B) in washed platelets stimulated with the indicated agonists (n=5). (C) Quantification of secreted ^{3}H-serotonin from δ-granules of washed platelets stimulated or not with Par4p at the indicated concentrations (n=3). (D) Quantification of main adhesion receptors expressed on the platelet surface (n=6). (black bars/lines = control; red bars/lines = *Arpc2$^{fl/fl}$PF4-Cre*). * p<0.05, ** p<0.01, *** p<0.001