SUPPLEMENTARY METHODS

Reagents and antibodies. Rabbit polyclonal antibodies against SHARPIN and OTULIN were from Proteintech Group (Rosemont, IL), FITC rat anti-mouse CD41 (αIIb), rat anti-mouse P-selectin (CD62P) antibodies were from BD Biosciences (San Diego, CA), a rabbit polyclonal antibodies against Rap1, p65 and serine 536 of the p65 (RelA) subunit of NF-κB, were from Cell Signaling Technologies (Danvers, MA), and a mouse monoclonal against the carboxy terminal LDD domain of HOIP was from R&D Systems (Minneapolis, MN). A polyclonal rabbit antibody against the central region of talin (amino acids 1650-1750), and a mouse monoclonal antibody against the carboxy terminal end (rod domain) of talin were from Abcam (Cambridge, MA) and Merck-Sigma (Temecula, CA), respectively. A rabbit polyclonal antibody against kindlin-3 and a mouse monoclonal antibody against p65 subunit of NF-κB were from ThermoFisher. A mouse monoclonal antibody against linear ubiquitin chains was from Merck-Millipore, and a human IgG1 anti-linear ubiquitin antibody, 1F11, was a gift from Genentech (South San Francisco, CA). Rabbit polyclonal antibody 8053 against integrin β3 was a gift from Mark Ginsberg. Fibrinogen and Alexa-647-conjugated fibrinogen were purchased from Enzyme Research (South Bend, IN) and Thermo Fisher/Invitrogen (Waltham, MA), respectively. Alexa-conjugated secondary antibodies were from Thermo Fisher, and Atto-647N secondary goat anti-rabbit antibodies were from Merck-Sigma ((Burlington, MA) and Rockland Antibodies (Gilbertsville, PA). Protease Activated Receptor (PAR) 4 receptor agonist peptide (AYPGKF) was from Genscript (Piscataway, NJ), and ADP and epinephrine were from Merck-Sigma. Dextran sodium sulfate (DSS) MW 36,000-50,000 was purchased from MP Biomedical (Irvine, CA). Thioglycollate was from Difco/Becton-Dickinson (Sparks, MD). Casein western blot blocking reagent was from Merck-Millipore, and Selleckchem (Houston, Tx).

Generation of conditional Sharpin null mice. C57BL/6 SHARPIN floxed mice were generated by Ozgene Pty Ltd. (Bentley DC, Australia). Specifically, a targeting vector to prepare floxed Sharpin mutant mice was constructed by inserting loxP sequences flanking exon 2 of the Sharpin gene. FRT (recognition sequence for flp recombinase) sites were inserted flanking a neomycin resistance gene for colony screening, and the targeting vector was transfected into C57BL/6 embryonic stem (ES) cells. Appropriate homologous recombination was confirmed by Southern blot analysis. The targeted ES cells were injected into recipient blastocysts to generate germline-transmitting chimeras. Mice carrying the mutated Sharpin locus were crossed with Flp Deleter mice to delete the neomycin cassette and then backcrossed onto the C57BL/6 strain. Sharpin floxed mice were crossed with Pf4-Cre mice (Jackson Laboratories) or GPIbα-Cre mice, to delete...
SHARPIN by Cre recombinase, and backcrossed for at least six generations. Mice carrying the inserted flox mutation on each allele are designated as SHARPIN\(^{fl/fl}\) mice. Mouse ear DNA was used for genotyping mice with the following primers for polymerase chain reactions: Pf4-Cre, sense: TGC ACA GTC AGC AGG TT and anti-sense: CCC ATA CAG CAC ACC TTT TG, to yield a band of 450 bp. GPIbα Cre was detected using primers, sense: GAACACAACTCTCTCTTGCTGG and anti-sense: GAAGAGTTAATGGCAGGAAAGAG to yield a wild type band of 297 bp and GPIbα-Cre band of 372 bp. Primers for detection of SHARPIN were: sense: TTATTGAATATGTCCGGGCCACC, and anti-sense: TTCCATCTCCTGAATGACCTCTCTGGC, to yield bands of 281 bp for wild type, and 390 bp for loxP (floxed) sites. The PCR primers used to detect the Sharpin knockout deleted gene segment were: sense: GTACTCGACAGTCTCTCTATCACG and anti-sense: TTCCATCTCCTGAATGACCTCTCTGGC, to yield a band of 583 bp. All mouse studies were conducted with Institutional Animal Care and Use Committee approval from the University of California. In all experiments, both male and female mice were included. In vivo experiments utilized age and sex matched littermate controls. Mice were housed with freely available food and water.

**Fetal liver preparation.** Fetal livers were removed from day 13 post-fertilization mouse embryos and differentiated to megakaryocytes as previously described.\(^5,6\) Livers were added to 0.5 ml DMEM, aspirated up and down to dissociate cells, centrifuged at 500 g for 5 minutes, resuspended in 300 μL DMEM with 10 ng/ml murine thrombopoietin (ProspecBio, Ness-Ziona, Israel) at 1x 10^6 cells per 35 mm well of a 6-well plate and allowed to differentiate for five days. Megakaryocytes from one well (approximately 2.5 X10-6 cells) and cells from the carcass were processed to isolate DNA according to instructions in a blood DNA kit (Biomiga, San Diego, CA),\(^7\) and used in PCR reactions for SHARPIN and PF4-Cre genotyping.

**Western Blotting.** Washed platelets were lysed with Triton X100 lysis buffer containing inhibitors,\(^22\) subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with appropriate antibodies. To test for Met-1 ubiquitination or NF-κB activation, platelets were stimulated with a cocktail of 100 μM adenosine diphosphate (ADP; Merck/Sigma, Burlington, MA), 400 μM Protease-activated Receptor-4 (PAR4) agonist peptide (AYPGKF amide peptide; Genscript, Piscataway, NJ), and 50 μM epinephrine (Merck/Sigma). Western blots were probed with antibodies against Met-1 ubiquitin or phosphoserine 536 of the p65 (relA) subunit of NF-κB. Peritoneal lavage cells prepared as below, were lysed with RIPA buffer plus inhibitors and western blots were probed for SHARPIN.
**Microscopy analysis.** Platelets attached to immobilized fibrinogen on glass coverslips were imaged by deconvolution microscopy with a 100x 1.4 NA oil objective at the UCSD core microscopy facility. Images were processed using Adobe Photoshop, and platelet area and perimeter were calculated using software from Image Pro (Media Cybernetics (Rockville, MD). To determine filopodial length, a single z-slice image of αIIb-positive platelets was used to set minimum and maximum gating thresholds (Imaris software; Bitplane Inc/Oxford Instruments; Concord, MA), for specific visualization of entire filopodia and to generate surfaces for calculation of the average total length and average filament number per cell. All pixels above threshold levels were included in the analysis and these thresholds were applied to all images in an experiment. For superresolution microscopy, platelets attached to fibrinogen-coated coverslips were stained with primary antibodies against αIIb and talin, and STED compatible secondary antibodies, then visualized on a LEICA STED SP8 confocal microscope with a tunable pulsed white laser to achieve a lateral resolution of 30-50 nm and axial resolution of 100-130 nm. Acquisition of 16 z-sections of 0.1 μm by STED microscopy covered the full platelet height. Images were processed using LASX software, with careful thresholding applied similarly to all slides to enable quantification of staining in 3D images. Maximum vertical pillar heights per cell for talin and αIIb were calculated from 3D images using the Clipping feature to provide a clean cross-sectional surface and permit measurement of 3D height. Pearson Colocalization and Overlap coefficients were determined from 3D images using Image Pro. Approximately 25-40 cells were analyzed for each group, and represent the sum of four individual experiments.

**Flow studies.** Individual channels of iBidi (Fitchburg, WI) μslide VI flow chambers were coated overnight with 100 μg/ml fibrinogen (Enzyme Research; location) or 200 μg/ml fibrillar bovine achilles tendon collagen, rinsed with PBS and connected to a KD Scientific perfusion pump (Holliston, MA), that was set to yield channel flow shear rates (G) between 350 s\(^{-1}\) and 1700 s\(^{-1}\). Blood was drawn from mice by cardiac puncture into syringes loaded with heparin to yield a final concentration of 10 U/ml, a concentration that is not expected to interfere with attachment and thrombus formation under flow. The flow chamber was mounted on a heating platform, whole blood was aspirated through channels at low and high shear rates (G) (350, 700 and 1700 s\(^{-1}\)), with volumes adjusted to maintain flow for 5, 3 or 2 minutes respectively, then perfusion-fixed with 3.7% formaldehyde for 10 minutes. Slides were then stained for αIIb as described for platelet spreading studies, and images acquired with a deconvolution microscope fitted with a 20x objective. For fibrinogen the total platelet-covered area per field of view was calculated from single optical sections while for collagen, the total surface area for platelets and platelet aggregates attached to collagen was calculated from 3D rendered images of 6 optical sections of 0.2 μm per experimental condition using Image-Pro 10 software (Media Cybernetics, Rockville, MD).
Thresholds were established to report on αIIb positive fluorescence, separately confirmed for specificity using IgG species control antibody, and applied to all images. The sum of all fluorescence in each field of view was calculated and the average surface area per field was calculated from n = 5 experiments.

**Blood cell counts.** Blood was collected by submandibular bleed directly into EDTA-coated tubes. Cell counts were analyzed using the IDEXX Procyte Dx Hematology Analyzer.

**DSS induced acute colitis.** In a DSS model of acute colitis,\(^\text{10,11}\) 8-10 week-old SHARPIN\(^{fl/fl}\) GPIbα-Cre- or Cre+ littermate mice were provided with autoclaved tap water with or without 2.4% DSS in cage bottles. On day seven, all mice were switched to autoclaved water. Mice were weighed daily, and the remaining water monitored regularly to confirm similar consumption. Nine days from the start of DSS administration, mice were euthanized, the body cavity exposed, and the colon segment between the rectum and caecum removed and measured using precision calipers. Colons were opened, cleared of stool, and rapidly fixed in 10% formalin. The next day, tissue was transferred to 70% ethanol, paraffin embedded and H & E stained at the La Jolla Institute of Allergy and Immunology Histology Core. Rectal bleeding was scored: 1-none; 2-slight; 3-significant but one day only; and 4-significant, bleeding multiple days. Histology slides were scored for epithelial damage and leukocyte infiltration according to Erben et al.\(^\text{12}\) as follows. Epithelial damage: 1- focal erosions; 2- erosions with focal ulcerations 3- erosions with extended ulcerations. Leukocyte invasion of tissue layers: 1- mild, mucosal; 2- moderate, mucosal and submucosal; and 3- marked, transmural invasion. The two latter scores were added for each mouse and the sum was recorded.

**Peritonitis model.** To assess leukocyte recruitment into the peritoneum following a sterile inflammatory stimulus,\(^\text{27}\) one ml of autoclaved 4% thioglycollate medium (Difco\(^\text{TM}\); BD, Sparks, MD) or sterile saline was injected intra-peritoneally into 6-8 week-old mice, and the mice were euthanized after 4 or 24 hours. The peritoneum was carefully exposed, injected with 6.5 ml ice-cold phosphate-buffered saline with 2 mM EDTA and the lavage fluid was withdrawn after two minutes and kept on ice. The cell suspension was centrifuged at 450g for 8 minutes, the pellet was incubated with a red blood cell lysis solution for 5 minutes, re-centrifuged and finally resuspended in 1 ml of cold phosphate-buffered saline.
REFERENCES


Supplementary Figure S.1. Generation and characterization of Sharpin flox/flox mice. A. Design strategy incorporating two lox P sequences bracketing exon 2 (black arrowheads) and a neomycin resistance gene flanked by FRT sites for selection (blue rectangles) in the mouse Sharpin gene. Embryonic stem cells with the correct inserts were used for mouse generation. The neomycin cassette was removed by crossing the generated mice with Flp Deleter mice. To delete SHARPIN in the megakaryocytic lineage, SHARPIN lox-P (SHARPIN^{f/f}) mice were crossed with Pf4-Cre or GPIbα-Cre mice. B,C. Polymerase chain reaction (PCR) for detection of wild type and floxed Sharpin (B,C; left panels), and for Pf4-Cre (B) and GPIbα-Cre (C) bands, from DNA prepared using mouse ear tissue. Arrows indicate expected PCR band sizes. D. PCR of DNA prepared from fetal liver differentiated megakaryocytes or corresponding embryo carcasses using primers reporting on excision of Sharpin exon 2. As expected, Sharpin was deleted in megakaryocytes, but not in embryo carcasses (arrow indicates 583 bp band corresponding to deleted exon).
Supplementary Figure S.2. Western blots of lysate from SHARPIN$^{fl/fl}$ Pf4-Cre (A-C) and SHARPIN$^{fl/fl}$ GPIbα-Cre (D-F) mouse platelets. Similar to global SHARPIN KO cpdm mice, HOIP was greatly reduced and SHARPIN protein was absent in platelets from SHARPIN$^{fl/fl}$ Pf4-Cre and SHARPIN$^{fl/fl}$ GPIbα-Cre (A,D) mice as determined by Western blotting. β-actin was used as a loading control. Western blots of integrin-regulatory proteins Talin and Rap1 (B,E). Kindlin-3 (C,F), showing similar expression in lysates from SHARPIN$^{fl/fl}$ Cre+ and SHARPIN$^{fl/fl}$ Cre- mouse platelets. Results are representative of at least three separate experiments.
Supplementary Figure S.3

Figure S.3. SHARPIN\textsuperscript{fl/fl} GPIb\textalpha-Cre+ platelets show increased spreading on immobilized fibrinogen. A.-C. SHARPIN\textsuperscript{fl/fl} GPIb\textalpha-Cre platelets were allowed to attach and spread for 30 minutes on fibrinogen in the absence of exogenous agonist, then stained with an FITC-conjugated anti-\alphaIIb antibody. Images were acquired on a deconvolution microscope with a 100x oil objective, and C. the average area was quantified using Image-Pro (n = 4). D.-F. Platelets were allowed to attach and spread in the presence of 300 \textmu M PAR4 agonist peptide (D,E), and the average area is shown in F. (n=3).
Figure S4. SHARPIN\textsuperscript{fl/fl} GPIb\textalpha-Cre\textsuperscript{+} platelets show increased agonist-dependent binding to fibrinogen. A, B. Flow Cytometry of fibrinogen bound to agonist-stimulated platelets. Washed platelets prepared from blood drawn from SHARPIN\textsuperscript{fl/fl} GPIb\textalpha-Cre\textsuperscript{-} and SHARPIN\textsuperscript{fl/fl} GPIb\textalpha-Cre\textsuperscript{+} mice and incubated for 30 minutes with Alexa-647-conjugated fibrinogen and the indicated concentrations of platelet agonists, A. ADP or B. PAR4 agonist peptide, AYPGFK. Results are expressed as specific fibrinogen binding, as described in Materials and Methods. Data are from five experiments with ADP and three experiments with AYPGFK. Asterisks indicate t-test comparisons between wild-type and SHARPIN-null platelets (* P < 0.05, ** P < 0.01), ANOVA comparison across all agonist concentrations yields P < 0.01, P < 0.05 for ADP and PAR4 respectively, reflecting the consistently increased binding of GPIb\textalpha-Cre platelets across agonist concentrations. C. \(\alpha\)IIb surface expression was determined on platelets prepared as in A,B, with or without the indicated agonists, and compared to non-specific IgG binding (n=3).
**Figure S.5. DSS induced colitis.** Mice were treated with water only (blue) or a DSS/water regimen (grey, orange) as indicated. Note however that both unfloxed SHARPIN$^{WT}$ GPIbα-Cre+ (grey) and floxed SHARPIN^{fl/fl} GPIbα-Cre- (orange) mice lost similar amounts of weight when treated with DSS, indicating the lack of protection offered by GPIbα-Cre alone. Floxed (fl/fl) GPIbα-Cre- mice without DSS treatment did not lose weight (blue).