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Supplementary Information for

SHARPIN at the Nexus of Integrin, Immune and Inflammatory Signaling in Human Platelets

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Supplementary Information Text:

Supplemental Materials and Methods.

Reagents and antibodies. Antibodies 8275 against the C-terminal 20 amino acids of integrin β3 (1), and D57 against human integrin αIIbβ3 (2) have been described. A sheep polyclonal antibody against SHARPIN and a mouse monoclonal against the carboxy terminal LDD domain of HOIP were from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against SHARPIN, NF-κB, Otulin, β-tubulin, IκBα, and SPATA2 were from Proteintech Group (Rosemont, IL). Mouse monoclonal and rabbit polyclonal antibodies against NEMO were from Santa Cruz Biotechnology (Dallas, TX). A mouse monoclonal antibody against IKKα/β was from BD Biosciences (San Diego, CA). A rabbit antibody against phosphoserine-536 of the p65 RelA subunit of NF-κB and a mouse antibody against total RelA were from Cell Signaling Technology (Danvers, MA). A PE-conjugated mouse antibody against human MHC Class I HLA-ABC, and an Alexa-647-conjugated rat antibody against human CD11b were from Biolegend (San Diego, CA). Fibrinogen was purchased from Enzyme Research (South Bend, IN) and Alexa-647-conjugated fibrinogen was from ThermoFisher/Invitrogen (Waltham, MA). Mouse and rabbit monoclonal antibodies against linear ubiquitin chains and HOIL-1 were from Millipore (Temecula, CA), and a human Fc anti-linear ubiquitin antibody, 1F11, was a gift from Genentech (South San Francisco, CA) (3). A mouse monoclonal β-actin antibody was from Millipore/Sigma (St. Louis, MO). An Elisa kit for sCD40L was from R&D Systems. Other reagents were from ThermoFisher (Waltham, MA).

GST-fusion protein preparation and pulldown assay. GFP-human SHARPIN DNA was a gift from J. Ivaska (University of Turku, Turku, Finland) (4). New EcoRI sites were added 5' and 3' of SHARPIN with the Clontech InFusion kit (Clontech, Mountain View, CA), using primers:
5': GGGATTCCCCGGAATTCATGGCGCCGCCAGCGGGC, and
3': GTCGACCCGGGAATTCCTAGGTGGAAGCTGCAGC, and the fragment was cloned into the EcoRI site of PGEX-6P-1 (GE Healthcare Life Sciences, Marlborough, MA). GST-fusion proteins were prepared from bacterial cultures (5) (6). In order to test for tight association of SHARPIN with the cytoplasmic tail of integrin αIIb, GST-SHARPIN coupled to glutathione Sepharose beads (GE Healthcare, City ST)(7) was used to pull down αIIb from human platelets that had been lysed in Radioimmunoprecipitation Assay (RIPA) buffer (8).

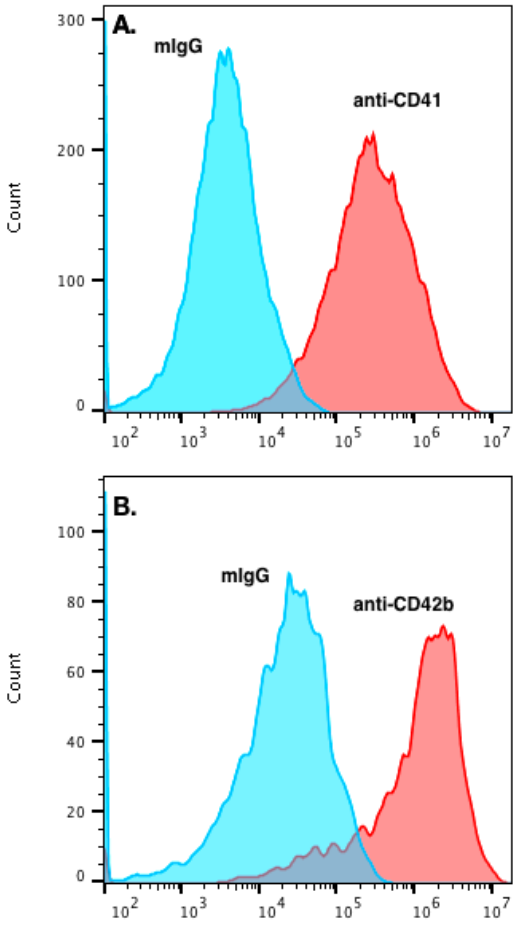
Harvest of iPS cell-derived megakaryocytes and platelets. Cells at maturation days 6/7 and 8/9 post doxycycline removal were used for megakaryocyte and platelet experiments, respectively. iPS cell-derived megakaryocytes were harvested by transfer to 15 ml conical tubes, large cells were allowed to settle for an hour at 37° C with 5% CO_2, and the supernatant was then spun at 150g for 5 minutes in the presence of 1:6 ACD anticoagulant. The pellet was resuspended in Tyrodes buffer and combined with large cells for use in experiments. For harvest of iPS-derived platelets, supernatant was spun at 400g for 12 minutes. Differentiation to bulk iPS-derived megakaryocyte lineage cultures did not induce broad myeloid differentiation as indicated by the lack of neutrophils in the preparation (Figure Supplementary S5). We explored various methods and timings for genetic modification of these cells and found that lentiviral infection at the immature megakaryocyte stage was optimal. Thus, to knock down SHARPIN, immature megakaryocytes were infected with lentivirus encoding GFP and SHARPIN shRNAs or scrambled control shRNA. Following cell maturation in culture, SHARPIN depletion in mature megakaryocytes and platelets was verified by Western blot.

Production of shRNAs and packaging into lentivirus. shRNA mature antisense sequences were used to produce two knockdown sequences of SHARPIN:
(shSHARPIN 1:
TTTTTAATTAAAAAACTCAGACCAGGACTACCCACCAATGGGCAGTCCTAGTCCGAGGGTGT
TTCGTCCTTTCCACA;
shSHARPIN 2:
TTTTTAATTAAAAAATCTCCAAGTATGACCCGCAGCCCAAGCTGCAGGTCACACTTGAAGAG
GTGTTTCGTCCTTTCCACA)
that were inserted into the XbaI- and PacI-digested pIRES-GFP lentiviral vector, FG12 (9). Lentiviruses encoding either of the two SHARPIN shRNAs or a control, scrambled shRNA (shControl) were packaged in 293T cells (10), and viral titers of $5x10^6$ transduction units/ml were used to infect immature megakaryocytes.

Western blotting and immunoprecipitation. Human platelets or iPS-derived cells in Walsh's buffer were unstimulated or stimulated with either 1 U/ml thrombin, 1μg/ml LPS, 1 μg/ml sCD40L, or an agonist cocktail (50 μM ADP, 50 μM epinephrine, 100 μM SFLLRN PAR1 activation peptide), as indicated in the Figure Legends. For NEMO immunoprecipitation and assay of linear ubiquitination, samples were processed according to Sasaki et al. (11), but with 5 mM N-ethyl maleimide added to the lysis

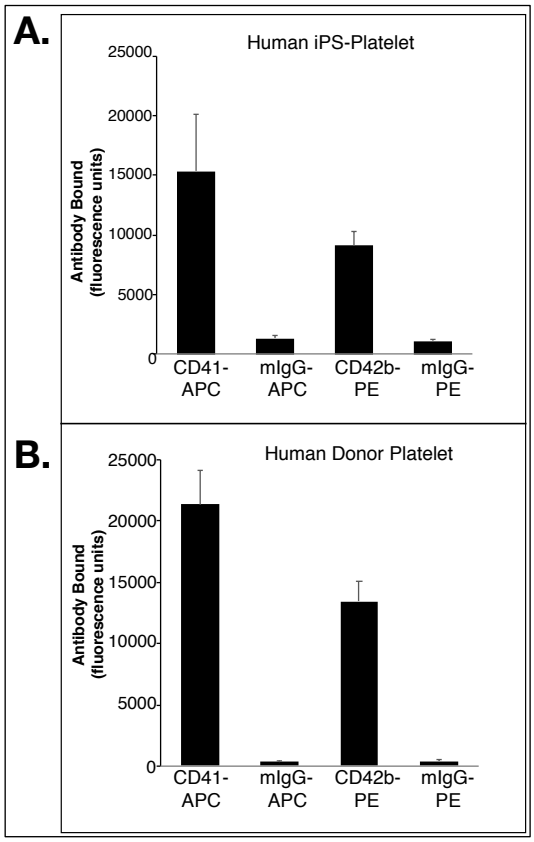
buffer, and blots were probed with antibodies specific for Met-1 ubiquitin. To examine αIIb or SHARPIN associations, platelets were lysed with Nonidet P-40 (NP-40) buffer (0.75% NP-40, 100 mM NaCl, 50 mM Tris [tris(hydroxymethyl)aminomethane], pH 7.4) containing "cOmplete" protease inhibitor cocktail, 1 mM sodium fluoride, 0.5 mM leupeptin, 100 μg/ml aprotinin, 2 mM PMSF, phosphatase inhibitor cocktail (Selleckchem, Houston, TX) and 20 μM calpeptin. Cleared lysates were immunoprecipitated with antibodies against αIIb or SHARPIN, and blots were probed for proteins of interest. Phosphorylation of serine-536 in the RelA subunit of NF-κB in platelets and megakaryocytes was assayed in cells that were resting or stimulated for 15 minutes with thrombin, or the above agonist cocktail. Signal intensity for SHARPIN and pS-536 RelA (NF-κB) was normalized using β-actin and total NF-κB, respectively. Immunoreactive bands were detected with the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Microscopy image acquisition. Platelets in Walsh's buffer were incubated with vehicle or stimulated with 0.5 U/ml thrombin and allowed to attach to fibrinogen-coated coverslips (100 μg/ml) for one hour at 37° C. Cells were fixed with 3.7% formaldehyde and processed for imaging as described (12). Platelets were stained with antibodies against SHARPIN and αIIbβ3 or control IgG antibodies. Images were acquired using a Leica SP8 confocal microscope with Lightning deconvolution (Wetzlar, Germany), and equipped with a High Contrast Plan-Apochromatic 63x 1.4 NA oil objective and 1.7x zoom, allowing a resolution of 120 nm. All cells within an experiment were imaged under identical acquisition conditions. Acquired images were minimally processed using Adobe Photoshop and Image J, with linear manipulations applied identically for all images. Megakaryocytes plated on fibrinogen were similarly processed, but, fixed with 2% paraformaldehyde for 30 minutes, and stained with antibodies against linear ubiquitin (1E3 antibody), αIIb, and Hoechst dye to stain DNA. Megakaryocyte images were acquired on an OMX microscope (GE Healthcare Bio-Sciences, Pittsburgh, PA) with a 100x 1.4 NA objective, and linear ubiquitin signal within cells was calculated using Volocity Image Analysis software. Mature, suspension megakaryocytes were cytospun onto glass slides, and stained with Wright Giemsa for imaging with a Keyence BZX-700 (Itasca, IL) microscope fitted with a Plan Apo 40x objective (UCSD Core Microscopy facilities, supported by NIH grants NS047101 and P30 2P30CA023100-28).



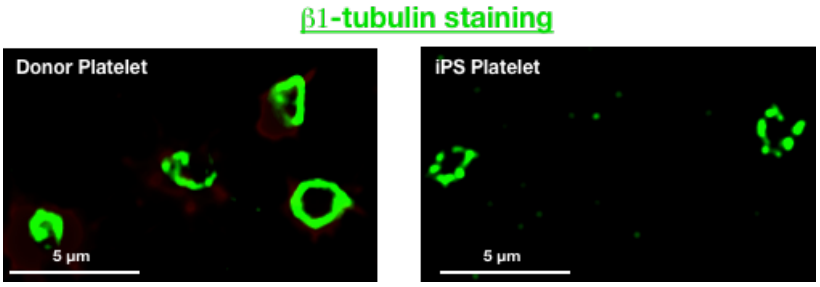
Supplementary Figure S1

Figure S1. Mature human megakaryocytes derived from iPS cells express CD41 (αIIb) and CD42b (GPIbα). In A, megakaryocytes were incubated with APC-conjugated mIgG or anti-CD41. In **B,** cells were incubated with PE-conjugated mIgG or anti CD42b, and binding was determined by flow cytometry.



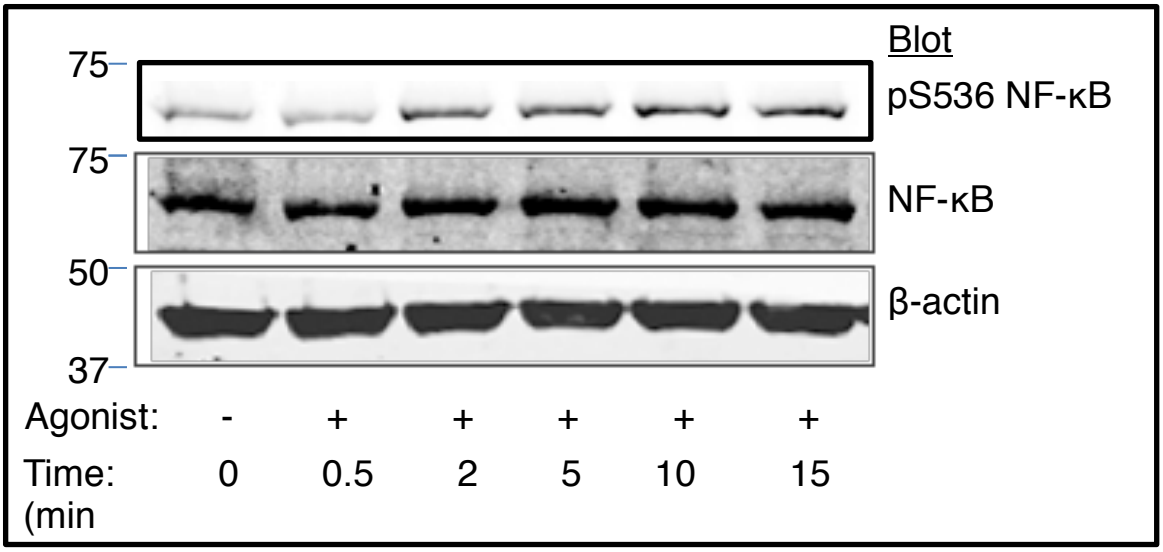
Supplementary Figure S2

Figure S2. Platelet surface expression of adhesion receptors. iPS-derived platelets (A) or (B) human donor-derived platelets were incubated with fluorescently-conjugated antibodies against CD41 (αIIb), CD42b (GPIbα), or IgG control antibodies, and platelet-associated fluorescence was determined by flow cytometry. Expression of αIIb and GPIbα was comparable between the cells.



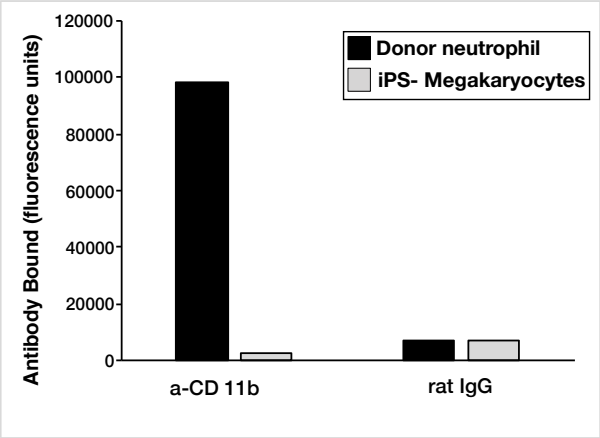
Supplementary Figure S3

Figure S3. Staining of β1-tubulin rings in platelets. Human platelets (left) and iPS cell-derived platelets (right) were incubated with a rabbit anti-β1-tubulin antibody, then with an Alexa-488 conjugated anti-rabbit secondary antibody, and images were acquired by microscopy.



Supplementary Figure S4.

Figure S4. Time course of NF-κB phosphorylation. Human platelets were incubated for 15 minutes in the absence or presence of an agonist cocktail (ADP, epinephrine and PAR1 agonist peptide as in Figure 4). Cells were lysed at the indicated times and blots were probed with an antibody to phosphoserine-536 of the p65 (RelA) subunit of NF-κB and reprobed for total RelA and β-actin.



Supplementary Figure S5.

Figure S5. Myeloid lineage cells are not found in iPS cell-derived megakaryocyte/platelet lineage preparations. Buffy coat neutrophils or iPS cell-derived megakaryocytes were incubated with anti-CD11b, or rat IgG control Alexa-647-conjugated antibodies, and antibody binding was determined by flow cytometry.

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