Methods

Animals

Nbeal2$^{-/-}$ mice were generated as previously described.[1] *Unc13d*^{Jinx} mice on a C57BL/6 background were purchased from the Mutant Regional Resource Center at the University of California (Davis), USA.

Megakaryocyte culture

Primary mouse bone marrow cell cultures were established and MKs grown to maturity as described.[1] Human MKs were cultured under serum-free conditions from peripherally-mobilized CD34+ cells as described. [2] For both species the final stage of culturing involved seeding onto coverslips coated with Matrigel (BD Biosciences) diluted 1:6 with DMEM in 12-well plates. After 2+ days of growth, cells were fixed (4% paraformaldehyde in PBS) and stained *in situ* (i.e. without cytospinning) to preserve their natural structure.

Platelet preparation

Anticoagulated blood was obtained from healthy, drug-free, human volunteers and a Gray platelet syndrome patient, for which approval was obtained from the University of Reading Research Ethics Committee or the Research Ethics Board at The Hospital for Sick Children. Informed consent was provided according to the Declaration of Helsinki. Platelet-rich plasma (PRP) was prepared by centrifugation at 150g at room temperature for lumiaggregometry, flow cytometry and fixation for immunofluorescence staining as described.[3]

Blood from C57BL/6, Nbeal2$^{-/-}$ and *Unc13d*^{Jinx} mice was collected into 3.2% sodium citrate and PRP obtained as described above. Washed platelets were prepared from PRP via two cycles of pelleting at 1000g and resuspension in PBS/ACD (85mM $Na_3C_6H_5O_7$, 71mM $C_6H_8O_7$ and 110mM $C_6H_{12}O_6$) pH 6.4 prior to resuspension in PBS plus protease inhibitor for lysate preparation, or in Tyrode's HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM $Na_2HPO_4{\cdot}12H_2O$,12mM $NaHCO_3$, 20mM HEPES and 1mM $MgCl_2$, pH 7.3) for experiments where platelets were stimulated with U46619 (Sigma) in the presence of abciximab (20 μg/ mL, ReoPro, Eli Lilly and Co.) with or without latrunculin A (200 μM, Calbiochem), then fixed and stained.

Immunostaining and confocal fluorescence microscopy

Fixed platelets and MKs were stained without or with permeabilization (0.2% Triton

X-100) using one or more primary antibodies: rat polyclonal anti-mouse CD41 (eBioscience), rabbit polyclonals against VWF (Dako) or ERp57 (Abcam), rabbit monoclonal to α-tubulin (Cell Signaling), goat anti-P-selectin (Santa Cruz Technology), mouse monoclonals against α-tubulin (Sigma), SERCA3 (PL/IM430; Santa Cruz Biotechnology), TGN46, CD71/TF, CD61 (Hybridoma Bank), CD41 (Pharmingen), calnexin, PDI (Abcam) or CD42B (BD Biosciences). DNA was stained with Hoechst 33342. Primary antibodies were either fluorescently tagged (rabbit anti-tubulin) or detected with secondary antibodies of donkey or goat origin specific for mouse, rabbit, goat or rat primary antibodies, conjugated with Alexa Fluor 647, 568, 555, 488 or 405 (Life Technologies). Cells were mounted with fluorescent medium (Life Technologies or Dako).

Confocal fluorescence microscopy images were acquired with 250 nm Z-stepping through oil immersion objectives (Olympus UPLSAPO 60x/1.35 and 100x/1.4) using a Quorum spinning disc confocal inverted fluorescence microscope equipped with a 1.5x internal magnification lens (Spectral Applied Research) and 4 solid- state lasers (Spectral Applied Research): 405 nm, 491 nm, 561 nm, 642 nm, an Improvision Piezo Focus Drive, 1.5x magnification lens (Spectral Applied Research), a Hamamatsu C9100-13 back-thinned EM-CCD camera and Yokogawa CSU X1 spinning disk confocal scan head with Spectral Aurora Borealis upgrade. Immunostaining conditions, laser intensity, camera and exposure settings were established with minimal/undetectable levels of autofluorescence, channel crosstalk and non-specific primary/secondary background fluorescence. Acquisition, image deconvolution, registry correction (maximum one z-pixel required in our system) and colocalization analysis were performed with Perkin Elmer Volocity software (versions 5.5 – 6.1) and videos were prepared with Imaris 7.6. Images were exported to Adobe Photoshop or Illustrator for final preparation. Colocalization analysis was performed using the thresholded Pearson's correlation coefficient (PCC).[4] All images shown in the text are representative of at least 3 independent preparations (e.g. MK cultures) and >100 immunostained cells over >10 fields.

Sucrose gradient sub-cellular fractionation

Platelet fractionation was performed as previously described [5] with minor modifications. Red blood cells were removed from the PRP by centrifugation at 100 x g for 10 min. Platelets were pelleted at 1000 x g for 10 min, washed twice and resuspended in non aggregating conditions in Tris-Citrate buffer (63 mM Tris/HCl, pH 6.5, 95 mM NaCl, 5 mM KCl, 12 mM citric acid) containing 1mM EGTA, 10 μM

indomethacin and 2U/mL apyrase. Platelets were then moved into a cell-disruption bomb (Parr 4639, Parr Instrument Co.) and homogenized by nitrogen cavitation. A pressure of 1200 psi was applied with N_2 to the platelet suspension and after 15 min the pressure was quickly released. This procedure was repeated three times and the final platelet homogenate was cleared from the cell debris and partially disrupted cells by centrifugation at 500 x g for 10 min. The platelet homogenate was fractionated over a linear sucrose gradient (from 60 to 30%, w/v in 5mM EDTA) by centrifugation at 284,061 x g for 2 hours at 4C. Fractions were collected from the top of the tube and aliquots analyzed by immunoblotting.

Immunoblot analysis

PRP was prepared from mouse or human blood as above, and washed 3 times in PBS/ACD pH 6.1 by centrifugation at 1000g for 10 minutes. After counting (Z1 Coulter Counter; Beckman Coulter Canada), 1×10^9 platelets/mL (final) were resuspended in PBS pH 7.4 containing protease inhibitor cocktail (5 mM EDTA; Complete; Roche Diagnostics) and lysed by the addition of Triton X-100 (0.5% v/v). SDS-PAGE and immunoblotting of plasma, cell lysates and sub-cellular fractions were performed using standard protocols. Goat polyclonal anti-actin C-1 (sc-1615) and mouse anti-**β**-actin (Thermo Scientific) and anti-GAPDH (Santa Cruz Biotechnology) were used for protein loading controls. Anti-PDI and ERp57 antibodies were as described above, the others used were rabbit polyclonal anti-CD289/TLR9 (Acris Antibodies) and mouse anti-TSP-1 (Thermo Scientific), anti-TLR9 (clone 26C593.2; Imgenex), goat polyclonal anti-integrin β3 (N-20, Santa Cruz), rabbit polyclonal anti calreticulin (Merck Millipore), anti-RabGDI and anti-Munc 13-4 (Santa Cruz Biotechnology); secondary antibodies were from Invitrogen or Jackson Laboratories.

Flow cytometric measurement of surface mobilization of P-selectin and thiol isomerases

Washed human platelets ($4x10^8$ cells/mL) were incubated with PE/Cy5 anti-human CD62P/P-selectin (BD Accuri flow cytometers, USA), with anti-PDI (Abcam) or anti-ERp57 (Abcam) for 10 minutes in presence of GPRP (0.5 mg/mL) and then with goat anti-rabbit or goat anti- mouse Alex Fluor 488 (Life Technologies Corp.) in the presence or absence of latrunculin A or its vehicle DMSO (< 0.2% v/v). Platelets were stimulated with 1 U/mL thrombin for 20 minutes and diluted 10-fold in PBS. Data for 10 000 gated events were collected and analysis performed by means of a BD Accuri C6 flow cytometer (BD Accuri flow cytometers, USA).

Mouse whole blood diluted with HBS (Hepes Buffered Saline) buffer was incubated with PE/Cy5 anti-human CD62P/P-selectin (BD Accuri flow cytometers, USA), or with anti-PDI (Abcam) for 10 minutes in presence of GPRP (0.5 mg/mL) and then with goat anti-rabbit Alex Fluor 488 (Life Technologies Corp.). Platelets were stimulated with PAR4-AP (AYPGKF-NH2) 300 μM in presence or absence of ADP (10 μM), and after 20 minutes the reaction was terminated by 10-fold dilution in PBS. Flow cytometric acquisition was performed using a FACS Canto II flow cytometer (BD Biosciences) and data were analysed using FACSDiva software (10,000 events were collected per sample). Negative controls were set using an appropriate IgG1 κ-isotype matched control for the anti-CD62P antibody (BD Accuri flow cytometers, USA) or using the secondary fluorescent antibody but omitting the primary in the case of thiol isomerases. For statistical analysis, GraphPad Prism 4.0 was used to perform t-test, one-way ANOVA analysis followed by Dunnett's test or two-way ANOVA followed by Bonferroni's test. P values < 0.05 were considered statistically significant.

References

1. Kahr WH, Lo RW, Li L, Pluthero FG, Christensen H, Ni R, Vaezzadeh N, Hawkins CE, Weyrich AS, Di Paola J, Landolt-Marticorena C, Gross PL. Abnormal megakaryocyte development and platelet function in nbeal2(-/-) mice. *Blood*. 2013;122:3349-3358
2. Lo B, Li L, Gissen P, Christensen H, McKiernan PJ, Ye C, Abdelhaleem M, Hayes JA, Williams MD, Chitayat D, Kahr WH. Requirement of vps33b, a member of the sec1/munc18 protein family, in megakaryocyte and platelet alpha-granule biogenesis. *Blood*. 2005;106:4159-4166
3. Licht C, Pluthero FG, Li L, Christensen H, Habbig S, Hoppe B, Geary DF, Zipfel PF, Kahr WH. Platelet-associated complement factor h in healthy persons and patients with atypical hus. *Blood*. 2009;114:4538-4545
4. Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S. Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophysical journal*. 2004;86:3993-4003
5. Jonnalagadda D, Sunkara M, Morris AJ, Whiteheart SW. Granule-mediated release of sphingosine-1-phosphate by activated platelets. *Biochimica et biophysica acta*. 2014;1841:1581-1589