Supplemental Data

**VPS33B regulates protein sorting into and maturation of α-granule progenitor organelles in mouse megakaryocytes**

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Supplemental Methods

Tamoxifen administration
There are different administration routes, dosing and timing for tamoxifen induction and the effectiveness depends on the gene of interest and the specific tissue.¹ The tamoxifen inducible Rosa26-CreERT² system has been successfully used by the Birmingham Platelet group to induce gene deletion in megakaryocytes for CLEC-2 deficient mice, with complete loss of protein after 5 days, which is the duration of platelets in the blood stream (unpublished data). In addition, the fact that the Vps33b⁰⁄⁻-PGKCre mouse was lethal prior to mid-gestation suggested that Vps33b is expressed early in development and might be more difficult to target than genes restricted to the megakaryocyte lineage. Thus, we decided to use intraperitoneal (i.p.) injections of tamoxifen for 5 consecutive days to achieve maximum effectiveness of the drug. Then, a blood sample was collected once a week to assess P-selectin expression in response to 0.05 U/mL thrombin as a marker of α-granules. A consistent level of reduction in P-selectin expression was detected after 4 weeks post-induction. The dry skin phenotype started to appear at the end of week 4-week 5 which is indicative of loss of VPS33B in other tissues (which is delayed relative to platelets due to the longer lived nature of these cells). Therefore we decided to perform all our experiments at 5 weeks post-induction.

Quantitative real-time polymerase chain reaction (q-PCR)
Total RNA was isolated using the NucleoSpin RNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was prepared using SuperScript III reverse transcriptase with first-strand synthesis primed using oligo (dT). Quantitative real-time PCR was performed using SYBR Green PCR master mix on a BioRad CTX Connect real-time PCR detection system (Life Science, Hertfordshire, UK). Levels of Vps33b amplified cDNA were normalized to the level of Hprt1 housekeeping control cDNA. Primers used: Vps33b forward – ACCTGAAAGTGCCCCGAGAC, Vps33b reverse – TTAGCCGTGTCTGTGAATGCAA, Hprt1 forward – TCAGTCAACGGGGGACATAAA, Hprt1 reverse – GGGGCTGTACTGCTTAACCAG.

Immunohistochemistry
Spleens and femurs from Vps33b⁰⁄⁻-ERT² and litter-matched Vps33b⁰⁄⁻ control mice were fixed in 10% formalin and embedded in paraffin (Sequani, Herefordshire, UK). Femurs were
decalcified prior to embedding. Five serial sections per sample (5 μm) were H&E stained and examined using a Zeiss Axiovert 200 inverted high-end microscope with a 40× objective.

**Platelet preparation**

Blood was collected from the descending thoracic aorta of CO₂-asphyxiated mice into 1/10 (v/v) acid-citrate-dextrose anticoagulant², and platelet count and mean platelet volume were measured using an ABX Pentra 60 whole blood counter (Block Scientific, New York, USA). Washed platelets were prepared by centrifugation as published³ and were resuspended at a density of 2×10⁸/mL.

**Platelet aggregation and secretion**

Murine washed platelets were stimulated with different agonists and platelet aggregation and adenosine triphosphate (ATP) secretion were measured simultaneously using a lumi-aggregometer (Chrono-Log, Havertown, PA, USA).

**Platelet half-life assay**

Platelet half-life was performed, as previously described⁵. Whole blood was collected at various times post-intravenous injection of 150 μL of 4 mg/mL biotin-N-hydroxysuccinamide into buffer containing 10% fetal bovine serum (FBS) and 5 mM ethylenediaminetetraacetic acid (EDTA). Platelets were stained with anti-mouse αIIbβ3-FITC and streptavidin-PE for 1 hour on ice and the percentage of biotin-labelled+ αIIbβ3+ platelets was measured by flow cytometry.

**Platelet adhesion under flow**

To study platelet aggregation at arteriolar shear, blood was collected into 5 U/mL heparin and 40 μM phenylalanyl-L-prolyl-L-arginine chloromethylketone (P-PACK). Whole blood was perfused through collagen-coated (100 μg/mL) glass microslide capillaries (0.1 mm) at a shear rate of 1000s⁻¹ for 4 minutes at 37°C as described⁶.

**Bleeding time assay**

Hemostasis was monitored by removal of the tail tip and blood loss was recorded⁷.

**Megakaryocyte culture**

Mature MKs were cultured from murine bone marrow hematopoietic stem cells as previously described⁸. MKs were left in culture for 5 days in StemPro media supplemented with 20 ng/mL murine stem cell factor (SCF) and 100 ng/mL murine thrombopoietin (TPO). Mature
MKs were isolated using a BSA density gradient (3%-1.5% BSA) and spread on fibrinogen-coated glass coverslips (100 µg/mL) for 1 hour (initial attachment) or 6 hours (proplatelet formation).

**Electron Microscopy**

For transmission electron microscopy (TEM) platelet-rich plasma (PRP) was prepared by centrifugation at 200×g for 10 minutes and fixed with an equal volume of 0.1% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 15 minutes. The platelets were postfixed with 3% GA in 0.1 M sodium cacodylate buffer at 4°C for 30 minutes. Native bone marrow and bone marrow-derived MKs in culture were directly fixed in 3% GA. All samples were fixed further with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at 4°C. Cells were rinsed in distilled water, dehydrated in a graded series of ethanol and embedded in Epon resin. Ultrathin sections (70-90 nm) were stained with uranyl acetate and lead citrate. Platelet whole-mount electron microscopy was performed by placing 10 µL of PRP onto carbon-coated formvar copper grids (Elektron Technology, Cambridge, UK) for 5 minutes. Grids were rinsed with distilled water and excess liquid was removed with a filter paper. For immunoelectron microscopy (IEM) the Tokuyasu method was followed. PRP was initially fixed with an equal volume of 4% PFA/0.4% GA in 0.1 M phosphate buffer (pH 7.4) followed by postfixation with 2% PFA/0.2% GA in phosphate buffer. Bone marrow-derived MKs in culture were directly fixed in the latter buffer. Immunogold labeling of ultrathin cryosections was performed at room temperature by floating the grids on drops containing mouse anti-CD63 (kindly gifted by Prof. Paul Saftig) and rabbit anti-VWF (Dako, Cambridgeshire, UK) antibodies all in a dilution of 1:20. Immunogold double-labeling was performed using 10-nm and 15-nm protein A gold conjugates (CMC Utrecht, Netherlands) in a dilution of 1:50. All imaging was done in a Tecnai G2 Spirit microscope (FEI, Eindhoven, Netherlands) using a Morada EM CCD digital camera (Olympus SIS, Münster, Germany).

**Flow cytometry**

Surface glycoprotein expression was measured in whole blood by flow cytometry using rat anti-mouse fluorescein isothiocyanate (FITC)-conjugated GPVI, Integrin α2 (CD49b, GP1α), αIIbβ3 (CD41/61), GP1bα (CD42b) and IgG (negative control) antibodies (Emfret, Würzburg, Germany). P-selectin levels were measured in resting and thrombin activated platelets (2×10⁷/mL) using a FITC-conjugated CD62P antibody (Emfret, Würzburg,
Germany). Polyploidy of mature MKs isolated by BSA density gradient was analyzed after DNA staining with propidium iodide. Samples were acquired using FACSCalibur flow cytometer and analyzed using CellQuest software (Becton Dickinson, Oxford, UK). For the immunophenotyping single cells were isolated from spleen and stained on ice for 15 minutes with LIVE/DEAD Fixable Aqua Dead Stain Kit followed by 20 minute incubation with the specified antibodies. Subsets were defined using the following markers: T cells, CD4⁺CD8⁻; B cells, CD19⁺; Myeloid cells, CD19⁺CD3⁻CD11b⁺; Dendritic cells, CD19⁻CD3⁻CD11c⁺; Neutrophils, CD19⁻CD3⁻Ly6C⁻GR1⁺⁺; Monocytes, CD19⁻CD3⁻Ly6C⁺GR1⁻; Macrophages, CD19⁻CD3⁻GR1⁻CD64⁺. Samples were acquired on MACSQuant flow cytometer and analyzed using FlowJo10.6 software. For the fibrinogen uptake assay bone marrow-derived MKs were cultured for 5 days prior to use and mature MKs were enriched following a BSA density gradient. MKs were given a 2 hour pulse of Alexa Fluor 488-conjugated fibrinogen (Life Technologies) in serum free DMEM at 37°C and internalization of fibrinogen was assessed by flow cytometry.

**Immunoblot analysis**

Washed mouse platelet or cultured MK whole cell lysates were prepared and western blotted as previously described. The following antibodies and dilutions were used: rabbit anti-VWF primary, 1:1000 (Santa Cruz Biotechnology, Heidelberg, Germany) and goat anti-rabbit HRP conjugate secondary, 1:1000 (Life Technologies, Paisley, UK); rat anti-P-selectin 1:500 (M130-0, Emfret, Eibelstadt, Germany) and goat anti-rat HPR conjugate secondary, 1:1000 (Life Technologies, Paisley, UK). Loading was confirmed by immunoblotting for β-actin using mouse anti-β-actin primary, 1:15000 (Sigma-Aldrich, Dorset, UK) and rabbit anti-mouse HRP conjugate secondary, 1:20000. Densitometry was carried out using ImageJ.

**Immunofluorescence microscopy**

Bone marrow-derived MKs were fixed with 4% PFA at room temperature for 10 minutes and permeabilized by 0.1% Triton X-100 at room temperature for 5 minutes. The fixed cells were blocked with 1% BSA and incubated with rabbit anti-VWF 1:8000 (Dako, Cambridgeshire, UK), and mouse anti-α-tubulin 1:500 (Sigma-Aldrich, Dorset, UK) primary antibodies. Secondary antibodies were 488-phaloidin 1:500, goat anti-rabbit Alexa Fluor 488 (green) or anti-mouse Alexa Fluor 568 (red) 1:300 (Life Technologies, Paisley, UK). DNA was stained with TOPRO-3 iodide (blue) (Life Technologies, Paisley, UK). Samples were mounted with ProLong gold antifade mountant (Life Technologies, Paisley, UK). Images
were obtained with a Leica SP2 confocal microscope (Leica, Milton Keynes, UK) and were analyzed with 7.6 IMARIS Cell software for VWF-containing vesicle quantification (Bitplane, Zurich, Switzerland).

Supplemental Tables

Table S1. Spleen Immunophenotyping in Vps33b^{0/0}\text{-ER}^T^2 mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Vps33b^{0/0} (Mean ± SEM; n = 7)</th>
<th>Vps33b^{0/0}\text{-ER}^T^2 (Mean ± SEM; n = 6)</th>
</tr>
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<tbody>
<tr>
<td>CD4 (%)</td>
<td>13.5 ± 1.1</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>8.1 ± 0.8</td>
<td>7.8 ± 0.9</td>
</tr>
<tr>
<td>CD19 (%)</td>
<td>36.6 ± 1.5</td>
<td>33.8 ± 2.2</td>
</tr>
<tr>
<td>CD11b myeloid cells (%)</td>
<td>18.8 ± 1.5</td>
<td>19.9 ± 2.7</td>
</tr>
<tr>
<td>CD11c dendritic cells (%)</td>
<td>11.4 ± 1.7</td>
<td>5.61 ± 1.2*</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>0.4 ± 0.2</td>
<td>1.12 ± 0.5</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>17.8 ± 3.6</td>
<td>9.1 ± 2.0</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>1.3 ± 0.2</td>
<td>4.3 ± 0.9**</td>
</tr>
</tbody>
</table>

T cells, CD4^+^CD8^−^, CD4^+^CD8^+^; B cells, CD19^+^; Myeloid cells, CD19^+^CD3^+^CD11b^+^; Dendritic cells, CD19^+^CD3^+^CD11c^+^; Neutrophils, CD19^+^CD3^+^Ly6C^+^GR1^+^; Monocytes, CD19^+^CD3^+^Ly6C^+^GR1^+^; Macrophages, CD19^+^CD3^+^GR1^+^CD64^+. *P < .05; **P < .01.
Supplemental Figures

Figure S1. Vps33b^{fl/fl}-ER^{T2} mouse targeting strategy. (A) Vps33b^{fl/fl} mice with LoxP sites flanking Vps33b exons 2-3 were developed in order to model ARC syndrome. Subsequent removal of exons 2-3 by CreER^{T2}-recombinase results in frameshift and premature termination of transcription. (B) CreER^{T2}-recombinase mediated Vps33b excision in the Vps33b^{fl/fl} mouse induced by intraperitoneal injections of 100mg/kg/day tamoxifen on 5 consecutive days between 6-8 weeks of age, and subsequent analysis 5 weeks post-induction. (C) External appearance of Vps33b^{fl/fl} and Vps33b^{fl/fl}-ER^{T2} mice. Note the presence of macerated skin lesions in Vps33b^{fl/fl}-ER^{T2} mice.
Figure S2. Platelet Half-life. The percentage of biotin$^+$αIIbβ3$^+$ platelets in the circulation of $Vps33b^{	ext{fl/fl}}$-ER$^{T2}$ mice were quantified every other day post-injection of biotin-$N$-hydroxysuccinamide by flow cytometry (n = 6 mice/genotype per time point). Mean ± SD
Figure S3. Determination of platelet granules. (A) Wild-type population of platelets in Vps33b<sup>fl/fl</sup>-ERT<sup>2</sup> mice. 25% of Vps33b<sup>fl/fl</sup>-ERT<sup>2</sup> platelets have similar number of α-granules (2.6 ± 0.3 per platelet) to that of littermate controls (2.2 ± 0.1 per platelet). (B) Quantification of δ-granules by whole mount electron microscopy (n = 30 platelets/genotype) showed similar numbers between platelets from Vps33b<sup>fl/fl</sup> and Vps33b<sup>fl/fl</sup>-ERT<sup>2</sup> mice. (C) Representative whole mount electron micrographs of platelets from Vps33b<sup>fl/fl</sup> (top panels) and Vps33b<sup>fl/fl</sup>-ERT<sup>2</sup> (bottom panels) mice at low and high magnifications. Scale bars: left panels 2 μm, right panels 1 μm. All values are Mean ± SEM; ns = not significant.
Figure S4. Quantification of α-granules in three ARC patients. Two patients with mutations in VPS33B (1) Compound heterozygous for c.745G>T; p.G249C and c.1235_1236delinsG; p.Pro412Argfs*7 and (2) Compound heterozygous for c.1225+5G>C and c.440_449del; p.Pro147Argfs*4; and one patient containing the homozygous p.Arg270* VIPAS39 mutation. Over 70% of platelet sections (n = 70-100 platelets per sample) in all three patients were devoid of α-granules when compared to the control.
Figure S5. ATP secretion in response to PAR1 in two ARC patients. Two patients with classical features of ARC and mutations in VPS33B (homozygous for p.Trp534*) and VIPAS39 (homozygous for p.Arg270*) showed reduced ATP secretion upon PAR1 stimulation. Circles (●) represent individual people.
Figure S6. Proplatelet formation in bone marrow-derived MKs. Representative confocal images of proplatelet forming MKs after 5 days in culture stained with (A) a mouse anti-α-tubulin primary antibody and an anti-mouse Alexa Fluor 568 secondary antibody and (B) a 488-phalloidin antibody (green) and TOPRO-3 iodide (blue). Images were obtained with a Leica SP2 confocal microscope. Scale bar 20 μm.
References


