Supplemental Data

Materials and Methods

Materials

BSA, Saponin, Triton X-100, Brefeldin A, anti-γ tubulin (C7604) and anti-β tubulin 1 (T816) were from Sigma-Aldrich (Rueil-Malmaison, France). The goat anti-rabbit and anti-mouse, DAPI (4’6-diamidino-2-phenylindole, dilactate), Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, and glutamine were from Invitrogen (Cergy-Pontoise, France). Rabbit giantin antibody was from AbCam (Cambridge, UK). Anti-PRC1 (sc-8356) was from Santa-Cruz (Texas, USA). Anti-TGN38 (AHP499G) was from Serotec. Glutaraldehyde and paraformaldehyde were from Euromedex (Souffelweyersheim, France). The Lin’ Easy Step and the recombinant mTPO were from StemCell Technologies (Vancouver, BC). Mouse antibodies against mouse integrin αIIbβ3 (RAM.2-488) and glycoprotein (GP)Ibβ (RAM.1-488) were produced and labeled in our laboratory.

Megakaryocyte culture

The mouse bone marrow progenitor cells were obtained after Lin selection (Stem Cell Technologies). The Lin- cells were prepared obtained as described [1]. Briefly, mouse BM was flushed from femurs and tibias with the DMEM medium and the cells were dissociated. After Lin’ selection, the cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Stem Cell Technologies), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 ng/mL TPO and 100 U/mL hirudin. At different days of cultures (day 0 to 4), the cells were prepared for confocal microscopy. In experiments focusing on the most early developmental stages of DMS formation, fresh bone marrow samples and progenitor cells were cultured for 1 - 2 - 4 hours respectively and fixed directly after (see below).

Confocal microscopy on cultured MKs

Lin- cells were permeabilized with 0.5% Saponin in PBS for 30 min and then incubated for 30 min with the primary antibodies followed by secondary Alexa 633-conjugated goat anti-mouse or anti-rabbit antibodies. In some experiments, single labeling was performed with an
anti-GPIbβ antibody, RAM.1-488 (10 µg/mL, 30 min), followed by DAPI counterstaining of the nuclei. The slides were observed under a confocal microscope (TCS SP5, Leica Microsystems). No fluorescence was detected with isotype-specific control IgG. In some experiments, brefeldin A (1 µg/mL) or vehicle (DMSO) was added to the cultures on day 1. The cells were fixed after treatment for 4 hours and prepared for immunofluorescence as described above. The number of MKs with pre-DMS was quantified in three different MK cultures and expressed as the percentage of the total number of GPIbβ-positive cells. The DNA staining was used to estimate the cell ploidy. The fluorescence was quantified on Z-stack of whole MKs (z-size 0.25 µm) using the Amira software. Six cells were included for each DMS stage and the results were expressed as a mean±SEM.

**Electron microscopy**

*Standard electron microscopy*

BM obtained by flushing femora with 0.1 M sodium cacodylate buffer were fixed in 2.5% glutaraldehyde and embedded in Epon as described [3]. Thin sections were stained with uranyl acetate and lead citrate and examined under a CM120 transmission electron microscope (FEI, The Netherlands). In some experiments, small BM blocks (1mm³) were fixed for 1 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were then incubated at room temperature with 1% tannic acid and processed for transmission electron microscopy. When stated in the text, serial thin sectioning has been performed to obtain a 3D map of the pre-DMS.

*Immunoelectron Microscopy*

Fresh BM was fixed with 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer for 1 h. The fixed samples were infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Ultrathin cryosections of ~70 nm were obtained at −110°C with a LEICA Ultracut UCT cryo-ultramicrotome (Leica Microsystems). Thin sections were rinsed in PBS containing 150 mM glycine (15min), PBS, PBS containing 0.1% acetylated BSA (BSAc, Aurion, 30 min), followed by 1 h incubation with primary antibody anti-GPIbβ or anti-TGN38 at 10 µg/ml in PBS /0.1% BSA or a corresponding non-immune antibody, 1 h incubation with
protein-A coupled with 10 nm colloidal gold particles (PAG) (Cell Microscopy Center, University Medical Center Utrecht, the Netherlands), post-fixation in 1% glutaraldehyde and embedding in 1.8% uranyl acetate/0.2% methylcellulose.

**Dual axis electron tomography**

About 300 nm thick sections were stained with uranyl acetate and lead citrate. Dual-axis tilt series of selected MKs were recorded using a Tecnai 20 transmission electron microscope (FEI) equipped with a slow scan CCD camera (charge-coupled-device camera, Temcam F214; TVIPS, Gauting, Germany) as previously described [3]. Tilt series were acquired with a single-axis high-tilt tomography holder (model 670; Gatan, Munich, Germany) or a Fischione tomography holder (model 2020; Fischione Instruments, K-Vision BV, Huizen, The Netherlands). After a first series of tilts, the specimens were manually rotated over an angle of 90° and a second series was acquired. Subsequently, 1000×1000 pixel images were recorded automatically with Xplore 3D software (FEI) using an angular range of −60° to +60° with a 1° increment. The tilt series were aligned with IMOD (Boulder Laboratory, Colorado) [4-5] using the fiducial markers and combined into one 3D volume. Firstly, aligned single-axis tomograms were computed by resolution-weighted back-projection. These two volumes were then combined into one final volume. After reconstruction, each tomogram was unpacked into tiff format and adjacent tomograms were manually stitched together using Adobe Photoshop CS3. The tiff files were stacked back into a 3D volume (one per section) using IMOD.

**FIB/SEM**

The principle of this approach is that epon-embedded samples are serially sectioned using a focused ion beam (FIB) integrated into a scanning electron microscope (SEM) and the milled face is then imaged with the electron beam at high resolution. Iteration of these two “milling-imaging” steps generates a series of images which describe the 3D structure of the sample at regularly spaced intervals and can be computationally assembled into a volume representing a reconstruction of the sample. The specific advantage of this technique is that large cell volumes (up to 100×100×100 µm) can be investigated. For FIB/SEM, samples were prepared as described previously [6]. Briefly, BM cells were processed as for TEM except that a contrast-enhancing step, consisting of incubating the cells in 1.5% potassium ferrocyanide and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, was added directly after the fixation.
The blocks were mounted on SEM stubs, coated with platinum/palladium and examined under a Helios NanoLab dual beam microscope (FEI). Samples were milled with the FIB (30 kV, 700 pA) at a thickness of 20 nm per section and images were acquired in backscattering mode (3 kV, 1 nA). Tomograms and 3D models were computed using Amira software.

**Confocal microscopy on BM tissue**

Confocal analysis of BM tissue was performed according to an adaptation of the method described by Takaku T [7]. Briefly, 1 mm thick transversal sections from 4% paraformaldehyde (PFA) fixed femurs were incubated with Alexa 555-conjugated anti-GPIbβ antibody (RAM.1-555; 10 µg/ml, overnight) followed by DAPI counterstaining (1/20000, overnight). The fluorescently labeled BM tissue was placed cut-face down onto a 8 well µ-slide (IBIDI). The BM tissue beneath the bone edge was located and positioned using differential interference contrast (DIC) illumination. Series of x-y-z images of typically 455.9x455.9 µm x-y size were collected along the z-axis at 0.5 µm step size through 100 µm of BM tissue, using a HCX PL APO lambda blue 20.0x/0.70 IMM objective and resonant scanner from a Leica SP5 confocal microscope. For quantitative analysis of MK number having a pre-DMS, BM specimens from 3 mice, each representing a typical volume of 456 x 456 x 100 µm³ was examined.

Figure S1: alphaIIbbeta3 immunostaining in cultured megakaryocytes. (A) DMS expansion in cultured MKs. Confocal images from MK cultures fixed after 1-4 days of culture. Cells were stained with anti-GPIbbeta (green) and DAPI (blue) and representative developmental stages of the DMS are shown. (Right panel) Intermediate DMS, (Middle panel) late DMS and (Left panel) MKs forming pro-platelets. Bars, 10 µm. (B) alphaIIbbeta3 immunostaining in cultured Mks. Confocal images from MK cultures fixed after respectively 4h and 4 days culture. Cells were stained with anti-alphaIIbbeta3 (green) and DAPI (blue). Representative developmental stages of the DMS are shown. (a) Distinct alphaIIbbeta3-positive territory is localized in the center of the cell, close to the nucleus (arrow). (b) alphaIIbbeta3-positive pre-DMS with multiple tubular connections with the cell surface (arrows). (c) Intermediate DMS, late stage DMS (d) and (e) MK forming pro-platelets. Bars, 10 µm.

Figure S2: Pulse-chase kinetics of anti-GPIbβ in cultured MKs in presence (B) or not (A) of cycloheximide (100µg/ml). Lin- BM cells were pulse-labeled for 15 min with Alexa488-conjugated anti-GPIb (green), washed, and subsequently fixed after 5 min, 1, 2, and 4 hours respectively. The images show maximal projections of whole cells. Bars, 10 µm.

Figure S3: Correlative light-electron microscopy of a cell displaying a surface GPIb cap. Cells were pulse-labeled for 1h using an anti-GPIb antibody, fixed and allowed to settle on pre-patterned Aclar supports. (A-C) Light microscopy: A cell showing a GPIb-positive cell surface patch was located by bright field microscopy (arrow in A) and by fluorescence microscope (box in B) with the help of the “L1 pattern” and of two adjacent GPIb-positive cells (green and red arrows in B). Higher magnification of the cell is shown using full Z-stack confocal microscopy (box in C). (D-F) Correlative EM images showing the same cell (boxed in C) at low magnification. Neighboring MKs are indicated by red and green arrowheads and the still visible pattern is shown by red puncta. (G-H) Higher magnification electron micrographs of the boxed areas in F and G. Shown is the first section of a series of 26 sections. No intracellular pre-DMS was detected in this section (H) and the others (not shown).

Figure S4: Ultrastructural characterization of the pre-DMS in bone marrow MKs. (A-D) TEM pictures showing the initial stage of DMS formation. (A) In situ stage I MK showing a round membrane network located between the lobulated nucleus. Bar, 2 µm (B) Higher
magnification of the boxed area in A. Bar, 500 nm (C) IEM with the anti-GPIb-beta showing that the membrane network is positive for GPIb-beta indicating that these membranes correspond to the early stages of DMS formation. Bar: 500 nm (D) Tannic acid staining reveals that the pre-DMS is connected to the cell surface. Bar: 500 nm. The arrow indicates the pre-DMS. n: nucleus, pm: plasma membrane.

**Figure S5: Contribution of the Golgi-derived vesicular transport to DMS biogenesis.** (A) IEM experiments showing an overview of the TGN38-positive area (arrowheads) in close position to the pre-DMS. Bar, 1 µm (B) High magnification example showing small TGN38-positive vesicles (arrowheads). Bar, 200 nm

**Figure S6: The intracellular GPIb-positive membranes represent the Golgi and TGN-derived membrane vesicles.** Cells displaying a GPIb-positive cell surface patch were analyzed by IF and IEM. Maximum intensity projections of the Z stacks showing a faint intracellular GPIb staining in the perinuclear region (inset). Immunogold labeling demonstrates that this represents Golgi stacks (g) and TGN-derived vesicles (tgn). Bar: 200 nm.

**Figure S7: Large volume 3D characterization of the completed DMS using FIB/SEM.** At late stages of MK development, the DMS expands throughout the cytoplasm forming numerous branching points (arrow). DMS membranes are tubular with alternating small neck regions and it is continuous with the cell surface at multiple sites (arrowheads).
Supplemental Figure S3