Supporting Information

Hematopoietic stem cell differentiation promotes the release of prominin-1/CD133–containing membrane vesicles – A role of the endocytic-exocytic pathway

Supporting Information – MATERIALS AND METHODS

Primary cells and cultivation

Primary HSPCs and MSCs were collected from healthy donors after informed consent and approval of the local ethics committee. Isolation of MSCs was performed as described (Freund et al, 2006). Mobilized peripheral blood was obtained by subcutaneous injection of 7.5 μg/kg granulocyte colony-stimulating factor per day (Granocyte, Chugai Pharma, Frankfurt, Germany) for 5 days. CD34+ MACS-immuno-isolated HSPCs from leukapheresis products (Freund et al, 2006) were cultured on MSCs growing on either fibronectin-coated 100-mm Petri dishes (12 x 10^4 HSPCs in 12 ml medium) or fibronectin-coated 12-mm coverslips (1 x 10^4 HSPCs in 1 ml medium) or Transwell filters (0.4 μm pore size; Corning) (4 x 10^4 HSPCs in 2.5 ml medium) in serum-free medium supplemented with early-acting cytokines (50 ng/ml stem cell factor, 50 ng/ml fetal liver tyrosine kinase-3 ligand, 15 ng/ml interleukin-3) (Freund et al, 2006). Unless otherwise stated, the HSPCs/MSCs were incubated at 37°C in a humidified 5% CO_2 atmosphere for 7 days before use. For the PMA treatment, HSPCs pre-cultured for 2 days on either MSCs or directly on fibronectin-coated 6-well plates (3 x 10^5 HSPCs in 3 ml medium) were incubated in medium containing 160 nM of PMA for 3 additional days. Alternatively, HSPCs were pre-cultured for 4 days before 1 day of PMA treatment.

Differential centrifugation

Conditioned HSPC/MSC medium was subjected to differential centrifugation as follows (all steps at 4ºC): 5 min at 300 x *g*; supernatant, 20 min at 1,200 x *g*; supernatant, 30 min at 10,000 x *g*; supernatant, 1 hour at 200,000 x *g*; supernatant, 1 hour at 400,000 x *g*. The

resulting pellets were resuspended in Laemmli buffer and analyzed by immunoblotting. An aliquot ($1/10^{th}$) of the 400,000 x *g* supernatant was analyzed in parallel.

Sucrose gradient centrifugation

The 200,000 x *g* pellet was resuspended in 30 μl of PBS plus 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and protease inhibitors (Complete, Roche Diagnostics), placed on top of a equilibrium sucrose gradient (0.1-1.2 M) and centrifuged at 65,000*g* for 5 hours, as described previously (Huttner et al, 1983). After centrifugation, 500 μl fractions were collected from the top to the bottom of the gradient using a pipette whereas the pellet was resuspended in 500 μl buffer A (150 mM NaCl, 2 mM EGTA, 50 mM Tris-HCl pH 7.5, 10 $\mu g\ ml^{-1}$ aprotinin, 2 $\mu g\ ml^{-1}$ leupeptin and 1 mM PMSF) containing 1% Triton X-100. 450 μl aliquots of each fraction were concentrated using methanol/chloroform (2:1) precipitation and analyzed by immunoblotting. The remaining 50-μl aliquot of each fraction was used to determine the sucrose concentration by measuring the refractive index.

Detergent lysis

HSPCs and the 200,000 x *g* pellet recovered after differential centrifugation were lysed for 30 min on ice in 60 μl or 30 μl, respectively, of ice-cold buffer A containing either 0.5% Triton X-100 or Lubrol WX (Lubrol 17A17, Serva). Detergent lysates were centrifuged at 4°C for 1 hour at 100,000 x *g*. The entire supernatant and pellet were analyzed by SDS-PAGE followed by immunoblotting.

Immuno-isolation of prominin-1–containing membrane vesicles

The immuno-isolation of prominin-1–CMV was performed at 4°C using immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The conditioned medium collected from either HSPCs growing on MSCs (24 ml) or, as control, MSCs alone (12 ml) was centrifuged at 1,200 x *g* for 20 min to remove cells. Supernanants were concentrated to 400 μl by Centricon PL-30 (Millipore) and 400 μl of ice-cold PBS were added prior centrifugation at 10,000 x *g* for 30 min. The 10,000 x *g* supernatants were pre-incubated with 150 μl of goat-anti-mouse IgG-magnetic-beads

(Miltenyi Biotec; Cat. Num. 130-048-401) per 12 ml of initial starting material for 1.5 hours at 4°C end-over-end. The supernatants were then applied to ice-cold PBS conditioned MS-columns (Miltenyi Biotec) placed into a magnetic field, and the flow-through material was collected. Half of the HSPC/MSC medium fraction and the control fraction were then incubated each with 50 μl of mouse mAb CD133-magnetic-beads (Miltenyi Biotec; Cat. Num. 130-050-801). The other half of HSPC/MSC medium fraction was incubated with 150 μl of goat anti-mouse IgG-magnetic-beads as an additional control. After 1 hour incubation at 4°C end-over-end, samples were subjected to a magnetic separation. The MS-columns were washed 3 times with 3 ml ice-cold PBS. Vesicles retained in the MS-columns were eluted by taking out the column from the magnet, and flushing with 100 μl PBS. Laemmli buffer (4x) was added and the samples were stored at –20°C. The recovered flow-through fractions were centrifuged at 200,000 x *g* for 1 hour to obtain the unbound materials. The pellets of the 200,000 x *g* centrifugation were resuspended in Laemmli buffer (1x) and stored at –20°C.

SDS-PAGE and immunoblotting

Proteins were analyzed by SDS-PAGE (7.5 or 10%) and transferred to poly(vinylidene difluoride) membranes (Millipore Corp., Belford, MA; pore size 0.45 μm) using standard procedures (Corbeil et al, 2001). Immunoblotting was performed using as primary antibody either mouse mAb 80B258 (1 μg/ml) directed against human prominin-1 (Karbanová et al, 2008), mouse mAb CD34 (2 μg/ml; BD Biosciences), mouse mAb anti-flotillin-1 (1 μg/ml; BD Biosciences), mouse mAb anti-flotillin-2 (0.05 μg/ml; BD Biosciences), mouse mAb actin (0.3 μg/ml; clone AC-40, Sigma, Dartmouth, Germany), rat mAb α-tubulin (1:500; clone YOL1/34, Serotec, Oxford, UK) or rabbit antiserum directed against syntenin (1 μg/ml; Abcam, Cambridge, UK) followed by appropriate horseradish peroxidase-conjugated secondary antibody. Antigen-antibody complexes were detected using enhanced chemiluminescence (ECL system, Amersham Biosciences) and quantified after scanning the Hyperfilm (Amersham) using the MacBas or ImageJ software.

Radioactive labelling, lipid extraction and thin layer chromatography

6-days-old HSPCs cultured on MSCs plated on Petri dishes were collected and centrifuged at 1,200 x *g* for 5 min. Recovered cells were resuspended and replated onto the same MSCs in fresh HSPC medium containing 10 μCi C^{14} / 100-mm Petri dish. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for 3 additional days.

Lipids from hematopoietic cells and immunomagnetic-isolated prominin-1–CMV (see above) were extracted as follows: cells and the prominin-1–containing vesicle fractions were each put into 2 ml of MeOH/$CHCl_3$ (2:1), mixed, and centrifuged for 5 min at 3,200 x *g*. The supernatants were transferred to a fresh tube, to which 0.5 ml of 20 mM acetic acid and 0.5 ml of $CHCl_3$ were added, mixed and centrifuged again for 2 min at 3,200 x *g*. The lower phase was transferred to another tube, while 25 μl of 1 M citric acid and 1 ml of $CHCl_3$ were added to the upper phase, mixed and centrifuged for 2 min at 3,200 x *g*. The lower phase was transferred to the same tube as the previous lower phase and the $CHCl_3$ was evaporated under a stream of nitrogen.

Dried lipids were dissolved in MeOH/$CHCl_3$ (1:2) and applied to silica TLC plates (Merck) using a capillary. To separate the lipids, the plates were run in chloroform/ethanol/water/triethylamine (35/50/10/35) until 2/3 of the distance, then the plates were dried and subsequently placed and run in iso-hexane/ethylacetate (5:1) for the full distance and dried again. TLC plates were exposed to image plates (Fuji), which were analyzed by the BAS 1800 II phosphoimager after 14 days. Bands were identified by comparison with lipid standards and quantified using ImageGauge V4.23.

Immunofluorescence and confocal microscopy

One-week-old co-cultured HSPCs/MSCs growing on fibronectin-coated coverslips were gently washed with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min, quenched with 50 mM NH_4Cl for 10 min and then permeabilized with 0.2% saponin in blocking buffer (PBS containing 2% fetal calf serum (FCS)) for 30 min. Cells were sequentially incubated for 30 min with mouse mAb CD133/1 (1 μg/ml; anti-prominin-1; Miltenyi Biotec) at room temperature (RT) followed by 1 hour incubation with Cy™3-conjugated AffiniPure donkey-α-mouse IgG (H+L) Fab fragment (1:20; Jackson ImmunoResearch Laboratories) at 4°C. All reagents were diluted in blocking buffer containing 0.2%

saponin. Remaining mouse epitopes were saturated by incubation with unconjugated AffiniPure rabbit-α-mouse IgG (H+L) Fab fragment (1:10; Jackson ImmunoResearch) overnight at 4°C. After a post-fixation step with 0.2% PFA (10 min) and quenching (NH_4Cl, 10 min), cells were incubated for 30 min with mouse mAb MX-49.129.5 (0.6 μg/ml; anti-CD63; Santa Cruz Biotechnology) followed by Cy™2- conjugated goat-α-mouse antibody IgG (H+L) (Jackson ImmunoResearch) at RT.

In the case of cell surface versus intracellular labelling, cells were first cell surface labelled with mAb CD133/1 (1 μg/ml) in the cold prior to 4% PFA fixation, as described previously (Corbeil et al, 1999), followed by the saturation of remaining mouse epitopes with unconjugated AffiniPure rabbit-α-mouse IgG (H+L) Fab fragment. Afterwards, cells were post-PFA-fixed, saponin-permeabilized, and finally labelled with mAb CD133/1 as described above for anti-CD63. As controls, no signal was observed when either anti-CD133 and anti-CD63 primary antibodies were omitted or only the CD133 immunoreactivity was detected when the second primary antibody was omitted, indicating that the first mouse primary antibody was fully saturated (data not shown).

When primary antibodies were derived from different species, fixed, saponin-permeabilized cells were sequentially incubated for 30 min at RT with mouse mAb CD133/1 (1 μg/ml) alone or with either sheep antiserum against TGN46 (1:25; Serotec) or rabbit antiserum against GM130 (1:150; Abcam) or rabbit antiserum against SARA (1:100; clone H-300, sc-9135, Santa Cruz Biotechnology) or rabbit antiserum against EEA1 (1:300: a generous gift from Marino Zerial, MPI-CBG) followed by appropriate secondary antibodies; Cy™2/3-conjugated goat anti-mouse IgG (H+L) alone or with either Cy™2-conjugated donkey anti-sheep IgG (H+L) or Cy™2/3-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch), all in blocking buffer. Alternatively, CD133-labelled cells were co-stained with BODIPY 493/503 (Invitrogen Molecular Probes, USA).

In all cases, nuclei were labelled with either Hoechst 33258 (Invitrogen, Karlsruhe, Germany) or 4,6-diamidino-2-phenylindole (DAPI; 1 µg/ml; Molecular Probes). The cells were mounted in Mowiol 4.88. Images were captured using a Leica SP5 upright confocal microscope or a Zeiss LSM 510 META. Settings were such that photomultipliers were within their linear range and the same optical section thickness was

set for all channels. Individual sections (0.5/1.0 μm interval) or a composite of 10/16 optical sections are shown. The images shown were prepared from the confocal data files using Adobe Photoshop software. Ratio of intracellular versus surface immunofluorescence of prominin-1 was calculated from total image pixel intensity using CellProfiler 2.0 software (Broad Institute, Cambridge, MA).

Immunoelectron microscopy

Membrane vesicles – The 200,000 x *g* pellet obtained after differential centrifugation (see above) of 12 ml conditioned HSPC/MSC medium was resuspended with 1 ml PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and protease inhibitors (Complete, Roche Diagnostics) and re-centrifuged at 200,000 x *g*. The resulting pellet was resuspended in 40 μl of 4% PFA in phosphate buffer. The suspension was applied for 2 min onto 400-mesh grids with formvar- and carbon-coating prior to washing with PBS. The samples were then blocked for 5 min with 0.1% glycin in PBS and twice for 10 min with 0.2% gelatine, 0.5% bovine serum albumin in PBS (PBG). The grids were incubated for 1 hour at RT with either mAb AC141 (0.46 mg/ml, Miltenyi Biotec) directed against human prominin-1 (Yin et al, 1997) or mAb PeliCluster CD63 (1:15, Sanquin) and after washing with PBG, samples were incubated for 1 hour with goat anti-mouse IgG coupled to 10-nm gold (British Biocell). After subsequent washing steps with PBG and PBS the grids were post-fixed for 5 min in 2% glutaraldehyde (GA) in PBS. The samples were negatively contrasted with 0.15% uranyl acetate in 1.9% methylcellulose and viewed in a Morgagni electron microscope (FEI Company). Micrographs were taken with a MegaviewII camera and AnalySIS software (Soft Imaging Systems).

HSPCs – HSPC/MSC co-cultures grown for 5 days on Transwell filters were gently washed with PBS and fixed with 4% PFA in PBS for 15 min at RT. Filters were then rinsed with PBS and incubated for 10 min in 50 mM NH_4Cl. The fixed cells were permeabilized and blocked with 0.2% saponin, 2% FCS in PBS (blocking solution) for 15 min at RT. Cells were incubated for 30 min at RT with mAb CD133/1 (1 μg/ml) followed by goat anti-mouse secondary antibody coupled to ultra-small gold (Aurion, Wageningen, The Netherlands) in blocking solution. Filters were rinsed sequentially with blocking

solution and PBS followed by fixation in 2% GA. The samples were rinsed in water and processed for silver enhancement using R-GENT SE-EM silver enhancement kit (Aurion) for 90 min at RT prior to post-fixation in 1% osmium tetroxide for 15 min at RT. Dehydration was performed by a graded series of ethanol. Samples were infiltrated in EMBed resin (Science Services). 70-nm ultrathin sections were cut on a UCT ultra-microtome (Leica Microsystems) and post-stained with uranyl acetate and lead citrate. For the CD63 labelling, ultrathin cryosections were prepared as described (Dubreuil et al, 2007), and immunolabelled with mAb PeliCluster CD63 (1:15).

Flow cytometry

HSPCs co-cultured on MSCs on fibronectin-coated Petri dishes for either 4, 9 or 14 days were recovered by centrifugation at 300 x *g* for 5 min and double-labelled with mouse mAb CD133/1-PE (Miltenyi Biotec) and mAb CD45-FITC (BD Biosciences). [Note that all recovered cells were $CD45^+$ indicating their hematopoietic origin]. Alternatively, HSPCs derived from a one-week-old co-culture were triple-labelled with mouse mAb CD133/1-APC, mAb CD42b-PE (BioLegend, San Diego, CA., USA) and mAb CD61-FITC (BD Biosciences). For the PMA-treated HSPCs and the corresponding controls, samples were triple-labelled with a combination of the following mAb CD133/1-PE or APC, mAb CD3-APC (clone UCHT-1), mAb CD11c-APC (clone B-ly-6), mAb CD14-pacific blue (clone TüK4), mAb CD15-FITC (clone HI98), mAb CD19-FITC (clone HIB19), mAb CD34-APC (clone 581), mAb CD41a-APC (clone HIP8), mAb CD61-FITC (clone VI-PL2), mAb CD235a-PE (clone GA-R2 HIR2; all from BD Biosciences except mAb CD133/1). In case of co-culture experiments, MSCs were excluded from analysis based on CD140a expression using mAb CD140a-PE (clone 16A1; BioLegend, San Diego, CA). 100 μl of the cell suspension (2 x 10^5 cells) were incubated with 10 μl of antibodies for 30 min at RT. Samples were analyzed by acquiring 10,000 events on a FACS LSR II (BD Biosciences). Instrument settings and gating strategies were established using appropriate controls, and data were analyzed using either DIVA software (BD Biosciences) or FlowJo software (TreeStar, Ashland, USA).

Double labelling of HSPCs for annexin V and prominin-1

HSPCs co-cultured on MSCs on fibronectin-coated Petri dishes for either 9 or 14 days were recovered by centrifugation at 300 x *g* for 5 min and resuspended in 100 μl Annexin V Binding Buffer (AVBB; BD Bioscience) containing 2% FCS. Cells were incubated in the dark with annexin V-APC (1:25; BD Bioscience) for 30 min at RT. Cells were washed twice in AVBB, fixed with 4% PFA for 30 min, quenched with 50 mM NH_4Cl for 10 min and blocked with blocking buffer (see above) for 30 min. Cells were labelled with mAb CD133/1 (1:50) for 30 min at RT followed by incubation with Cy™3-conjugated goat anti-mouse IgG (H+L). Both antibodies diluted in blocking buffer. Nuclei were visualized with Hoechst 33258. Cells were washed twice with PBS, once with H_2O and mounted in Mowiol 4.88. Samples were observed on a Zeiss LSM 510 Meta microscope. The numbers of annexin V–positive cells, CD133–positive and double–positive cells were scored.

Internalization of prominin-1–containing membrane vesicles

The materials recovered in 200,000 x *g* fraction were resuspended in 300 μl of PBS, and incubated with 1,0 μM 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Vybrant Cell-Labeling Solution, Molecular Probes) for 15 min at 37ºC. Afterward, DiO-labelled vesicles were immuno-isolated based on prominin-1 as described above. Isolated DiO-labelled prominin-1–CMV were then incubated with co-cultured HSPCs/MSCs growing on fibronectin-coated coverslips either for 1 or 6 hours. Samples were processed for immunofluorescence for SARA. Cells were observed using a Leica SP5 upright confocal microscope.

Supporting Information – References

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Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90**:** 5002-5012

Supporting Information – FIGURE S1-S6

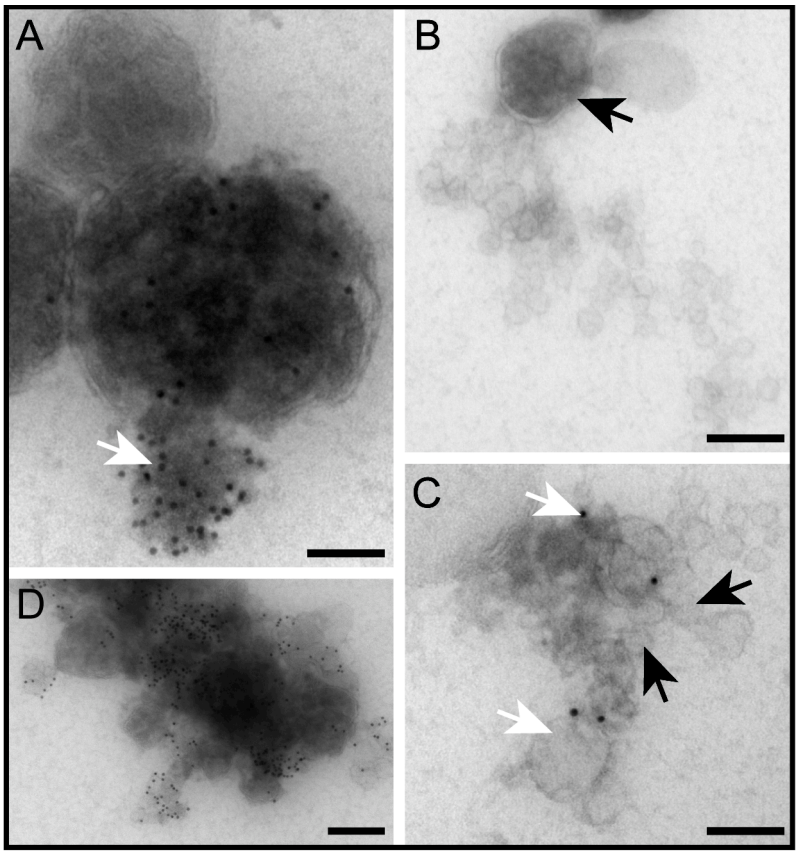

Figure S1. Extracellular membrane vesicles are not all prominin-1–positive.

A, B, C, D. One-week-old conditioned co-culture HSPC/MSC medium was subjected to differential centrifugation for 5 min at 300 x *g*, 20 min at 1,200 x *g*, 30 min at 10,000 x *g* and 60 min at 200,000 x *g*. The materials recovered in the 200,000 x *g* pellet were processed for prominin-1 (A-C) or CD63 (D) immunogold labelling followed by negative staining EM analysis. White and black arrows indicate the prominin-1–positive and –negative membrane particles, respectively.

Scale bars, 100 nm [(A), (B), (C), (D)].

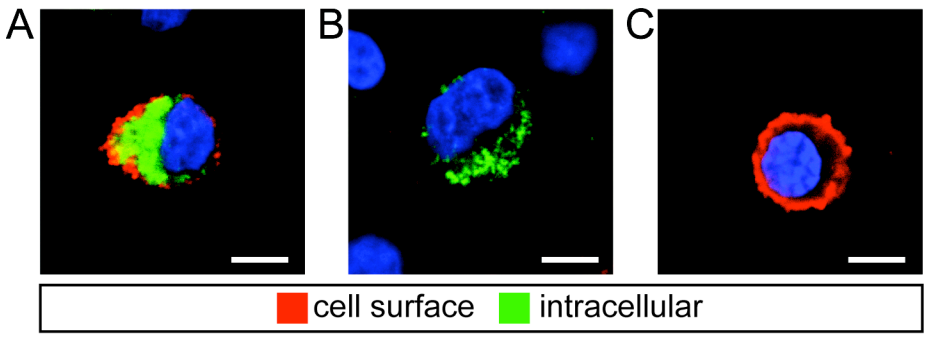

Figure S2. Rare cells exhibited solely an intracellular or cell surface staining of prominin-1.

A, B, C. HSPCs growing for one week on MSCs were first cell surface-labelled for prominin-1 prior to PFA-fixation and saponin-permeabilization followed by a second round of prominin-1 labelling. The nuclei were stained with DAPI (blue). The labelled cells were analyzed using confocal laser-scanning microscopy. Single optical x-y-plane sections are shown. In addition to the dual labelling of prominin-1 (A, cell surface, red; intracellular, green), some rare cells (<1% of prominin-1–positive cells) exhibited only an intracellular (B, green) or cell surface (C, red) staining.

Scale bars, 5 μm [(A), (B), (C)].

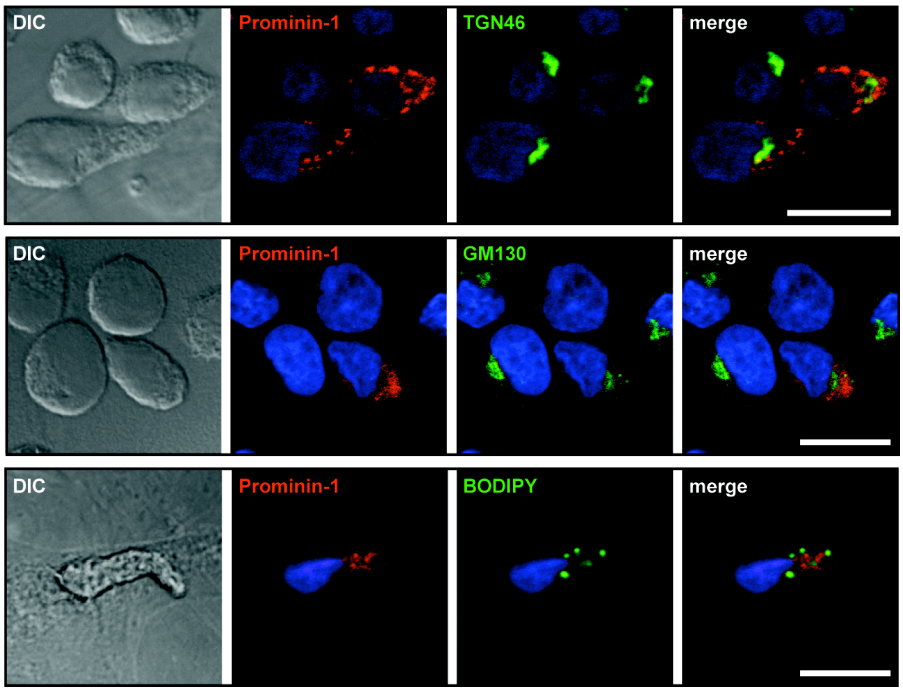

Figure S3. Intracellular prominin-1 is concentrated neither in the cis-Golgi apparatus, TGN nor lipid droplets.

PFA-fixed, saponin-permeabilized HSPCs growing on MSCs were double-immunolabelled for prominin-1 (red) with either GM130 or TGN46 (both in green) followed by appropriate secondary antibodies. Lipid droplets and nuclei were visualized by BODIPY (green) and Hoechst (blue) staining, respectively. Labelled cells were analyzed using a confocal laser-scanning microscope. Single optical x-y-plane confocal sections revealed that prominin-1 is not concentrated in either the cis-Golgi apparatus (GM130), the TGN (TGN46) or lipid droplets (BODIPY). DIC, differential interference contrast.

Scale bars, 10 μm.

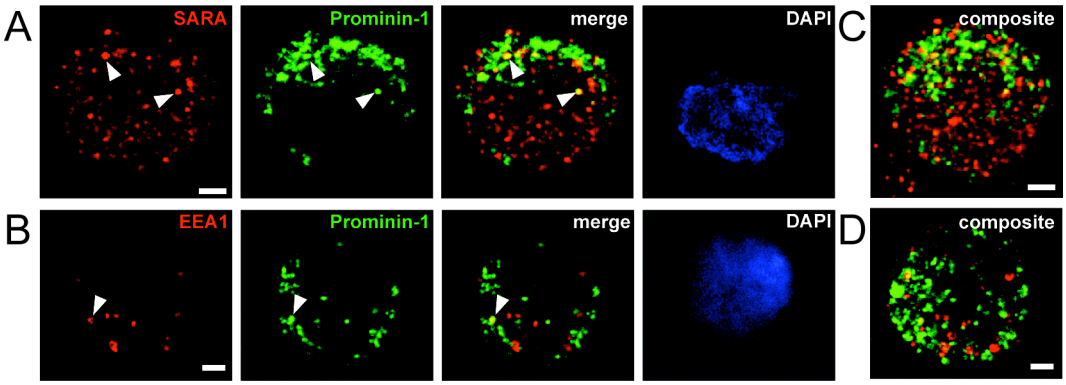

Figure S4. Partial localization of prominin-1 in signaling and early endosomal compartments.

A, B, C, D. PFA-fixed, saponin-permeabilized HSPCs growing on MSCs were double-immunolabelled for prominin-1 (green) with either Smad anchor for receptor activation protein (SARA; A, C) or early endosomal antigen 1 (EEA1; B, D; both in red) followed by appropriate secondary antibodies. Nuclei were visualized by DAPI (blue). The labelled cells were analyzed using confocal laser-scanning microscopy. A single optical x-y-plane section at the middle of the cell (A, B) or a composite of 10 optical sections (C, D) is shown. Arrowheads, double–immunoreactive structures.

Scale bars, 2 μm [(A), (B), (C), (D)].

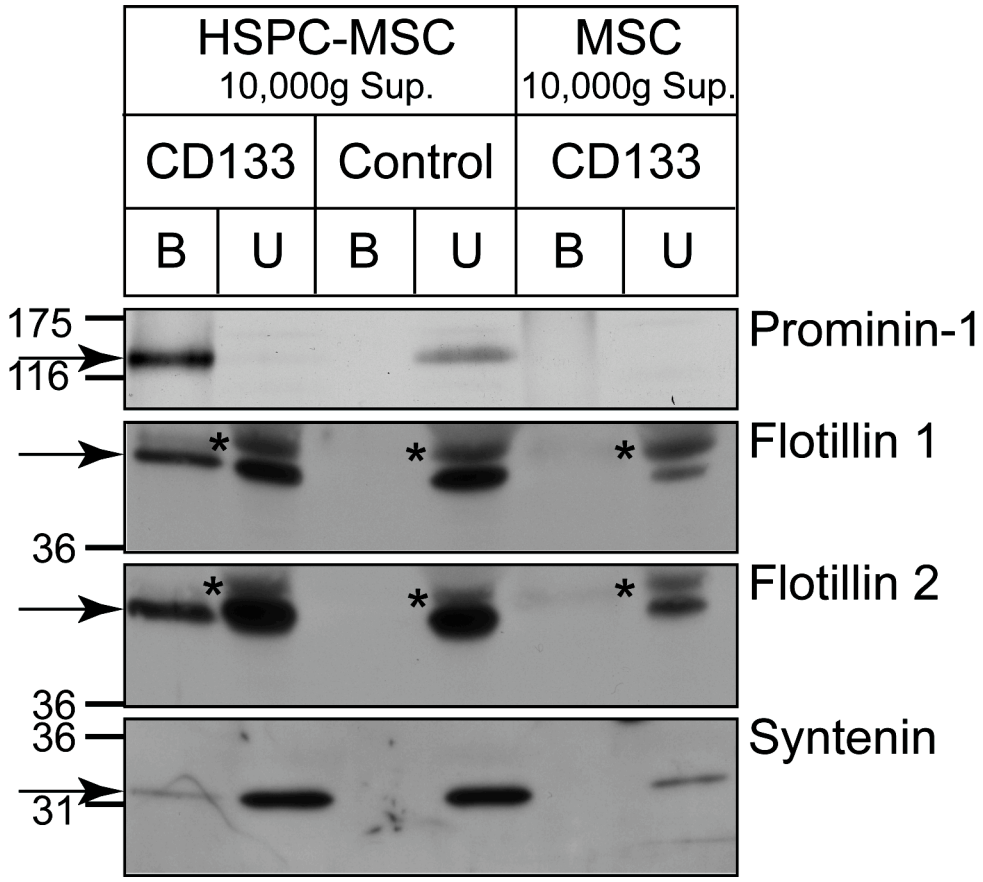

Figure S5. Immuno-isolated prominin-1–containing membrane vesicles contain exosomal protein markers.

The conditioned medium collected from either HSPCs growing on MSCs (HSPC-MSC) or MSCs alone (MSC) was centrifuged at 10,000 x *g* for 30 min, and the resulting supernatants were subjected to immuno-isolation using mouse mAb anti-human CD133 coupled to paramagnetic-beads (CD133) or, as a negative control, goat anti-mouse IgG-conjugated paramagnetic beads (Control). Bound (B) and unbound (U) fractions were analyzed by immunoblotting for prominin-1, flotillin 1, flotillin 2 and syntenin. Arrows, immunoreactive bands of interest; asterisks, unspecific bands observed without the primary antibody.

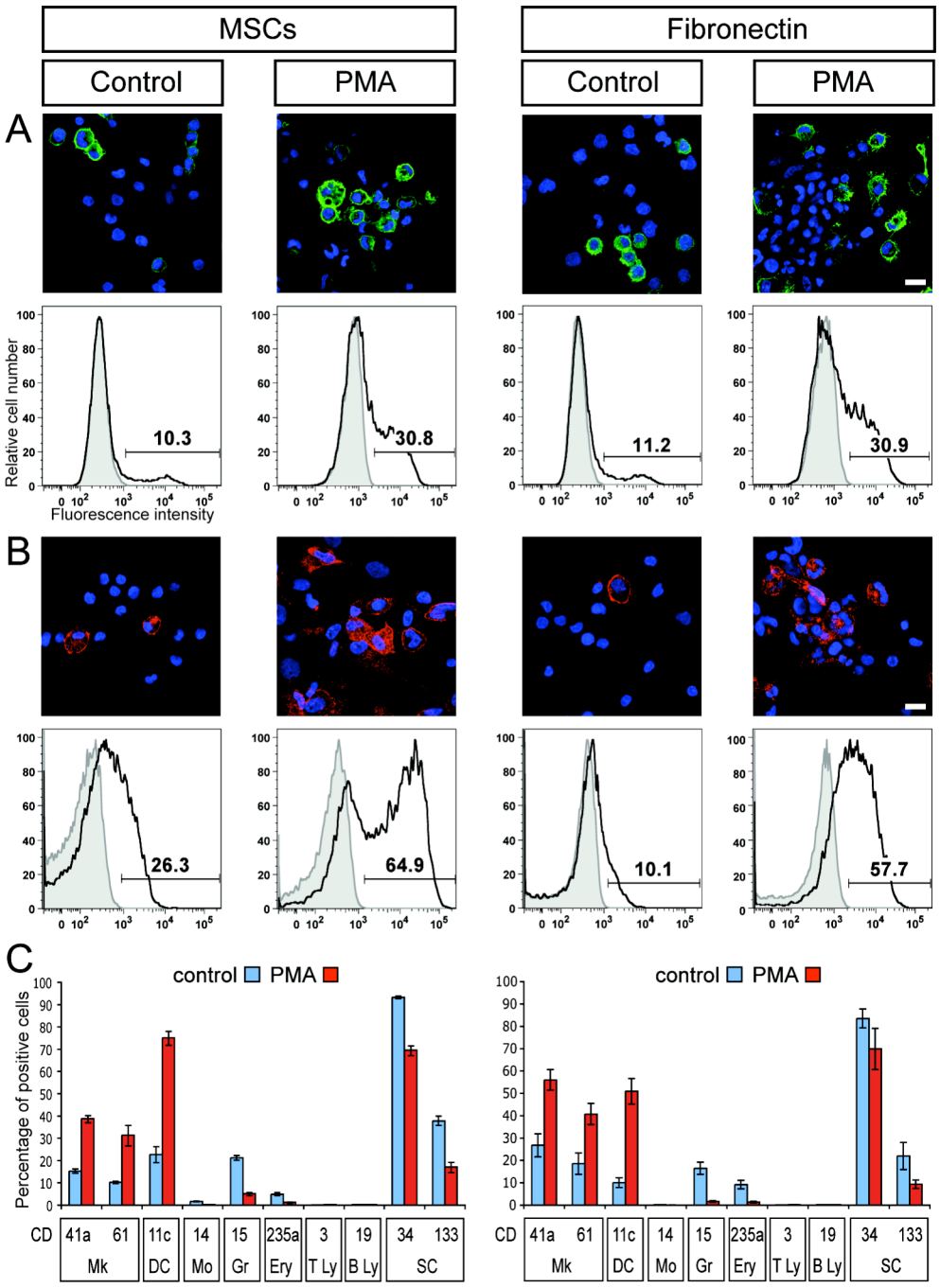

Figure S6. Phenotypic characterization of PMA–treated hematopoietic cells.

A, B, C. HSPCs pre-cultured either on MSCs or fibronectin-coated dishes for 2 days were incubated in the absence (Control) or presence of phorbol ester PMA (PMA). After 3 days, cells were analyzed either by confocal laser-scanning microscopy (A, B, top panels) or flow cytometry (A, B, bottom panels; C) using as marker either CD61 (A, C),

CD11c (B, C) or other as indicated (C). For the confocal microscopy, cells were PFA-fixed, and saponin-permeabilized prior the labelling. The nuclei were stained with DAPI (blue). Single optical x-y-plane sections are shown. For the flow cytometry analysis, the antigen expression (open areas) and appropriate isotype-matching control (grey filled areas) are depicted. The number of positive cells is indicated above the bar (A, B). Data obtained from five independent experiments are shown. CD markers used are characteristic for megakaryocytes (Mk), dendritic cells (DC), monocytes (Mo), granulocytes (Gr), erythrocytes (Ery), lymphocytes (Ly) and stem cells (SC). Note that upon PMA treatment, the expression of lineage specific markers for megakaryocytic and dendritic cells was increased whereas stem cell markers, specifically prominin-1 (CD133) decreased. Under these experimental conditions, cells were negative for lymphoid lineage markers.

Scale bars, 10 μm [(A), (B)].