SUPPLEMENTAL METHODS, TABLES AND FIGURES

The VPS33B binding protein VPS16B is required in megakaryocyte and platelet α-granule biogenesis

Running title: VPS16B required for platelet α-granule biogenesis

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Supplemental Methods

Mass spectrometry analysis

Human VPS33B was cloned into the p3xFLAG-CMV-14 expression vector (Sigma-Aldrich, Oakville, ON, Canada). Forward and reverse primers were designed to include the native start codon, with added restriction sites (EcoRI at 5' end and KpnI at the 3' end), allowing ligation into the p3xFLAG-CMV-14 vector. The construct was used to transfect HEK293 cells with Lipofectamine 2000 (Invitrogen**,** Life Technologies Inc., Burlington, ON, Canada**).** Stably transfected cells expressing VPS33B-3xFLAG fusion protein were generated by selection for neomycin resistance using 2.5 mg/mL G418. G418 resistant colonies were isolated and selected based on VPS33B-3xFLAG expression levels. HEK293 cells stably expressing VPS33B-3xFLAG fusion protein were lysed and pre-cleared with mouse IgG-Agarose (Sigma-Aldrich), followed by immunoprecipitation as per manufacturers recommendation for the anti-FLAG M2 affinity gel (Sigma-Aldrich). Protein elution was performed by competition with 3xFLAG peptide (Sigma-Aldrich), and subjected to mass spectrometry analysis at the Hospital for Sick Children mass-spectrometry facility. Results were visualized using Scaffold software (version 3.0.9, Proteome Software Inc.).

Antibodies

All antibodies used were against human antigens. The following mouse monoclonal antibodies were used: Anti-adaptor protein 1 (AP-1; clone 100/3; Sigma-Aldrich), anti-CD63 (clone H5C6; BD Biosciences Pharmingen, Mississauga, ON), anti-Golgi matrix protein of 130 kDa (GM130; clone 35; BD Biosciences Pharmingen), anti-human lysosome-associated membrane protein 1 (LAMP1; clone H4A3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-mannose-6-phosphate receptor (M6PR; clone 2G11; Calbiochem, San Diego, CA), anti-transferrin receptor (TfR; clone DF 1513; Sigma-Aldrich), anti-thrombospondin-1 (TSP1; clone D4.6; Lab Vision Corporation, Fremont, CA), anti-osteonectin (clone AON-5031; Haematologic Technologies, Inc. Essex Junction, VT) anti-green fluorescent protein (GFP; clone 3E6; Invitrogen, Burlington, ON), anti-c-Myc (clone 9E10; Covance, Laval, QC), anti-HA (clone 16B12; Covance), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; clone 6C5; Millipore, Etobicoke, CA), and anti-β-actin (clone AC-15; Sigma-Aldrich). The rabbit monoclonal anti-early endosome antigen 1 (EEA1; clone C45B10) and anti-Rab7 (clone D95F2) were from Cell Signaling (New England Biolabs Ltd., Pickering, ON). The following polyclonal antibodies were used: Rabbit anti-von Willebrand factor (VWF; Dako Canada Inc., Burlington, ON). goat anti-fibrinogen (Rockland Immunochemicals Inc., Gilbertsville, PA), rabbit anti-NAP-2/CXCL7 (PeproTech Inc., Rocky Hill, NJ) for detecting β-thromboglobulin (β-TG), rabbit anti-platelet factor 4 (PF4; Chemicon International Inc., Temecula, CA), goat anti-P-selectin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-GFP (Invitrogen). An antiserum was prepared against the human VPS33B peptide DTLTAVESKVSKLVTDKAAGKITDAFSSL (amino acids 450-478, RefSeq NP_061138.3). Peptide synthesis and rabbit immunization were performed by Alpha Diagnostic International (San Antonio, TX). Antibody was purified as previously described.[1] Immunoblotting indicated that both serum and purified antibody recognizes human VPS33B (not shown). Mouse antiserum was prepared against

human S-tagged VPS16B eluted in 2 M urea as per manufacturer's protocols (Novagen, Gibbstown, NJ) at the Hospital for Sick Children (Toronto, ON) monoclonal antibody facility. High specificity for human VPS16B was observed using immunoblotting (Supplemental Figure 1A,B), but not with immunofluorescence microscopy. Secondary antibodies for immunoblotting (horseradish peroxidase-conjugated) and immunofluorescence microscopy (Alexa fluorophore-conjugated) were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and Invitrogen respectively.

Molecular evolutionary analysis of VPS16B

Identification of VPS16B proteins and delineation of VPS16 domain

The previously reported VPS16B proteins from *D. melanogaster* (flybase - FBpp0084899),[2] *H. sapiens* (ensembl - ENSP00000313098) and *C. elegans* (wormbase - CE07594) were used in BLAST searches[3] of the UniProt database and an in-house collection of complete eukaryote genomes[4] using an E-value cut off of e^{-10}. To identify boundaries of the VPS16 domain, we compared VPS16B proteins with VPS16A proteins using all-against-all pairwise Smith-Waterman local sequence alignments.[5] The subsequence shared between the two proteins across all species was designated as the VPS16 domain. Secondary structure prediction was performed using the PSIPRED server.[6] The pairwise alignment of the VPS16 domains from *H. sapiens* (Supplemental Figure 3B) was performed using the Needleman-Wunsch global alignment algorithm.[5]

Multiple sequence alignment and phylogenetic reconstruction

The PROMALS3D web server[7] was used to generate both the full length VPS16A alignment (Supplemental Figure 3A) and the alignment that underlies the VPS16 domain phylogeny (Figure 2). This tool improves alignment accuracy by including secondary structure information and homologues with three dimensional structures. The PROMALS alignment algorithm was chosen as the 'first alignment stage' and default parameters were chosen for all other options. Minor edits were subsequently incorporated using the Geneious software suite.[8] Maximum likelihood reconstructions were calculated using the proml algorithm, part of the phlylip package (v. 3.69).[9] A total of 10000 bootstrap replicates were generated.

Bright field and electron microscopy

Romanowsky (Wright-Giemsa) stained blood films were visualized and captured on a Nikon TiU inverted microscope (Nikon Canada Inc. Instruments, Mississauga, ON, Canada) equipped with a digital camera (Nikon Digital Sight DS-U3) using a 60x/1.4 (magnification/numerical aperture, NA) oil immersion objective. Digital images were saved using the Nikon NIS-Elements Basic Research software. Platelet whole-mount electron microscopy was done by placing 2-3 drops of platelet rich plasma (PRP) on to Formvar-coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA) for 5 minutes whereby excess liquid was removed with a filter paper, followed by a 5-minute fixation with 2.5% glutaraldehyde in PBS pH 7.4. After rinsing with distilled water (dH_2O) the grids were placed into a JEOL JEM-1011 electron microscope (EM, with a 300/20 mm condenser/objective aperture, Mississauga, ON, Canada) and the number of dense granules counted in a minimum of 50 platelets (15000-100000x magnification), to obtain the mean number of dense granules per platelet. For TEM of platelets, PRP was centrifuged at 800g for 10 minutes and fixed with 2.5% glutaraldehyde in PBS pH 7.4 at

4°C for 1 hour. The supernatant was discarded and platelets were washed with 0.1 M phosphate buffer (pH 7.4), followed by dH_2O. Platelets were then postfixed with 2% osmium tetroxide, dehydrated, embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-1011 electron microscope at 80 kV. Digital images were captured with a side-mounted Advantage HR CCD camera (Advanced Microscopy Techniques, Danvers, MA), saved in TIFF format and trimmed for publication using Adobe Photoshop (CS3). All digital images were imported into Adobe Photoshop® CS3 in RGB format and assembled in Adobe Illustrator® CS3 for labeling using an Apple Mac Pro computer.

Confocal immunofluorescence microscopy

Dami cells stably expressing GFP-hVPS16B were grown on glass coverslips in the presence of PMA and TPO for 3-5 days. Cells were fixed in 4% paraformaldehyde for 30 min at RT followed by six two-minute washes in PBS, treatment with 10 mM glycine for 20 min, followed by three additional washes with PBS. Fixed cells were permeabilized with 0.1% TX-100 for 30 min, followed by washes with PBS, and blocking in 3% bovine serum albumin (BSA) in PBS for 1 hr. Cover slips were incubated with 100 µL of primary antibody solution (1% BSA) for 1 hr, washed with PBS, and incubated with 150 µL of secondary antibody solution (1% BSA; Alexa Fluor-488 or -568, 1:1000, Invitrogen) for 1 hr. Cover slips were washed once in PBS, then treated with Hoechst stain (1:12000, PBS) for 2 min, and washed once again. Cells were mounted using mounting medium (Dako Canada Inc.). Laser fluorescence images were captured with a Leica DMIRE2 inverted fluorescence microscope using a 63x/1.4 oil immersion objective lens, equipped with a Hamamatsu C9100-12 back-thinned EM-CCD camera and Yokogawa CSU 10 spinning disk confocal scan head (with Spectral Aurora Borealis upgrade). Representative z-stack digital images were acquired using Volocity 3D imaging software (PerkinElmer, Waldham, MA, version 5.1.1). Final figures were made as described in the microscopy section above. Colocalization analysis was performed using the thresholded Pearson's correlation coefficient (PCC) according to Costes et al.[10] measuring a number of individual cells (n in Supplementary Table 1) where the data was processed by Volocity 3D imaging software.

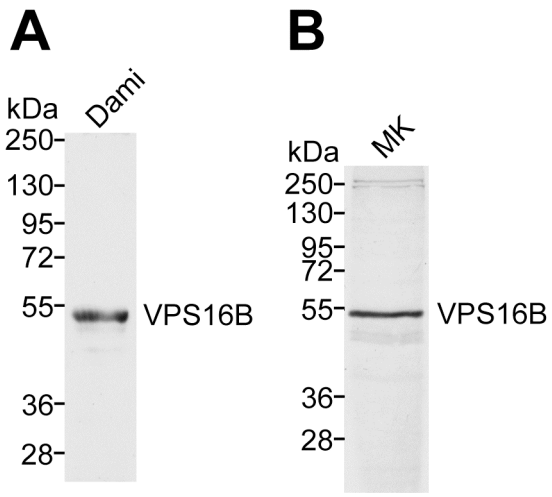
Supplemental Table S1

Protein	n	Pearson's correlation coefficient (PCC), mean ± SD (range)
AP-1	17	0.514±0.88* (0.425-0.602)
CD63	17	0.072±0.041 (0.031-0.113)
EEA1	18	0.218±0.089 (0.129-0.307)
GM130	16	0.101±0.038 (0.063-0.139)
LAMP1	20	0.532±0.098* (0.434-0.630)
M6PR	18	0.188±0.060 (0.128-0.248)
Rab7	20	0.483±0.103* (0.380-0.587)
TfR	20	0.156±0.085 (0.071-0.241)
VWF	28	0.449±0.080* (0.369-0.529)

Proteins are red and GFP-VPS16B green as shown in Figure 7 and Supplemental Figure 4. n = number of cells analyzed. *PCC representing colocalization.

Supplemental Figures

Supplemental Figure S1



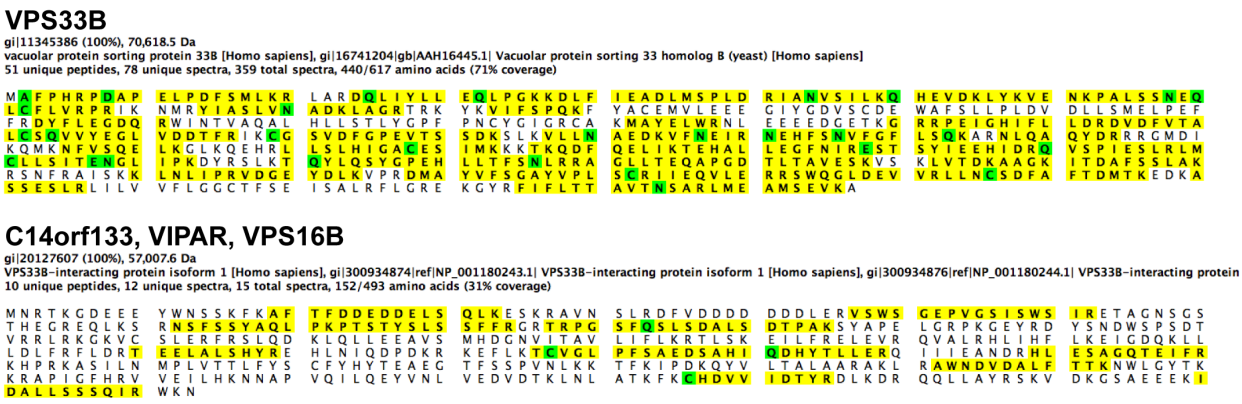
Supplemental Figure S1. Specificity of polyclonal mouse anti-VPS16B serum in immunoblots. (A) Expanded images of the input lanes from Figure 1B in Dami (upper immunoblot; Dami) and **(**B) megakaryocyte cells (lower immunoblot; MK). Both Dami and megakaryocyte lysates probed with anti-VPS16B mouse serum showed a single band migrating at ~57 kDa (reduced 10% SDS-PAGE).

Supplemental Figure S2

A

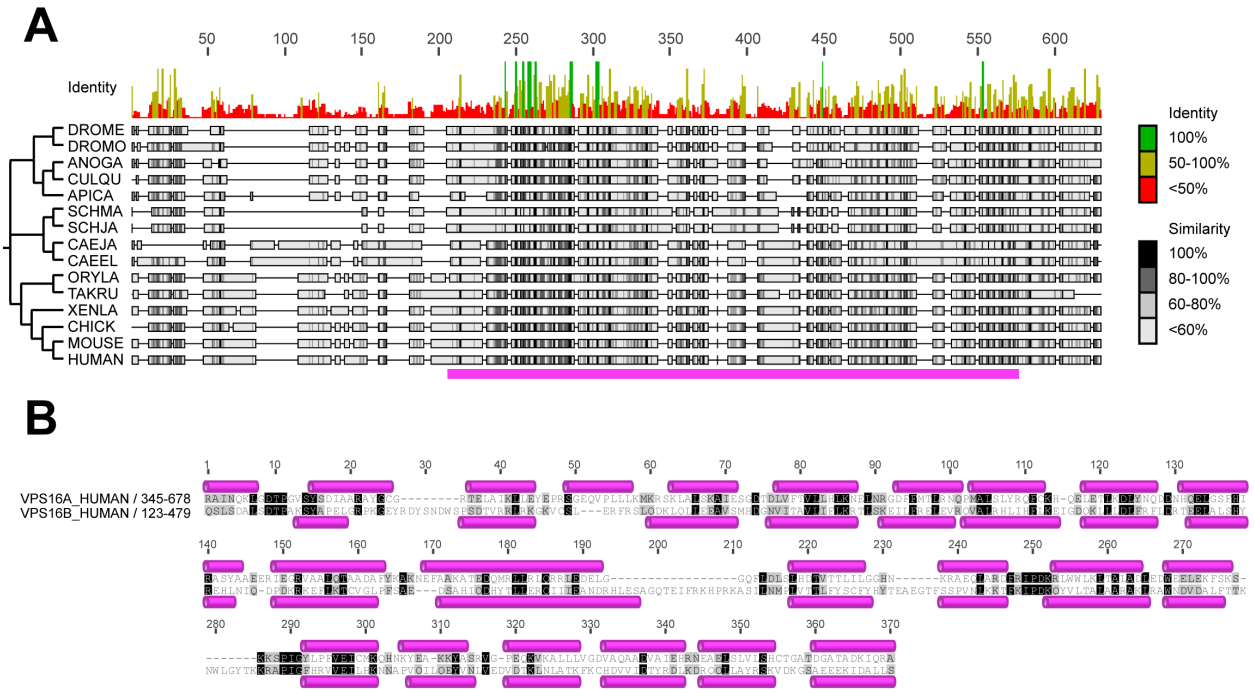
		MS/MS View: Identified Protein	Accession number	Molecular weight	Control HEK293 cells	VPS33B-Flag	
1	☑	vacuolar protein sorting protein 33B [Homo sapiens]...	gi|11345386	71 kDa		51	VPS33B
2	☑	unnamed protein product [Homo sapiens]	gi|194387844...	70 kDa	16	14	
3	☑	unnamed protein product [Homo sapiens]	gi|189054900...	73 kDa	14	14	
4	☑	VPS33B-interacting protein isoform 1 [Homo sapien...	gi|20127607	57 kDa		10	C14orf133

B



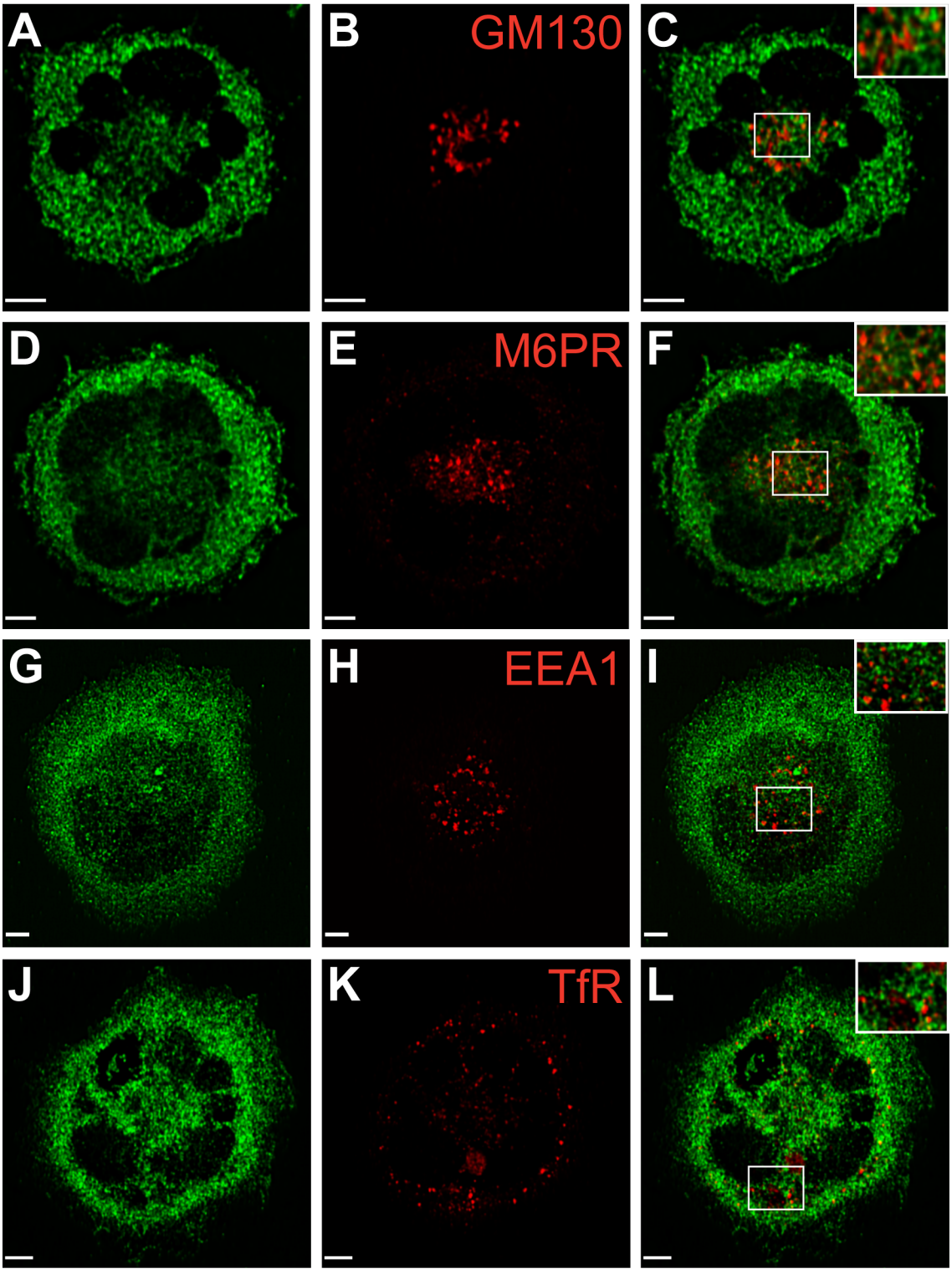
Supplemental Figure S2. Detection of C14orf133 binding to VPS33B in HEK293 cells using mass spectrometry. HEK293 cells stably expressing VPS33B-FLAG and control HEK293 cells were utilized to immunoprecipitate VPS33B-binding proteins using an anti-FLAG antibody. Identification of the immunoprecipitated proteins was by proteolytic fragmentation and mass spectrometry analysis. Detection of peptides in the eluates was visualized using Scaffold 3 (Proteome Software). (A) VPS33B and C14orf133 peptides were among the four top hits detected in the eluate of VPS33B-FLAG stable HEK293 cells (with over 95% probability) but not in the control HEK293 cells. (B) VPS33B was detected by 51 unique peptides (71% sequence coverage), and C14orf133 was detected by10 unique peptides (31% sequence coverage).

Supplemental Figure S3



Supplemental Figure S3. Alignment of VPS16. (A) Multiple sequence alignment of VPS16B proteins. The members of the VPS16B protein family were identified through sequence similarity searches (see Supplemental Methods). VPS16B proteins were confirmed in 35 species spanning the Bilateria; shown here are sequences from a representative 15 species. The N terminus (alignment positions 1-161) is more variable across the taxonomic lineages, although there is a highly conserved region at the start of the protein (positions 14-36). The Vps16_C domain is located between alignment positions 197 and 575 (indicated below the alignment). The conservation in the alignment is given as the level of identical amino acids at each position (coloured bars) and the similarity (grey-scale colouring). DROME – *Drosophila melanogaster*; DROMO – *D. mojavensis*; ANOGA – *Anopheles gambiae* ; CULQU – *Culex quinquefasciatus*; APICA – *Apis mellifera carnica*; SCHMA – *Schistosoma mansoni*; SCHJA – *Schistosoma japonicum*; CAEJA – *Caenorhabditis japonica*; CAEEL – *C. elegans*; ORYLA – *Oryzias latipes*; TAKRU – *Takifugu rubripes*; XENLA – *Xenopus laevis*; CHICK – *Gallus gallus*; MOUSE – *Mus musculus*; HUMAN – *Homo sapiens*. (B) Pairwise global alignment of the VPS16_C domain between VPS16A and VPS16B in humans. The VPS16 domain from the human copies of VPS16A and VPS16B were aligned using the Needleman-Wunsch and secondary structure predictions used to identify positions of alpha-helices (pink cylinders). Despite possessing only 20% sequence identity, VPS16A and VPS16B share a well conserved secondary structure (see Supplemental Methods), lending further support to sharing a common ancestor.

Supplemental Figure S4



Supplemental Figure S4. Localization of GFP-VPS16B within stable megakaryocytic Dami cells.

Dami cells stably transfected with GFP-VPS16B fusion protein (green) were co-stained with different intracellular markers (red) as indicated. Merge images are shown on the right of each row (C,F,I,L). Absent colocalization is observed between GFP-VPS16B and the *cis*-Golgi marker GM130 (A-C), the endosome/*trans*-Golgi marker M6PR (D-F), the early endosome marker EEA1 (G-I), and the recycling endosome marker TfR (J-L). Insets highlight areas showing absent colocalization. Multi-lobed nuclei are readily observed in Dami cells stimulated with phorbol ester and TPO. Representative z-stack images are shown. The white bar represents 5 μm. Images were obtained using a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu C9100-12 back-thinned EM-CCD camera and Yokogawa CSU 10 spinning disk confocal scan head (with Spectral Aurora Borealis upgrade), with 63x/1.4 objective lenses. Data was acquired and processed using the Perkin Elmer Volocity software v5.1.1.

References

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