Defective AP-3-dependent VAMP8 trafficking impairs Weibel-Palade body exocytosis in Hermansky-Pudlak Syndrome type 2 blood outgrowth endothelial cells

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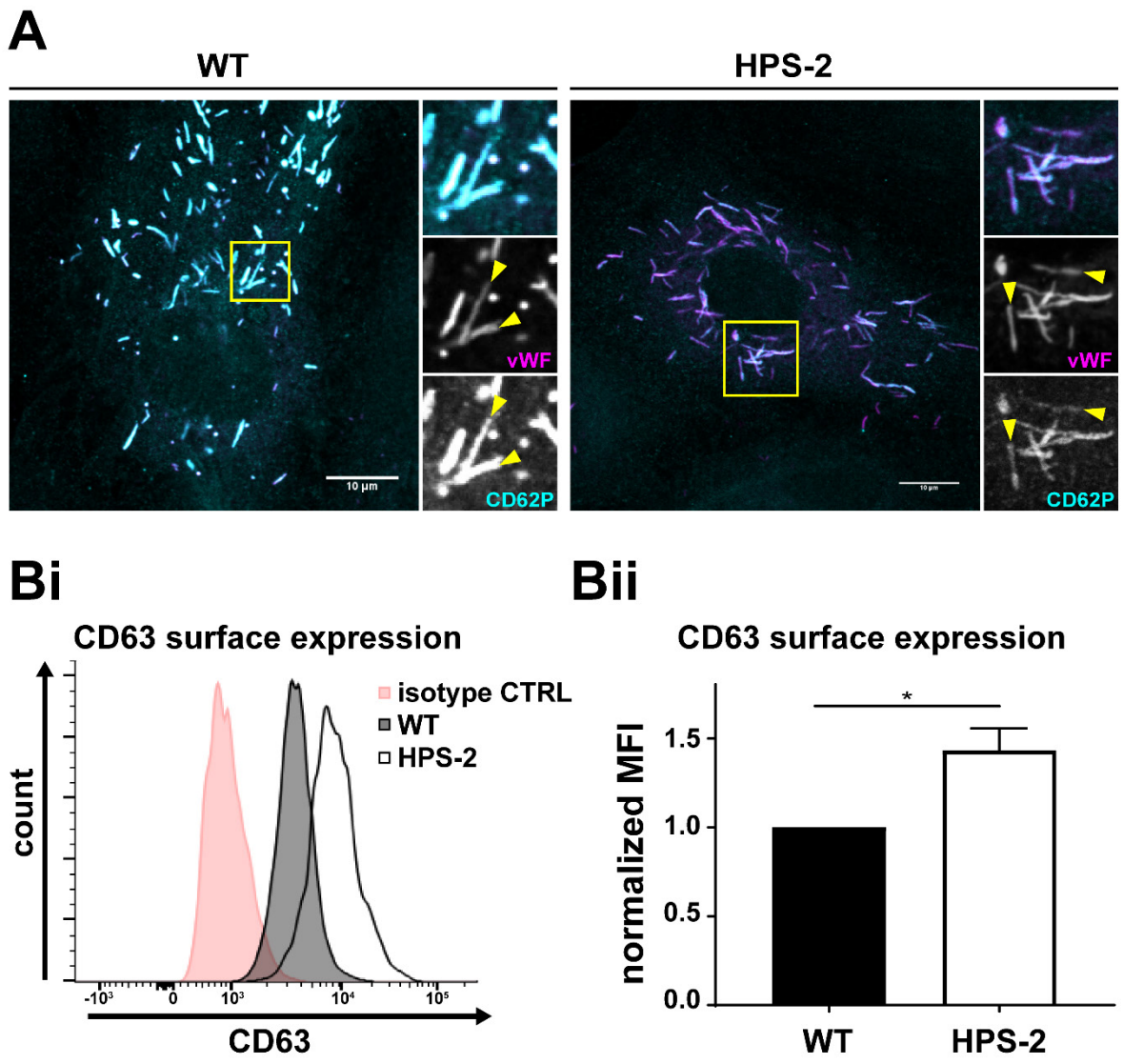
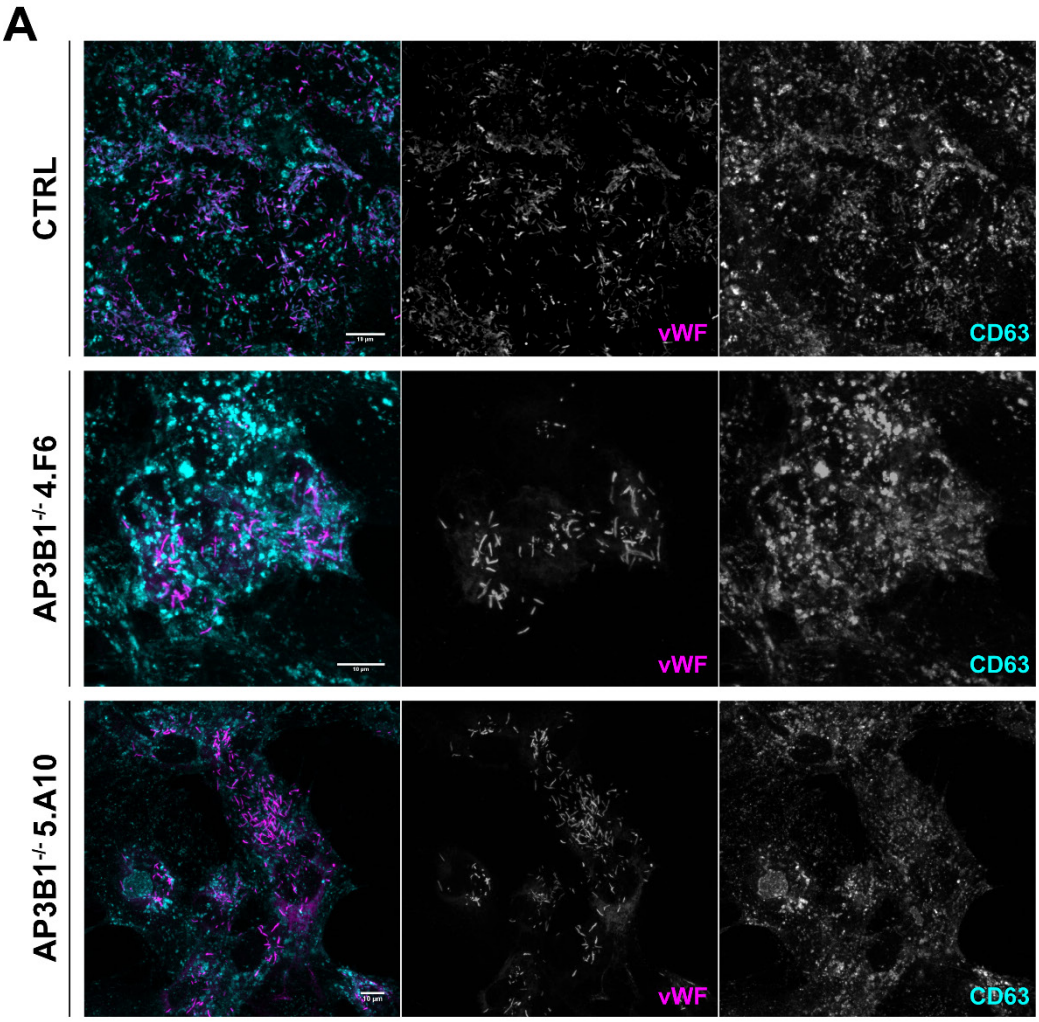
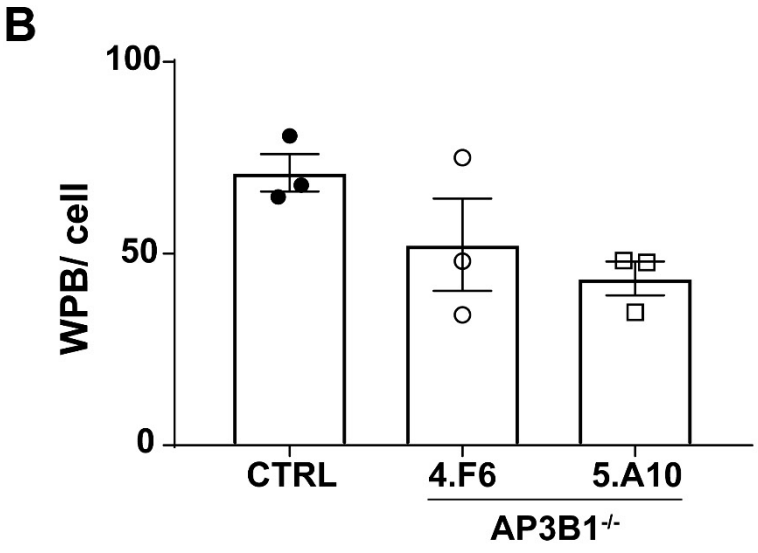
Supplementary Figure 1



Supplementary Figure 1: P-selectin (CD62P) and CD63 trafficking in HPS-2 BOECs. (A) WT and HPS-2 BOECs were immunostained for vWF (magenta) and CD62P (cyan). Boxed regions are magnified on the right. The yellow arrowheads show WPB in both channels. In both WT and HPS-2 BOEC WPBs are positive for CD62P. (B) Flow cytometric analysis of CD63 membrane expression under steady state conditions in WT and HPS-2 BOECs. (Bi) Representative histogram plot of WT and HPS-2 BOEC stained with mouse anti-CD63 (Bii) Quantification of 6 independent experiments. CD63 is significantly increased on the plasma membrane of HPS-2 BOEC. (Paired two-tailed Student's t-test, * $p<0.05$).

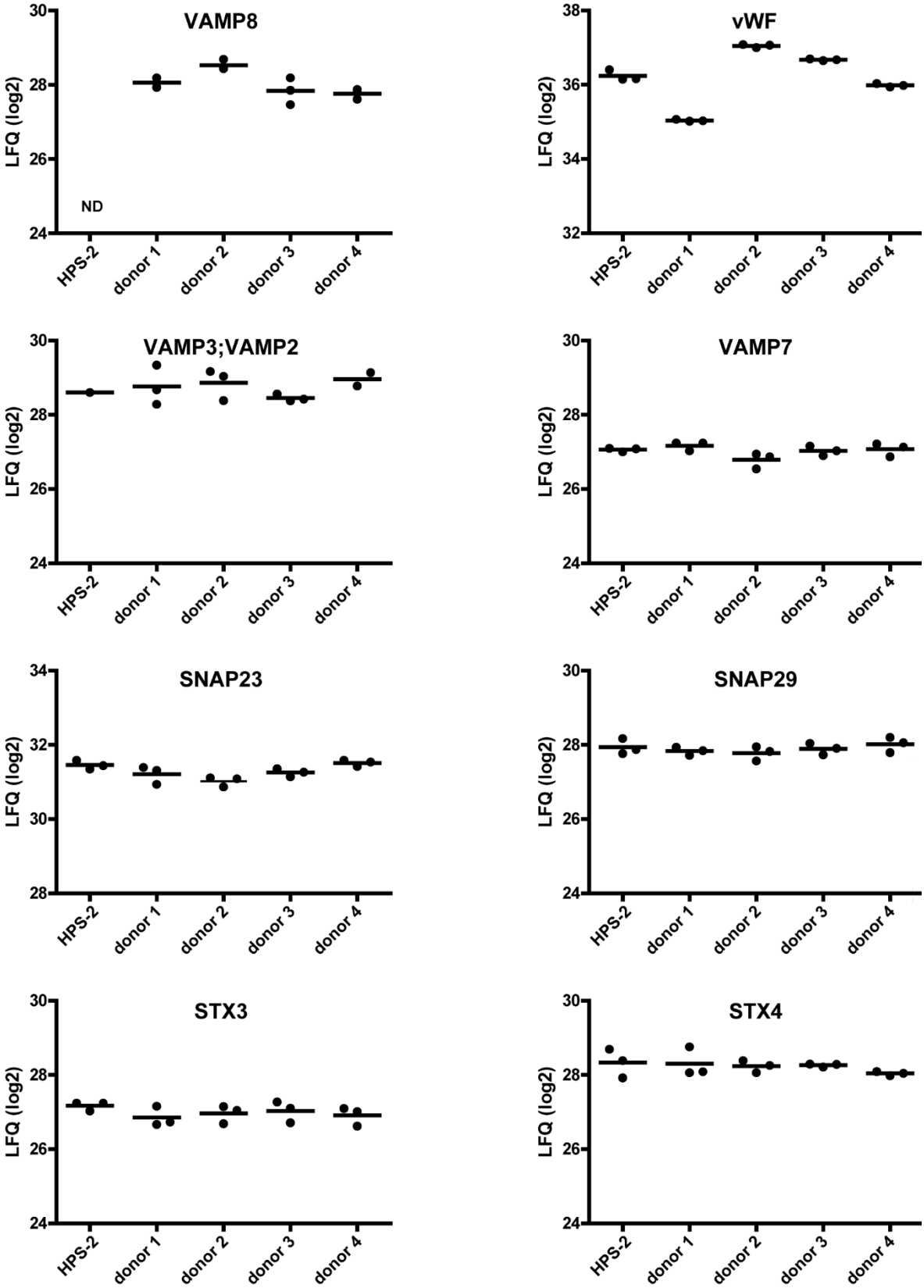
Supplementary Figure 2





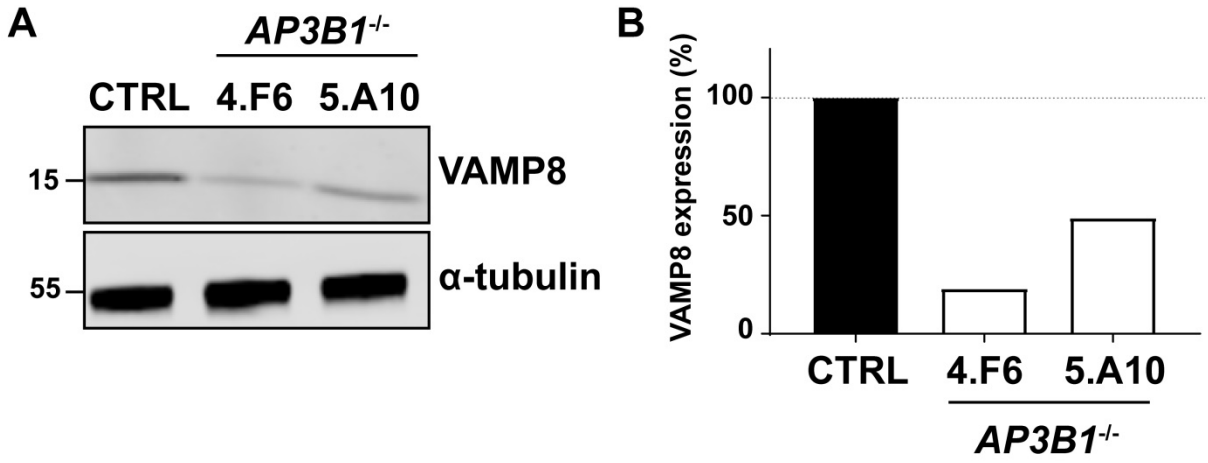
Supplemental Figure 2: WPB formation and maturation (CD63 delivery) in *AP3B1*$^{-/-}$ BOEC lines. (A) Control and *AP3B1*$^{-/-}$ BOEC were immunostained for VWF (magenta) and CD63 (cyan). (B) average amount of WPB per cell in control and *AP3B1*$^{-/-}$ BOEC.

Supplementary Figure 3



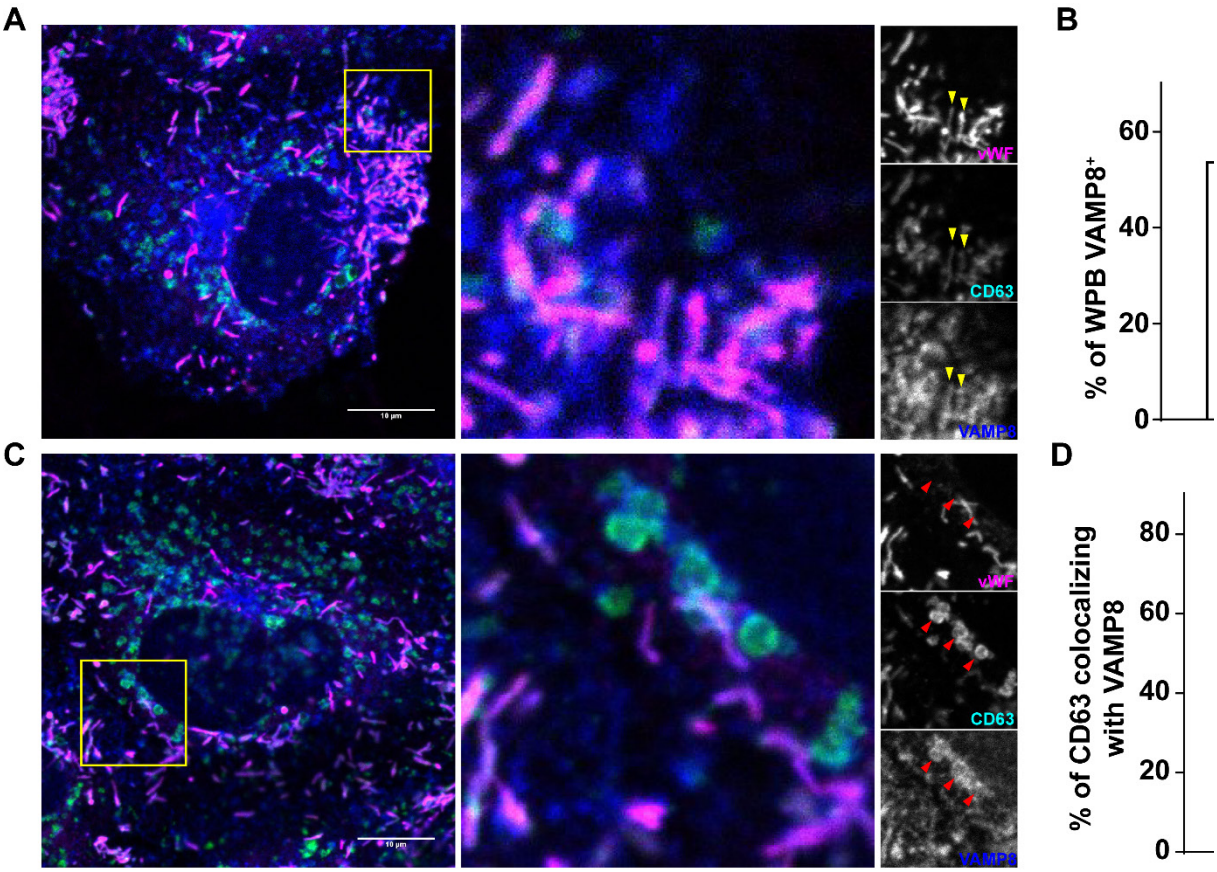
Supplemental Figure 3: Expression of exocytotic SNARE proteins in healthy control and HPS-2 BOECs. Graph representing LFQ (log2) values for VAMP8, VAMP3, VAMP7, SNAP23, SNAP29, STX3, STX4 and vWF.

Supplementary Figure 4



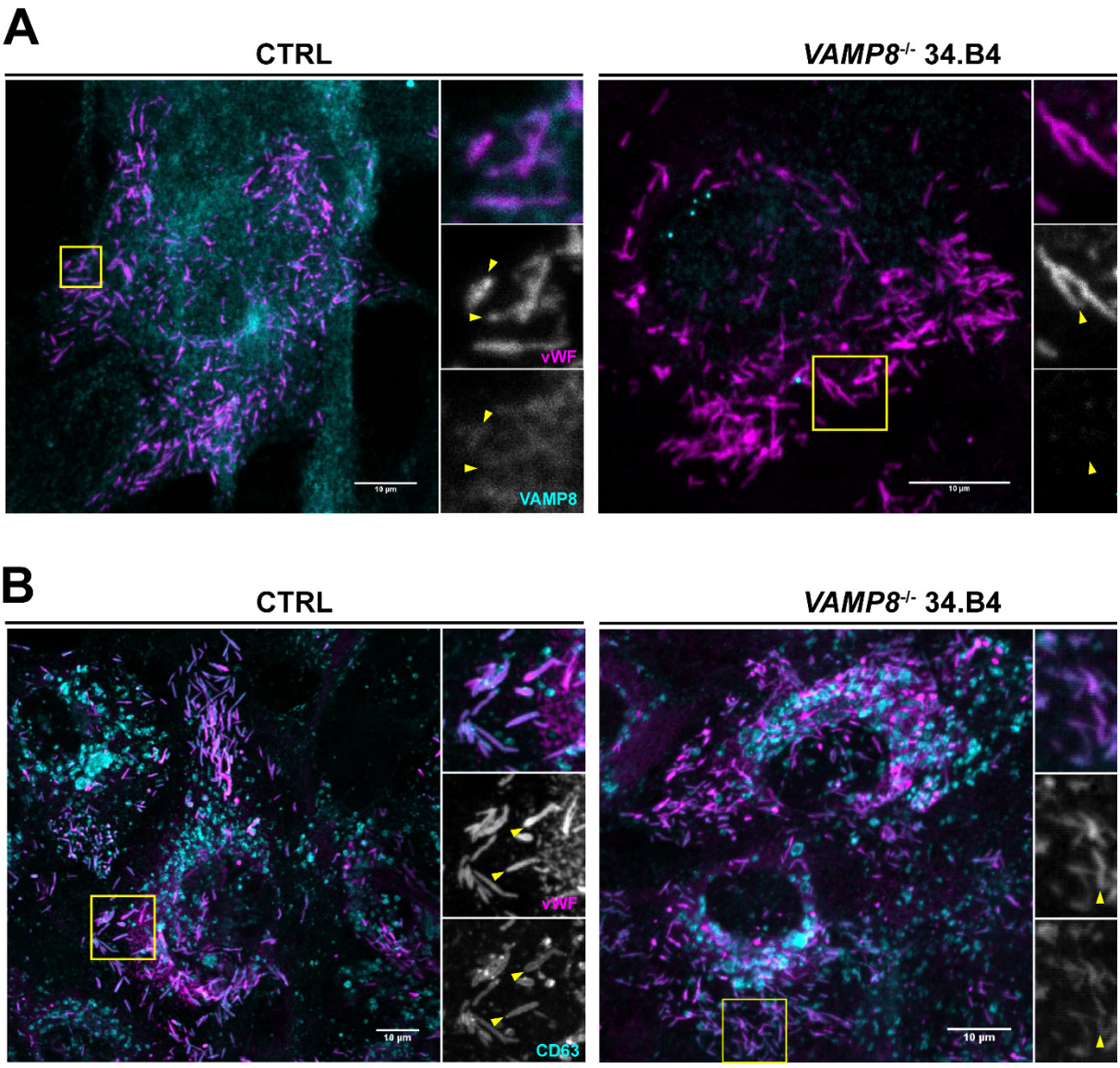
Supplementary Figure 4: Reduced expression of VAMP8 in *AP3B1*$^{-/-}$ BOEC lines. (A) Western blot analysis of VAMP8 expression in lysates from two clonal *AP3B1*$^{-/-}$ BOEC lines (4.F6 and 5.A10) compared to clonal control BOEC. α-tubulin is used as loading control. Molecular weight standards are indicated on the left (kDa). (B) Quantification of relative VAMP8 expression in clonal *AP3B1*$^{-/-}$ BOEC lines normalized to VAMP8 in clonal control BOEC (CTRL). VAMP8 expression is severely affected in both clones with one, 4.F6, expressing less than 20%, while the other, 5.A10 having expression below 50%.

Supplementary Figure 5



Supplementary Figure 5: VAMP8 and CD63 expression in healthy control endothelial cells. (A) Control BOECs were immunostained for vWF (magenta), CD63 (green) and VAMP8 (blue). Boxed regions are magnified on the right. WPBs are indicated by yellow arrowheads that are positive for both VAMP8 and CD63. (B) Proportion of WPB that contain CD63 immunoreactivity. (C) Control BOEC were immunostained for VWF (magenta), CD63 (green) and VAMP8 (blue). Boxed regions are magnified on the right. CD63 positive endosome-like structures are indicated by red arrowheads. These structures are only positive for CD63 and VAMP8, but lack VWF. (D) Proportion of CD63 that co-localizes with VAMP8. Scale bars represent 10 μm.

Supplementary Figure 6



Supplementary Figure 6: CD63 trafficking in VAMP8 knockout cell lines. (A) Control and *VAMP8*$^{-/-}$ BOECs were immunostained for vWF (magenta) and VAMP8 (cyan), demonstrating the lack of VAMP8 immunoreactivity on WPBs in *VAMP8*$^{-/-}$ BOECs. (B) Control and *VAMP8*$^{-/-}$ BOEC were immunostained for vWF (magenta) and CD63 (cyan). In both control and VAMP8 knockout BOEC WPBs are positive for CD63. Boxed regions are magnified on the right. The yellow arrowheads show WPB in both channels. Scale bars represent 10 µm.

Supplementary Table 1. Antibody reagents

Target	Species (isotype)	Label	Supplier	Cat.nr / clone	Use [concentration/dilution]
VWF	mouse (IgG_{2b})	-	described in [1]	CLB-RAg20	IF [1:1000]
α-tubulin	mouse (IgG_1)	-	Sigma-Aldrich	T9026	WB [1:1000]
VWF	rabbit	-	DAKO	A0082	ELISA [6 µg/ml]
VWF	rabbit	HRP	DAKO	A0082	ELISA [2 µg/ml]
AP-3 β3A	rabbit		proteintech	13384-1-AP	WB (1:500)
AP-3 µ3	rabbit		Abcam	ab201227	WB (1:1000)
GFP	sheep		BIORAD	4745-1051	WB [0.5 µg/ml]
CD63	mouse (IgG_1)	AF488	Sanquin	CLB-gran/12	IF [0.4 µg/ml]
CD62P	mouse (IgG_1)	AF488	AbD Serotec	MCA796	IF [2 µg/ml]
CD62P	mouse (IgG_1)	PE	BioLegend	304906	FCM (1:100)
VAMP8	rabbit		Synaptic Systems	104303	WB, IF [1 µg/ml]
rabbit IgG	donkey	680LT	Li-Cor	925-68023	WB [0.1 µg/ml]
mouse IgG	donkey	800CW	Li-Cor	925-32212	WB [0.1 µg/ml]
rabbit IgG	goat	AF633	ThermoFisher	A11011	IF [2 µg/ml]
mouse IgG	goat	AF568	ThermoFisher	A11004	IF [2 µg/ml]
mouse IgG	goat	AF488	ThermoFisher	A11004	IF [2 µg/ml]
rabbit IgG	chicken	AF647	ThermoFisher	A21443	IF [2 µg/ml]

Supplementary Materials and Methods

Antibodies

Antibodies used in this study are listed in Supplementary Table 1.

Lentiviral transfection and transduction

HEK293T cells, cultured in DMEM-F12 (Lonza, Basel, Switzeland) supplemented with 10% FCS, were seeded on collagen coated plates or flasks. HEK293T cells were transfected with lentiviral transfer (see in section DNA constructs, Materials and Methods), 3rd generation packaging (MDL and REV) and envelop (VGV-G) plasmids using *transit*-LT1 (Mirus Bio LLC, Madison, WI, USA) and following the supplier's protocol. Virus particles were collected 24 and 48 hours following transfection. Virus particles were filtered through 0.45 µm pore filters in EGM-18. Two batches of virus were used to transduce BOECs or cord blood BOECs (cbBOECs) for the period of 48 hours. Transduced endothelial cells were selected by puromycin (0.5 µg/ml) that was added to the medium for 72 hours after the second virus installment.

Immunoblotting

Endothelial cells were grown to confluency and lysed in NP-40 based lysis buffer (0.5% NP-40, 0.5 mM EDTA, 10 mM Tris HCl, 150 mM NaCl), supplemented with Complete protease inhibitor cocktail (Roche, 05056489001). Proteins were separated on a Novex® NuPAGE® 4-12% Bis-Tris gel (ThermoFisher, NP0321/NP0323) and transferred onto a nitrocellulose membrane (iBlot Transfer Stack, ThermoFisher, IB3010). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, USA,LI 927) and probed with primary antibodies and subsequently with IRDye conjugated secondary antibodies (see Supplementary Table I). Visualization of IRDye conjugated antibodies was performed by means of LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Blot analysis for

band intensities was done in Image Studio Lite (V4.0, LI-COR Biosciences) and when needed intensities were normalized to the intensity of α-tubulin which was used as a loading control.

Whole-proteome analysis

Whole proteome analysis was performed as described previously.[2] In short, BOECs were cultured in 10 cm culture dishes in triplicate. Upon confluency, cells were lysed and processed into tryptic peptides using the Filter Aided Sample Preparation method.[3] Tryptic peptides were desalted using Empore-C18 StageTips [4] and separated by nanoscale C18 reverse phase chromatography coupled online to an Orbitrap Fusion Tribid Mass Spectrometer via a nanospray Flex Ion Source (Thermo Scientific, Waltham, MA, USA) using the same settings as described previously.[5] All MS data were acquired with Xcalibur software (Thermo Scientific).The RAW mass spectrometry files were processed with the MaxQuant computational platform, version 1.5.2.8.[6] Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database, (downloaded February 2015). Standard settings with the additional options match between runs, label-free quantification, and unique peptides for quantification were selected. The generated proteingroups.txt table was filtered for reverse hits, only identified by site and potential contaminants using Perseus 1.5.1.6. The label-free quantification values were transformed in log2 scale. Samples were grouped per BOEC donor (HPS-2 patient (ID 8621), 4 healthy controls (IDs 7908, 7950, 7958 and 7977); 5 groups, 3 samples per group), and proteins were filtered for at least 3 valid values in at least 1 of the 5 groups. Missing values were imputed by normal distribution (width = 0.3, shift = 1.8), assuming these proteins were close to the detection limit. Global changes in protein levels were assessed using ANOVA (false discovery rate, 0.05, S0: 0.4). Z-scored LFQ (log2) values were represented using a heatmap combined with hierarchical clustering. The .raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange Consortium

(http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository [7] with the data set identifier PXD011294.

Fluorescence microscopy

Immunostaining and fluorescence imaging of fixed cells was performed as previously described.[2] Immunostained cells were mounted in MOWIOL mounting medium and images were acquired using a Leica SP8 confocal microscope (Leica, Wetzlar, Germany). Images were processed and analyzed using ImageJ or IMARIS Digital Imaging software (Bitplane AG, Zürich. Switzerland).

Secretion assay

Endothelial cells were grown in 6-well wells and cultured for the period of 7 days prior to the experiment. Unstimulated VWF release was determined for 1 and 24 hours in release medium [serum-free M199 (Thermofisher, 22340) supplemented with 0.2% (w/v) bovine serum albumin (BSA) (Merck, 112018)] and EGM-18 medium, respectively. Cells were pre-incubated in RM medium for 15-30 minutes prior to stimulated VWF secretion. Cells were stimulated in RM medium supplemented with 0.1-100 µM histamine (Sigma-Aldrich, H7125), 10 µM forskolin (Sigma-Aldrich, F6886) with 100 µM IBMX (Sigma-Aldrich, I7018), or vehicle (unstimulated) for 30 minutes, unless stated otherwise. Lysates were obtained in RM medium supplemented with 1% Triton X-100. VWF levels were determined by ELISA as described previously.[2] Secretion is expressed as relative proportion of intracellular VWF in lysates of unstimulated cells.

Data and statistical analysis

Flow cytometry data was analyzed using FlowJo version 10 (Ashland, OR, USA). Statistical analysis was by student's t-test and using GraphPad Prism 7.04 (Graphpad, La Jolla, CA, USA). Significance values are shown in the Figures or in Figure legends. Data are shown as mean ± SEM.

Supplementary References

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