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Chapter 3

Impaired VWF release in EIEE4 ex vivo endothelial cells identifies STXBP1 as a positive regulator of Weibel-Palade body exocytosis

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Abstract

Vascular endothelial cells contain unique rod-shaped secretory organelles, called Weibel-Palade bodies (WPBs), which contain the hemostatic protein Von Willebrand factor (VWF) and a cocktail of angiogenic and inflammatory mediators. We have shown that the Rab27A effector synaptotagmin-like protein 4-a (Slp4-a) plays a critical role in regulating hormone-evoked WPB exocytosis. Using a non-biased proteomic screen for targets for Slp4-a we now identify syntaxin binding protein 1 (STXBP1) and syntaxin-2 and -3 as endogenous Slp4-a binding partners in endothelial cells. Co-immunoprecipitations showed that STXBP1 interacts with syntaxin-2 and -3, but not syntaxin-4. siRNA-mediated silencing of STXBP1 expression impaired histamine- and forskolin-induced VWF secretion. To further substantiate the role of STXBP1 we isolated blood outgrowth endothelial cells (BOECs) from an early infantile epileptic encephalopathy type 4 (EIEE4) patient carrying a de novo mutation in STXBP1. STXBP1 haploinsufficient EIEE4 BOECs contained similar numbers of morphologically normal WPBs compared to control BOECs of healthy donors, however, EIEE4 BOECs displayed significantly impaired histamine- and forskolin-stimulated VWF secretion. Based on these findings, we propose that the Rab27A-Slp4-a complex on WPB promotes exocytosis through an interaction with STXBP1, thereby controlling the release of vaso-active substances in the vasculature.
STXBP1 promotes Weibel-Palade body exocytosis

**Introduction**

Endothelial cells line the lumen of all blood vessels, providing a highly dynamic barrier that plays a crucial role in maintaining vascular homeostasis. They contain specialized secretory organelles, called Weibel-Palade bodies (WPBs), which allow the endothelium to store and release, in a regulated fashion, a presynthesized cocktail of hemostatic, inflammatory and angiogenic mediators in response to endothelial activation, injury or stress[^1^-^3]. The main component of these organelles is von Willebrand factor (VWF), a multimeric glycoprotein crucial for platelet plug formation and stabilising coagulation factor VIII. In addition to VWF, several soluble chemokines (e.g. IL-6 and IL-8) as well as the integral membrane proteins CD63 and P-selectin are stored in these organelles[^4^-^9]. Coordinated expression of CD63 and P-selectin on the endothelial cell surface following WPB exocytosis is crucial for leukocyte extravasation at sites of inflammation[^10]. The presence of angiopoietin-2 and IGFBP7 in WPBs points towards a critical role for the organelle in regulation of angiogenesis[^11^-^13].

The precise composition of mediators stored in WPBs depends crucially on the physical, mechanical and chemical signals in the local microenvironment; for example targeting of eotaxin-3, IL-8 and IL-6 has been observed in response to pro-inflammatory mediators such as IL-1β or IL-4[^5,7,14,15] while the inclusion of angiopoietin-2 is modulated under conditions that mimic shear stress[^16].

Exocytosis of WPBs is triggered by a wide range of physiological secretagogues that trigger Ca$^{2+}$- and cAMP-dependent signalling pathways, such as histamine and thrombin or vasopressin and epinephrine respectively[^3,17^-^20]. Several key regulators of WPB exocytosis have been identified, including the small GTPases RalA, Rab3A, Rab3D and Rab27A[^21^-^25]. Rab27A plays a particularly crucial role in regulating WPB exocytosis through its ability to recruit multiple effector molecules[^26], including myosin Va-Rab interacting protein (MyRIP), synaptotagmin-like protein 4-a (Slp4-a) and Munc13-4. MyRIP-dependent recruitment of the actin motor protein myosin Va to the WPB is proposed to link the organelle to the actin cytoskeleton, preventing access to and fusion with the plasma membrane[^27,28]. In contrast, both Slp4-a and Munc13-4 promote WPB exocytosis[^25,29], although the mechanism remains unclear.

To investigate the mechanism by which Slp4-a regulates WPB exocytosis we used an unbiased mass spectrometry approach aimed at identifying endogenous Slp4-a interactors in human endothelial cells. Using this screen we identified syntaxin binding protein 1 (STXBP1, also known as Munc18-1) and syntaxin-2 and -3 as potential effectors of Slp4-a. RNAi mediated depletion of STXBP1
significantly impaired WPB release. Loss-of-function mutations in STXBP1 have been shown to be the cause of early infantile epileptic encephalopathy type 4 (EIEE4), a severe epileptic disorder\[30-32\]. Blood outgrowth endothelial cells (BOECs), isolated from peripheral blood of a patient with EIEE4 carrying a de novo mutation in STXBP1, displayed impaired stimulated WPB release, supporting a role for STXBP1 in regulating VWF secretion. Our findings provide a link between the Rab27A-Slp4-a complex on WPBs and a regulatory component of the SNARE-complex which controls the release of vaso-active substances in the vasculature.

**Materials and Methods**

**Antibodies and reagents**

Histamine, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and ionomycin were from Sigma Aldrich (Steinheim, Germany). Glutathione-Sepharose 4B was from GE Healthcare Europe GmbH (Diegem, Belgium). Trypsin, Fura-2/AM, Alexa 488- and Alexa 568- and Alexa 633-conjugated secondary antibodies were from Invitrogen (Breda, the Netherlands). Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics (Mannheim, Germany). All chemicals used were of analytical grade. Primary antibodies used in this study are shown in Supplementary Table 1.

**Cell culture, BOEC isolation and secretion assay.**

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Promocell (Heidelberg, Germany) and grown in EGM-2 medium (Lonza, Basel, Switzerland) supplemented with 18% fetal calf serum or as described\[5\]. BOECs were isolated and cultured as previously described\[56\]. 15 ml venous blood was drawn from one individual with EIEE4 and a de novo STXBP1 mutation (patient 4 in [30]) after parents signed an informed consent form for participation. The study was approved by the Sanquin Ethical Advisory Board and the Commission for Medical Ethics of the University of Antwerp. Cells were stimulated with serum free (SF) medium or SF medium containing 100 µM histamine or 10 µM forskolin supplemented with 100 µM 3-isobutyl-1-methylxanthine (IBMX). VWF secretion was assayed by ELISA as described previously\[19\]. VWF antigen levels in patient plasma were determined using ELISA.

**RNAi**

Downregulation of endogenous expression of STXBP1 was performed essentially as described in [29]. Pools of 4 siRNA oligo duplexes (ON-TARGETplus
SMARTpool, Dharmacon, Lafayette, USA) were used to deplete HUVEC of STXBP1 (#L019571). A pool of 4 non-targeting siRNAs (ON-TARGETplus Non-targeting pool, #D001810) was used as a control. Oligo sequences are given in Supplemental Table 2. Depletion of STXBP1 was assessed by immunoblot. Band intensities of immunoblots were analyzed using ImageJ (http://rsbweb.nih.gov/ij/) in order to determine STXBP1 knock down efficiency.

**DNA constructs**
EGFP-Slp4-a full length (FL) and Proregion-EGFP have been described previously[29,57]. Slp4-a fragments were amplified from IMAGE clone 3908307 (Geneservice, Nottingham, UK) containing human Slp4-a cDNA. The Slp4-a-SHD fragment containing residues 1-150 was amplified by PCR using Slp4-a-1F (5’-AGATCTCGAGCTATGTCGGAGTTACTGGAC-3’) and Slp4-a-150R (5’-ATAGAATTCTCTTTTACTCAGTGAGTT-3’); the Slp4-a ΔSHD fragment containing residues 151-671 was amplified using Slp4-a-151F (5’-ATACTCGAGCTACAGTGGGACAGTCCCTTTCATC-3’) and Slp4-a-671R (5’-GTTGGATCCTCCATAACCCAGGCT-3’). Both fragments where digested with XhoI and BamHI and cloned into XhoI-BamHI digested pmCherry-C1 (Clontech, Palo Alto, CA). The inducible bacterial GST-expression vector pGEX-C1 was created by PCR amplification of the pEGFP-C1 (Clontech) multiple-cloning site (MCS) with GEX-C1F (5’-GTCATAAGATCTAGGCCTCGAGCTCAAGCTTC-3’) and GEX-R(5’-TATGACGCGGCCGCTGGATCCCGGGCCCGGCTACC-3’), digestion of the PCR fragment with BglII and NotI and insertion into BamHI-NotI digested pGEX-6P-1 (GE Healthcare, Chalfont St Giles, UK). GST- and EGFP-tagged fusions of Slp4-a truncation variants were made by excising the Slp4-a fragments with XhoI and BamHI from pmCherry-Slp4-a-SHD and pmCherry-Slp4-a ΔSHD and inserting them into XhoI-BamHI digested pGEX-C1 or pEGFP-C1 respectively.

**Pull-down and mass spectrometry**
GST-Slp4-a-SHD and GST-Slp4-a ΔSHD were produced in IPTG-induced BL21 bacteria. Bacterial lysates were prepared in lysis buffer (1% Triton X-100, PBS and protease inhibitors) and incubated with Glutathione-Sepharose. Endothelial cells were rinsed twice with PBS, after which lysates were made in lysis buffer which were then pre-cleared for 10 minutes with Glutathione-Sepharose. Immobilized GST-fusion proteins were washed 2 times in lysis buffer and incubated overnight at 4°C with pre-cleared endothelial cell lysates. GST-fusion proteins and interactors were eluted with 10 mM glutathione and separated on
a precast 4-20% gradient SDS-PAGE gel under reducing conditions. Protein bands were visualized with colloidal coomassie. Following staining the gel was washed with ultrapure water and lanes containing the samples were cut in 7 pieces each. A Perkin Elmer Janus Automated Workstation was used for in gel trypsin digestion. Peptide mixtures were acidified to 0.1% TFA and injected onto a nanoACQUITY UPLC (Waters Corporation) coupled to a LTQ-Orbitap XL (Thermo Fisher Scientific) via an Advion Biosciences Nanomate. Peptides were eluted over a 30 min gradient (5-40% ACN). Peak lists were extracted using Mascot distiller and searched with Mascot v.2.4.1 (Matrix Science) against the *Homo sapiens* reference proteome. Oxidation of methionine was entered as a variable modification and the search tolerances were 5ppm and 0.8 Da for peptides and fragments respectively. Searches for each lane were combined and results compiled in Scaffold 4.0.3. For multi-experiment comparison the Mascot .dat file was parsed into a local version of the ProHits database. For each identified protein in the Slp4-a pulldown, the frequency of occurrence in the ProHits database was calculated. Proteins with high frequencies tend to be contaminants whereas proteins with low frequencies tend to be specific for a pulldown. After the addition of the Slp4-a pulldowns, the frequency of the bait (Slp4-a) was calculated as 0.0185 indicating that the protein had previously not been found in the database.

**Immunoprecipitation**

HUVECs were rinsed once in PBS and were then lysed in NP40 lysis buffer containing 1% NP40, 10% glycerol, 137 mM NaCl, 25 mM HEPES, 5 mM MgCl$_2$, 5 mM CaCl$_2$, pH 7.4 containing protease/phosphate inhibitors (Pierce HALT cocktail, Thermo Scientific, Rockford, IL). Total cell lysates were centrifuged for 10 minutes at 4°C at 20,000g. Supernatants were incubated with 50 ml NHS-activated magnetic beads coupled (Thermo Scientific) with 8 mg mouse anti-STXBP1 IgG (Synaptic Systems, Göttingen, Germany) or 8 mg control mouse IgG (Santa Cruz, Santa Cruz, CA) according to manufacturer’s instructions. Cell lysates were incubated with the antibody-coupled beads for 2 hours at 4°C and were washed 4 times with 1% NP40 lysis buffer and once with H$_2$O. Co-immunoprecipitates and/or lysates were resuspended in a final concentration of 2x sample buffer containing 100 mM dithiothreitol. Proteins were separated on pre-cast 4-12% NuPAGE gels (Invitrogen, Breda, The Netherlands) transferred to 0.2 μm nitrocellulose membrane and probed with primary antibodies, followed by the appropriate IR dye-coupled donkey secondary antibodies (LI-COR Biosciences, Cambridge, UK). Membranes
were scanned with the LI-COR Odyssey Infrared Imaging system (LI-COR Biosciences).

**Fluorescence microscopy**
Immunostaining and fluorescence imaging of fixed cells was performed as previously described\[5\]. Primary and secondary antibodies along with their dilutions for immunofluorescence and immunoblotting are given in Supplementary Table 1. Immunostained cells were mounted in MOWIOIL or Fluorsave mounting medium (Calbiochem, Nottingham, United Kingdom) and images were acquired by point scanning confocal microscopy using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) or a Leica SP1 (Leica Microsystems, Wetzlar, Germany).

Ionomycin induced exocytosis of Proregion-EGFP-containing WPBs or EGFP-Slp4-a FL or EGFP-Slp4-a-SHD expressing WPBs was determined as previously described\[29,59\]. The probability of release, \(P_r\), was determined as the mean percentage of degranulation of fluorescent WPBs after ionomycin stimulation.

**Statistical analysis**
Data were plotted in GraphPad Prism 5.04 (Graphpad, La Jolla, CA). Statistical analysis was by one-way ANOVA and Bonferroni post-test analysis using GraphPad Prism 5.04. Significance values are shown on the Figures or in Figure legends. Data are shown as mean ± SEM.

**Results**

The N-terminal synaptotagmin-like protein homology domain (SHD) mediates WPB localization but is not sufficient to enhance WPB exocytosis.

Previously, we have shown that Rab27A recruits its effector Slp4-a to the WPB, which coincides with the acquisition of secretion competence by this secretory organelle\[29\]. WPBs contain a cocktail of secretory Rabs capable of recruiting Slp4-a, including Rab27A and three isoforms of Rab3 (Rab3A, B and D)\[22,23,25,29\]. Slp4-a is reported to bind to these secretory Rabs via its N-terminal Synaptotagmin-like protein Homology Domain (SHD)\[33\]. We speculated that this region was responsible for recruitment of Slp4-a to WPBs and for this purpose we expressed epitope-tagged fusion proteins of Slp4-a truncations that consist of, or lack the SHD (Figure 1A). Full length mCherry-Slp4-a and mCherry-Slp4-a-SHD localized to WPBs, while a truncation mutant lacking the SHD domain (mCherry-Slp4-a-ΔSHD) did not. Instead a diffuse
cytoplasmic staining extending to the cell periphery and possibly the plasma membrane was observed (Figure 1B).

Having confirmed that the SHD domain of Slp4-a was essential for recruitment to WPBs, we next examined whether this region alone was sufficient to support the enhancement of hormone-evoked WPB exocytosis reported for EGFP-Slp4-a-FL\textsuperscript{[29]}. Exogenously expressed EGFP-Slp4-a-SHD labeled WPBs exclusively (Figure 2A). Data from live cell imaging of Ca\textsuperscript{2+}-driven WPB exocytosis in EGFP-Slp4-a-SHD expressing HUVEC (Figure 2B-D) was compared to previously reported data for Proregion-EGFP (which labels the WPB contents and serves as a control) and EGFP-Slp4-a FL expressing HUVECs\textsuperscript{[29]}. As for EGFP-Slp4-a FL, expression of EGFP-Slp4-a-SHD significantly increased the delay in the onset of WPB fusion (Figure 2Di) and reduced the mean maximal rate of WPB exocytosis, although like EGFP-Slp4-a FL, this difference did not reach statistical significance (Figure 2Dii). However, in contrast to EGFP-Slp4-a FL, EGFP-Slp4-a-SHD failed to increase the probability of release (\(P_r\)) of WPBs (Figure 2Diii). This suggested that the capacity to enhance Ca\textsuperscript{2+}-evoked WPB exocytosis must reside in the region of the molecule distal to the SHD.

Figure 1: Targeting of Slp4-a to WPBs depends on its Synaptotagmin-like protein homology domain. (A) Cartoon overview of mCherry-tagged Slp4-a truncation variants, indicating the boundaries of the SHD domain. (B) Immunocytochemical analysis of intracellular localization of Slp4-a variants. HUVECs were transfected with mCherry-tagged constructs and were fixed with paraformaldehyde 24 hours post-transfection. WPBs were visualized by immunostaining for VWF propeptide (green). Magnifications of boxed regions are shown on the rights and the bottom. Arrowheads point to WPBs devoid of mCherry-Slp4-a-ΔSHD. Size bars represent 10 μm.
Syntaxin binding protein 1 (STXBP1) and syntaxin-2 and -3 are targets for Slp4-a in endothelial cells.

To identify effectors of Slp4-a involved in regulated WPB release, we performed pull-downs in HUVEC lysates with GST-Slp4-a-ΔSHD and GST-Slp4-a-SHD. Putative interacting proteins were identified by mass spectrometry following their separation by SDS-PAGE and trypsin-mediated in-gel digestion. As expected, a substantial number of peptides derived from Slp4-a and GST were identified (Figure 3). Using the criteria outlined in Materials and Methods a total number of 324 candidate binding partners for Slp4-a-ΔSHD and 429 potential interactors for Slp4-a-SHD were identified (for full list see Supplementary Table 3A-B). Among the candidate partners for the SHD were Rab8A (Mascot score=176, ProHits frequency=0.0185) and Rab27A (Mascot score=74, ProHits frequency=0.0093) (Figure 3A). A prominent binding partner of Slp4-a-ΔSHD was syntaxin binding protein 1 (STXBP1; Mascot score=925, ProHits frequency=0.0093) (Figure 3A). Seventeen unique peptides corresponding to STXBP1 were identified; the annotated spectrum of

Figure 2: Slp4-a enhances WPB probability of release through a region distal to the SHD domain. (A) HUVEC expressing EGFP-Slp4-a-SHD (green) and immunolabeled for VWF (red). Grayscale images are from the region indicated by the white box. Scale bar represents 20 μm. (B) Fura-2 fluorescence ratio showing a representative record of the increase in fluorescence ratio (intracellular Ca²⁺) upon addition of 1 μM ionomycin. (C) Cumulative plot of WPB fusion times, normalized by their total number, in Proregion-EGFP- (black), EGFP-Slp4-a FL (grey) and EGFP-Slp4-a-SHD expressing cells (red). (Di-iii) Mean delays (seconds), maximal rates of exocytosis (WPBs/second), and probabilities of WPB exocytosis, \( P_r \) (%) for Proregion-EGFP (black), EGFP-Slp4-a FL (grey) and EGFP-Slp4-a-SHD expressing cells (red). Mean % degranulation WPB \( \text{Proregion-EGFP} = 65.8 \pm 1.5\% \), n=46 cells, 2151 fusion events; mean % degranulation WPB \( \text{EGFP-Slp4-a FL} = 85.8 \pm 2.6\% \), n=15 cells, 849 fusion events; mean % degranulation WPB \( \text{EGFP-Slp4-a-SHD} = 65.8 \pm 2.2\% \), n=22 cells, 694 fusion events.
peptide ISEQTYQLSR corresponding to residues 288-297 is displayed in Figure 3B. For Slp4-a-ΔSHD, we identified 6 unique peptides derived from syntaxin-3 (Mascot score=316, ProHits frequency=0.0093) and 1 peptide derived from syntaxin-2 (Mascot score 52, ProHits frequency=0.0278). Annotated spectra

![Figure 3: Proteomic identification of Slp4-a interactors by pull down of GST-tagged Slp4-a truncation variants.](image)

(A) Slp4-a interactors were pulled down from HUVEC lysates by incubation with purified GST-Slp4-a-SHD (left) or GST-Slp4-a-ΔSHD (right) coupled to Glutathione-Sepharose. Eluted proteins were separated by SDS-PAGE and analyzed by mass spectrometry using the criteria described in the Materials and Methods section. Mascot score of putative interactors (GST-Slp4-a-SHD: 429 identifications; GST-Slp4-a-ΔSHD: 324 identifications) was plotted versus number of unique peptides identified. Colored symbols indicate putative interactors that have a ProHits frequency ≤0.0278. Blue symbols represent bait (GST and Slp4-a), green symbols represent interactors shown in the table below which summarizes the Mascot score, number of unique peptides identified and the ProHits frequency. (B) Annotated spectrum of the ISEQTYQLSR peptide corresponding to residues 288-297 of STXBP1.
of Rab8A, Rab27A, syntaxin-2 and -3 are shown in Supplementary Figure 1-4. Identification of STXBP1, syntaxin-2 and syntaxin-3 as putative binding partners of Slp4-a led us to test whether these proteins might exist in a complex in which the interaction of syntaxin-2 or -3 with Slp4-a is mediated through STXBP1, as has been previously suggested. In order to analyze the interaction of STXBP1 with syntaxins in endothelial cells, we performed co-immunoprecipitation studies. Analysis of the co-immunoprecipitates of endogenous STXBP1 revealed that syntaxin-2 and syntaxin–3 associate with STXBP1 in HUVECs (Figure 4A). We also assessed the ability of syntaxin-4 to interact with STXBP1, because this syntaxin has previously been implicated in the regulation of WPB exocytosis. However, syntaxin-4 failed to co-immunoprecipitate with STXBP1 (Figure 4A). When approached from the other end, we could also demonstrate co-precipitation of STXBP1 with syntaxin-3 (Figure 4B). Syntaxin-3 immunoprecipitation did not co-precipitate syntaxin-2, ruling out the possibility that STXBP1 binds syntaxin-2 and -3 simultaneously. Taken together the data indicate that Slp4-a-STXBP1-syntaxin-2 and Slp4-a-STXBP1-syntaxin-3 complexes are present in endothelial cells.

**Figure 4: Interaction of STXBP1 with SNARE proteins in endothelial cells.** Endothelial lysates were incubated with magnetic beads covalently coupled with mouse anti-STXBP1 IgG or an equivalent amount of naïve mouse IgG (A) or with rabbit anti-syntaxin-3 IgG or an equivalent amount of naïve rabbit IgG (B). Lysates (input) and co-immunoprecipitates (IP) were separated by SDS-PAGE and probed with rabbit anti-STXBP1, anti-syntaxin-2, anti-syntaxin-3 or mouse anti-syntaxin-4 followed by incubation with IRdye-labeled donkey anti-rabbit or anti-mouse IgG. Syntaxin-2 and syntaxin-3 but not syntaxin-4 co-immunoprecipitated with STXBP1 (A). Immunoprecipitation of syntaxin-3 co-precipitated STXBP1 but not syntaxin-2 (B).
STXBP1 depletion decreases WPB exocytosis

To address whether STXBP1 is involved in regulating WPB exocytosis, we reduced STXBP1 in primary endothelial cells using siRNA mediated knockdown (Figure 5A). Endogenous expression of STXBP1 protein was decreased to approximately 30% of control levels (Figure 5B). STXBP1 depletion did not affect the number or cellular localization of WPBs (not shown), nor did it affect intracellular levels of VWF (Figure 5C). Slp4-a or Rab27A localization was also not notably altered after STXBP1 knockdown (not shown). However, depletion of STXBP1 impaired both histamine and forskolin-induced VWF secretion (Figure 5D), indicating that STXBP1 is a critical mediator for both Ca\(^{2+}\) and cAMP-triggered WPB exocytosis.

Figure 5: Impaired Ca\(^{2+}\)- and cAMP-mediated VWF secretion after STXBP1 depletion.
(A) HUVEC were nucleofected with siRNA oligos directed against STXBP1 (siSTXBP1) or with non-hybridizing control oligos (siCTRL) and were assayed 48 hours post-nucleofection. Lysates were separated by SDS-PAGE and were probed with rabbit anti-STXBP1 or mouse anti-actin antibodies followed by incubation with IRdye-labeled donkey anti-rabbit or anti-mouse IgG. (B) Quantification of STXBP1 expression in control cells (black) or after STXBP1 depletion (white) (n=3). (C) Endothelial cells were lysed in SF medium containing 1% Triton X-100 and were assayed for VWF content by ELISA (n=3). (D) Endothelial cells were incubated for 30 minutes with SF medium (basal), SF medium supplemented with 100 mM histamine (HIS) or with 10 mM forskolin and 100 mM IBMX (FSK). Supernatants were assayed for secreted VWF by ELISA. *** P<0.005 (n=3).
Impaired WPB exocytosis in BOECs derived from an EIEE4 patient.

To further test the function of STXBP1 we determined whether hormone-evoked secretion of VWF was impaired in endothelial cells derived from a patient with a known defect in STXBP1. Early infantile epileptic encephalopathy type 4 (EIEE4) is a rare but very severe epileptic disorder characterized by defective neurotransmitter release as a consequence of de novo loss-of-function mutations in the STXBP1 gene\cite{30-32}. As such, individuals with EIEE4 represent human models of STXBP1 dysfunction. We isolated blood outgrowth endothelial cells (BOECs) from a 16 year old EIEE4 patient carrying a de novo heterozygous 23 to 35.4 Kbp microdeletion in STXBP1 [c.963 +? (1967+?) del; p.Thr322_Glu603 del]\cite{30}. This deletion affects exons 12 to 20 of STXBP1.

Figure 6: Characterization of EIEE4 BOECs. (A) Healthy control donor and EIEE4 BOEC lysates were separated by SDS-PAGE and were probed with rabbit anti-STXBP1 or mouse anti-actin antibodies followed by incubation with IRdye-labeled donkey anti-rabbit or anti-mouse IgG. (B) Quantification of STXBP1 expression in healthy control donor (black) or EIEE4 BOECs (white) (n=3). (C) EIEE4 and control donor BOECs were fixed with paraformaldehyde and immunostained for VWF (green) and VE-cadherin (red). Bar represents 10 μm.
and is predicted to result in either a truncated protein spanning amino acid 1 – 321 (provided that the mRNA transcript undergoes translation) or in nonsense mediated decay, which would result in haploinsufficiency\textsuperscript{311}. In this patient circulating plasma levels of VWF:Ag (64 IU/dL) were at the low end of the physiological range (50-200 IU/dL). BOEC colonies were derived from peripheral blood and were pooled for further analysis. As shown in Figure 6A, endogenous STXBP1 protein expression was decreased in EIEE4 BOECs compared to BOECs derived from a healthy control individual (49.6 ± 5.4% residual STXBP1 protein level as estimated by immunoblotting, Figure 6B). Using two antibodies directed against STXBP1 we were unable to detect the predicted truncated form of STXBP1 in EIEE4 BOECs (data not shown), supporting the hypothesis that the aberrant transcript is degraded. The decrease in STXBP1 protein expression did not affect WPB morphology or numbers as shown by immunocytochemistry (Figure 6C). In addition, we did not observe changes in the recruitment of Slp4-a or Rab27A to WPBs (Supplemental Figure 5-6), suggesting that the upstream components of the exocytotic machinery were unperturbed. EIEE4 BOECs contained slightly more VWF when compared to healthy donor BOECs however this difference was not statistically significant (Figure 7A). We next tested whether agonist-induced release of VWF was impaired in EIEE4 BOECs. Unstimulated (basal) VWF secretion was slightly lower in EIEE4 BOECs but this was not significant compared to the control BOECs of a healthy donor. However, both histamine- and forskolin-stimulated VWF secretion were significantly reduced in the EIEE4 BOECs compared to the control (Figure 7B).

Figure 7: Impaired Ca\textsuperscript{2+} - and cAMP-mediated VWF secretion in EIEE4 BOECs. (A) Endothelial cells were lysed in SF medium containing 1% Triton X-100 and lysates of healthy control donor (black) or EIEE4 BOECs (white) were assayed for VWF content by ELISA. (B) BOECs were incubated for 30 minutes with SF medium (basal), SF medium supplemented with 100 mM histamine (HIS) or with 10 mM forskolin and 100 mM IBMX (FSK). Supernatants were assayed for secreted VWF by ELISA. *** P<0.005; n=3.
Discussion
The small GTPase Rab27A and its effectors MyRIP, Munc13-4 and Slp4-a play key roles as regulators of WPB exocytosis\cite{25,27-29}. Each of these effectors perform discrete steps in the sequence of events that follow secretory organelle formation until eventual exocytosis, with some of them exerting opposing effects (MyRIP - Myosin Va vs. Slp4-a and Munc13-4) on the probability of release of the WPB. A complicated picture emerges, in which the releasability of a WPB is determined by the balance of effectors present on its membrane\cite{29} and the efficacy of the individual effectors’ downstream mechanisms. While the mechanism of action of MyRIP, a negative-regulator of WPB exocytosis, has been partially elucidated\cite{28}, how Slp4-a and Munc13-4 function as positive regulators of WPB exocytosis remains unclear. Here we provide evidence that Slp4-a functions through the recruitment of STXBP1.

The SHD-domain of Slp4-a contains binding sites for several secretory Rabs, including Rab27A, Rab3 and Rab8. Consistent with this, our unbiased proteomic analysis identified Rab27A and Rab8A as binding partners for the SHD-domain of Slp4-a. Earlier work from our group has shown that WPB localization of Slp4-a depends on the small GTPases Rab27A and (primarily) Rab3B, however only Rab27A-bound Slp4-a was found to be functionally involved in exocytosis. Surprisingly, we did not identify peptides of any of the Rab3 isoforms in our pull down with Slp4-a-SHD, although FRAP studies suggest that the majority of WPB localized Slp4-a is in complex with Rab3B\cite{29}. The likely explanation is that the Slp4-a-Rab3 interaction is weaker or more labile than the corresponding interactions with Rab27A or Rab8. This idea is supported by both co-immunoprecipitation studies which show a very weak pull down of Rab3’s compared to Rab27A and by FRAP analysis of WPB-dissociation rates for Slp4-a in the presence of exogenously expressed Rab27A, Rab3B or Rab3D\cite{29}. In the latter case the dissociation rate for Slp4-a in the presence of Rab27A was an order or magnitude slower than in the presence of Rab3B or D, indicating a strong interaction between these molecules in living cells. Similar observations have been made in PC12 cells; while Rab3A was shown to interact with Slp4-a in an in vitro binding assay in the absence of Rab27A and Rab8A\cite{36}, only Rab27A and Rab8A could be detected after co-immunoprecipitation with Slp4-a\cite{37}. The strong interaction between Rab27A and Slp4-a might reflect in part the unique ability of this Rab protein to bind Slp4-a irrespective of its GTP/GDP status\cite{33}. Together these properties may have contributed to a competitive advantage for Rab27A during our pull down procedure. We also identified Rab8A in our screen, a protein that is localized to
the Golgi apparatus and is not recruited to WPBs, which makes it an unlikely candidate to contribute directly to WPB localization of Slp4-a\textsuperscript{[38]}. Rab8A-bound Slp4-a has been reported to exert different effects on secretion depending on the cell type studied\textsuperscript{[37,39,40]}. Nonetheless, depletion of Rab8A has been found to decrease phorbol-ester-induced VWF release from endothelial cells\textsuperscript{[38]}, whether this is mediated via Slp4-a remains to be established.

Live cell imaging of WPB exocytosis in EGFP-Slp4-a-SHD expressing cells showed that the SHD domain of Slp4-a alone was not sufficient to enhance hormone-evoked WPB exocytosis. This result suggests two things: 1) that the capacity of exogenously expressed Slp4-a to promote WPB exocytosis is unlikely to be due solely to displacement of the negative regulator MyRIP from the WPB\textsuperscript{[29]}, but must instead reflect an active property of Slp4-a; 2) that this activity requires the region of the molecule distal to the SHD. We identified STXBP1, syntaxin-2 and syntaxin-3 as endogenous molecules that interact specifically with this distal region of Slp4-a. STXBP and syntaxins are key components of the SNARE machinery, a multi protein complex that catalyzes the fusion of membranes\textsuperscript{[41]}.

Our data suggest a crucial role for STXBP1 in both Ca\textsuperscript{2+}- and cAMP-mediated VWF secretion from endothelial cells. In addition, stimulated VWF secretion of BOECs derived from an EIEE4 patient, a human model of STXBP1 haploinsufficiency, was impaired. Previous studies demonstrate that STXBP1 is essential for secretory vesicle release, illustrated by the fact that STXBP1 null mutant mice showed reduction of Ca\textsuperscript{2+} regulated synaptic vesicle exocytosis from neuroendocrine cells\textsuperscript{[42,43]}. Furthermore, insulin granule docking and secretion from pancreatic β-cells as well as catecholamine-containing large dense core vesicle docking and secretion from chromaffin cells are impaired by STXBP1 deletion\textsuperscript{[44-46]}. The severe neurological defects displayed by patients with EIEE4 are also attributed to abnormalities in synaptic vesicle release\textsuperscript{[30,31]}.

On this basis it is likely that STXBP1 also contributes to the process of WPB docking in endothelial cells, most likely by linking the WPB (through Rab27A-Slp4-a) to plasma membrane syntaxins.

The physiological significance of regulation of WPB exocytosis by SNARE proteins is also underscored by a number of recent genome wide association studies on VWF plasma levels and associated risk factors for cardiovascular diseases such as arterial and venous thrombosis. Single nucleotide polymorphisms (SNPs) in syntaxin-2 and also STXBP5 (a member of the same protein family as STXBP1) have been associated with circulating VWF levels, risk of venous thrombosis and severity of the bleeding phenotype in Von
STXBP1 promotes Weibel-Palade body exocytosis

Willebrand Disease type 1 patients\(^\text{[47-50]}\). It is intriguing to speculate that these deficits reflect disruption of the SNARE machinery regulating WPB exocytosis and VWF secretion.

In the EIEE4 patient studied, plasma VWF levels were found to be on the low side of the normal range. Such a mild quantitative deficiency in VWF does not qualify as VWD type 1 according to current laboratory criterions\(^\text{[51]}\) nor does it confer a high risk of bleeding, and indeed this patient has no such history. Circulating levels of VWF are determined primarily by unstimulated (basal) secretion of VWF from endothelial cells, which is now known to arise primarily through basal WPB exocytosis\(^\text{[52]}\). It is interesting to speculate whether the small (but non-significant) reduction in basal secretion of VWF identified in cultured BOECs isolated from the EIEE4 patient might reflect the function of the patients own endothelium, accounting for a slightly low circulating level of plasma VWF. Furthermore, based on our findings with EIEE4 BOECs we hypothesize that the effect of STXBP1 haploinsufficiency on VWF secretion would present itself more prominently upon acute endothelial stimulation.

Patients with EIEE4 display severe neurological defects including early onset epilepsy and intellectual disability, which have been attributed to abnormalities in synaptic vesicle release\(^\text{[30,31]}\). The neurological deficit suggests that regulation of synaptic vesicle release and hence neurodevelopment only properly operates within a critical window of STXBP1 levels\(^\text{[43]}\). It is striking that the neurological defects are not accompanied by considerable hemostatic complications given that they utilize the same aberrant secretory machinery.

STXBP1 has also been implicated in insulin release\(^\text{[44]}\). Although moderately elevated levels of blood glucose have been observed in mice heterozygous for STXBP1 deficiency as a result of slightly decreased insulin release\(^\text{[44]}\), no altered glucose levels or symptoms related to aberrant insulin secretion have so far been reported in patients with EIEE4. This suggests that the consequences of perturbation of the secretory machinery in pancreatic β-cells or endothelial cells caused by haploinsufficiency of STXBP1 are not as detrimental as in neurons. Rapid, coordinated release of a sufficient dose of neurotransmitters is required to support synaptic transmission from one neuron to the other and the maintenance of synaptic connections; in contrast release of systemically acting compounds such as hormones (insulin) or (hemostatic) components released from WPBs operate in a much broader time-frame and dosage bandwidth and may therefore be slightly more lenient to partial STXBP1 deficiency.
A growing number of molecules have been identified as components of the control mechanism for stimulated WPB exocytosis\textsuperscript{[33]}. Earlier work from Fu and colleagues has shown the involvement of Munc18c (STXBP3) and syntaxin-4 in thrombin stimulated P-selectin expression, which coincides with release of Munc18c – Syntaxin-4 interaction after protein kinase C dependent phosphorylation events on either partner\textsuperscript{[35]}. Syntaxin-4 associates with SNAP23 and the R-SNARE VAMP3 which, along with VAMP8 (which does not associate with syntaxin-4), is localized on WPBs\textsuperscript{[54]}. From our and these observations it becomes apparent that there is both redundancy within the SNARE molecules involved as well as a considerable resilience of endothelial cells to conditions that perturb SNARE complex formation\textsuperscript{[54,55]}. Together this may account for the lack of a clear hemostatic defect in the EIEE4 patient. The apparent redundancy of SNARE complexes operating in endothelial cells might allow for different upstream signaling cascades to target discrete exocytotic machineries, providing the endothelium with the possibility to fine tune its secretory response.

**Author Contributions**

DvB, BS, NH, KvH and RB performed research and analyzed data; SW, BC, PDJ, JE and KV contributed vital reagents; DvB, NH, MF-B, JV, MH, TC and RB designed the research; DvB, BS, JV, TC and RB wrote the paper.

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References


Supplementary tables and figures

**Supplemental Table 1**

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**Supplemental Figure 1:** Proteomic identification of Rab8A as interactor of Slp4-a-SHD. Annotated spectrum of peptide ANINVENAFFTLAR, corresponding to residues 152-165 of Rab8A.
Supplemental Figure 2: Proteomic identification of Rab27A as interactor of Slp4-a-SHD. Annotated spectrum of peptide FLALGDSGVGK, corresponding to residues 12-22 of Rab27A.

Supplemental Figure 3: Proteomic identification of Rab8A as interactor of Slp4-a-ΔSHD. Annotated spectrum of peptide NSIDKITQYVEEVKK corresponding to residues 41-55 of syntaxin-2.

Supplemental Figure 4: Proteomic identification of Rab8A as interactor of Slp4-a-ΔSHD. Annotated spectrum of peptide TKDDLEQLTTEIK corresponding to residues 73-85 of syntaxin-3.
Supplemental Figure 5: Rab27A expression in healthy control donor and EIEE4 BOECs. EIEE4 and control donor BOECs were fixed with paraformaldehyde and immunostained for VWF (mouse anti-VWF RAg20; Alexa 568 anti-mouse IgG; green) and Rab27A (rabbit anti-Rab27A; Alexa 488 anti-rabbit IgG; red). Boxed areas are shown in bigger magnification. Bar represents 10 μm.

Supplemental Figure 6: Slp4-a expression in healthy control donor and EIEE4 BOECs. EIEE4 and control donor BOECs were fixed with paraformaldehyde and immunostained for VWF (mouse anti-VWF RAg20; Alexa 568 anti-mouse IgG; green) and Rab27A (rabbit anti-Slp4-a; Alexa 488 anti-rabbit IgG; red). Boxed areas are shown in bigger magnification. Bar represents 10 μm.