Sec22b determines Weibel-Palade body length by controlling anterograde ER-Golgi transport

by Ellie Karampini, Petra E. Bürgisser, Jenny Olins, Aat A. Mulder, Carolina R. Jost, Dirk Geerts, Jan Voorberg, and Ruben Bierings

Haematologica 2020 [Epub ahead of print]

Citation: Ellie Karampini, Petra E. Bürgisser, Jenny Olins, Aat A. Mulder, Carolina R. Jost, Dirk Geerts, Jan Voorberg, and Ruben Bierings. Sec22b determines Weibel-Palade body length by controlling anterograde ER-Golgi transport. Haematologica. 2020; 105:xxx
doi:10.3324/haematol.2019.242727

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Sec22b determines Weibel-Palade body length by controlling anterograde ER-Golgi transport

Ellie Karampini¹, Petra E. Bürgisser², Jenny Olins¹, Aat A. Mulder³, Carolina R. Jost³, Dirk Geerts⁴, Jan Voorberg¹,⁵ and Ruben Bierings¹,²

¹Molecular and Cellular Hemostasis, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Center, University of Amsterdam, The Netherlands, ²Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands, ³Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands, ⁴Medical Biology and ⁵Experimental Vascular Medicine, Amsterdam University Medical Center, University of Amsterdam, The Netherlands.

Running head: Sec22b controls VWF trafficking and WPB size

Corresponding author:
Dr. Ruben Bierings, Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands. e-mail: r.bierings@erasmusmc.nl

Word count: 3563  Figure count: 5
Abstract

Von Willebrand factor (VWF) is a multimeric hemostatic protein that is synthesized in endothelial cells, where it is stored for secretion in elongated secretory organelles, so-called Weibel-Palade bodies (WPBs). Hemostatic activity of VWF is strongly tied to WPB length, but how endothelial cells control the dimensions of their WPBs is unclear. In this study we used a targeted shRNA screen to identify the longin-SNARE Sec22b as a novel determinant of WPB size and VWF trafficking. We found that Sec22b depletion resulted in loss of the typically elongated WPB morphology along with disintegration of the Golgi and dilation of rough ER (rER) cisternae. This was accompanied by reduced proteolytic processing of VWF, accumulation of VWF in the dilated rER and reduced basal and stimulated VWF secretion. Our data demonstrate that the elongation of WPBs, and thus adhesive activity of its cargo VWF, is determined by the rate of anterograde transport between ER and Golgi, which depends on Sec22b-containing SNARE complexes.
Introduction

ER-to-Golgi transport is the first step in the secretory pathway. As eukaryotic cells are vastly compartmentalized, ER is the first stop in protein production as well as the initially quality check point whether proteins are correctly folded. Correctly folded proteins are then trafficked to the Golgi where they are additionally modified before being directed to their appropriate destination: endo/lysosome, plasma membrane or secretion. At the trans-Golgi network (TGN), proteins will either enter the “constant” constitutive pathway for unimpeded release, or they are temporarily stored in secretory vesicles, often of the lysosome-related organelle (LRO) family, for regulated secretion. Storage and regulated secretion allows the immediate discharge of larger protein quantities in a correct physiological setting.

Biogenesis of LROs is crucial for the proper function of a wide variety of cells, their importance being well-highlighted by the fact that defective LRO formation results in a vast manifestation of clinical abnormalities that include bleeding, immunodeficiency, hypopigmentation and neurological defects. Within the LRO family, Weibel-Palade bodies (WPBs) are the storage organelles of endothelial cells (ECs). WPBs primarily contain von Willebrand factor (VWF), a large multimeric hemostatic protein that serves a critical role in platelet adhesion and as chaperone for coagulation factor VIII. WPB biogenesis is directly dependent on synthesis and correct post-translational processing of VWF. WPBs have a distinct, elongated morphology that is intrinsically linked to the inherent ability of VWF multimers to self-organize in tubules when exposed to the internal milieu of the TGN. Quantitative or qualitative defects in VWF, for instance due to mutations in VWF, cause von Willebrand disease (VWD), the most common inherited bleeding disorder. VWF mutations that affect its synthesis or processing often result in altered WPB morphology, with WPBs being either round or short. Upon regulated, explosive release from WPBs, VWF unfurls into strings of up to 1 mm long that are anchored on the apical side of the endothelium. VWF strings create an adhesive platform for platelets to initiate the formation of the initial platelet plug at site of vascular damage. The adhesive capacity of VWF towards
platelets and self-associating plasma VWF is proportional to WPB size 23. In turn, WPB size is determined before budding from the TGN by incorporation of so-called “VWF quanta” and it was previously shown that reduced VWF synthesis or unlinking of Golgi-stacks affects WPB length 24. However, how ECs control WPB size and thus hemostatic activity of VWF is largely unknown.

Due to the distinctive shape of their WPBs, ECs are an excellent model system for elucidating how cells manage formation and morphology of LROs. As WPB formation is VWF driven, monitoring intracellular VWF trafficking can be used as a tool to study the complex mechanisms involved. VWF undergoes extensive post-translational modification during its path through the endothelial secretory pathway 25. VWF enters the endoplasmic reticulum (ER) as a single pre-pro-polypeptide chain which forms tail-to-tail dimers by formation of disulfide bonds between the C-terminal cysteine knot (CTCK) domains of two proVWF monomers 26. After dimerization-dependent ER exit, proVWF dimers are transported to the Golgi, where VWF propeptide is cleaved from the proVWF chain. Inter-dimer disulfide bonds between cysteines in the D3 domains lead to formation of head-to-head VWF multimers 27. VWF multimers are then condensed into tubules that are packaged into newly forming WPBs that emerge from the TGN 28, 29.

Trafficcking of proteins during formation and maturation of subcellular organelles such as WPBs is dependent on membrane fusion, which is universally controlled by SNARE proteins 30. The SNARE complex consists of a v-SNARE on the vesicle membrane and t-SNAREs on the acceptor membrane which together form a four-helix bundle that allows the membranes to fuse. Although several SNAREs have been associated with WPB exocytosis 7, the SNAREs taking part in WPB and LRO biogenesis are not known. The subfamily of longin-SNAREs (VAMP7, YKT6 and Sec22b), which derives its name from an N-terminal self-inhibitory longin-domain that can fold back on the SNARE domain, controls membrane fusion events that traffic proteins to and from the Golgi 31.

In this study we addressed the role of longin-SNAREs in the formation of WPBs. Using a targeted shRNA screen of longin-SNAREs in primary ECs we identify Sec22b as a novel
determinant of WPB morphology. Sec22b silencing results in short WPBs, disintegration of
the Golgi complex, reduced proVWF processing and retention of proVWF in a dilated ER.
Our data suggest that the distinctive morphology of WPBs and thus the adhesive activity of
its main cargo VWF is determined by the rate of membrane fusion between ER and Golgi,
which is dependent on Sec22b-containing SNARE complexes.
Methods

Antibodies
Antibodies used in this study are listed in Supplementary Table S1.

Cell culture, lentiviral transfection and transduction
Pooled, cryo-preserved primary human umbilical vein endothelial cells (HUVECs) were obtained from Promocell (Heidelberg, Germany). HUVECs were cultured in EGM-18 medium; EGM-2 medium (Lonza, Basel, Switzerland, CC-3162) supplemented with 18% FCS (Bodinco, Alkmaar, Netherlands). Human embryonic kidney 293T (HEK293T) cells were obtained from ATCC (Wessel, Germany) and were grown in Dulbecco’s modified Eagle medium containing D-glucose and L-glutamine (Lonza, Basel, Switzerland) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293Ts were seeded on collagen coated plates or flasks and were transfected with 3rd generation lentiviral packaging plasmids pMD2.G, pRSV-REV and pMDLg/pRRE (Addgene, Cambridge, USA) using transit-LT1 (Mirus Bio LLC, Madison, WI, USA) following the supplier’s protocol. After 6-8h incubation, the medium was exchanged for EGM-18. Virus particles were collected 24 and 48 hours following transfection and were filtered through 0.45 μm pore filters in EGM-18. Two batches of virus were used to transduce HUVECs, cord blood BOECs (cbBOECs) or HEK293T cells for the period of 48 hours. Transduced ECs were selected by puromycin (0.5 μg/ml) that was added to the medium for 72 hours after the second virus installment.

DNA constructs for shRNA silencing of longin-SNAREs, CRISPR editing and mEGFP-Sec22b-ΔSNARE
The LKO.1-puro-CMV-mEGFP-U6-shC002 vector, which simultaneously expresses mEGFP and a non-targeting control shRNA from the CMV and U6 promoter respectively, was described previously 32. shRNAs targeting Sec22b, VAMP7 and YKT6 were obtained from the MISSION® shRNA library developed by TRC at the Broad Institute of MIT and Harvard
and distributed by Sigma-Aldrich (Supplementary Table S2). Fragments containing the shRNA expression cassette from the shRNA library were transferred to the LKO.1-puro-CMV-mEGFP-U6 vector by SphI-EcoRI subcloning. CRISPR mediated depletion of Sec22b in HUVEC was performed essentially as described previously 33. LentiCRISPR_v2 (a gift from Dr. Feng Zhang; Addgene #52961), a lentiviral vector which simultaneously expresses Cas9 endonuclease and guide RNAs (gRNAs) has been described previously 34. gRNAs were designed to target exon 1 of the SEC22B gene using the CRISPOR Design tool (http://crispor.tefor.net/)35 by submitting the DNA sequence of SEC22B exon 1 flanked by 100 bp up- and downstream (chromosome 1: 120,1501898-120,176,515 reverse strand; Supplemental Figure 2A). gRNA sequences were selected that have a high predicted efficiency with limited off-target effects. gRNAs used in this study are shown in Supplemental Table S3 and were cloned as hybridized complementary oligos (with BsmBI restriction site compatible overhangs on either side) into BsmBI-digested LentiCRISPR_v2 plasmid. LVX-mEGFP-LIC has been described previously 36. To construct a human Sec22b variant that lacks its SNARE domain (Gly135-Lys174), a synthetic Sec22b fragment was generated by gene synthesis in which codons 135-174 were removed from the 214 codon Sec22b coding sequence and which was flanked by BsrGI and NotI sites, respectively. The resulting Sec22b-ΔSNARE fragment was cloned in frame behind mEGFP in LVX-mEGFP-LIC by subcloning between BsrGI and NotI sites. All constructs were sequence verified. Lentiviral plasmids were produced in Stbl3 bacteria.

Further details on materials and methods are found in the Supplementary Data.
Results

Weibel-Palade body length is significantly reduced upon Sec22b silencing

To determine the role of longin-SNAREs in WPB biogenesis we performed a targeted shRNA screen against VAMP7, YKT6 and Sec22b in human umbilical vein endothelial cells (HUVECs) and evaluated WPB morphology by VWF immunostaining. As shown in Figure 1A, in shCTRL, shVAMP7 and shYKT6 transduced cells, VWF was primarily stored in typical cigar-shaped WPBs. However, upon Sec22b silencing (shSec22b) (Fig. S1), WPBs had lost their characteristic elongated morphology and appeared short and “stubby”. Quantification of WPB length in control and knockdown (KD) cells showed a significant reduction in WPB length after Sec22b KD, whereas no difference was found in the absence of VAMP7 or YKT6 (Fig. 1B). To further substantiate Sec22b’s role in WPB formation we used CRISPR-mediated SEC22B-editing of HUVECs (Fig. S2A-C). Cells depleted of Sec22b were identified by Sec22b staining (Fig. S2D). A similar reduction of WPB length was observed in ECs targeted with 3 separate gRNAs directed to exon 1 of Sec22b (Fig. 1B, S2E). As a third, independent strategy we also determined WPB morphology after expression of an mEGFP-tagged non-fusogenic Sec22b variant (mEGFP-Sec22b-ΔSNARE), which lacks the SNARE domain responsible for fusion and compared these with mEGFP expressing cells (Fig. 1D). We found that the ΔSNARE construct had a dominant negative effect on WPB size, with WPBs significantly shorter compared to mEGFP control (Fig. 1E). To investigate whether the size reduction extends to other (post-Golgi) organelles, we determined the localization of the tetraspanin CD63. CD63 normally cycles between plasma membrane, endo-lysosomal organelles and WPBs in an AP-3-dependent manner 33. Silencing of Sec22b did not lead to apparent changes in the morphology of CD63+ endolysosomal organelles, nor did it impede the trafficking of CD63 to shorter WPBs (Fig. S3). Together these results identify Sec22b as a determinant of secretory organelle size in ECs.
Sec22b silencing results in unlinked Golgi ribbon

Since the size of nascent WPBs is regulated by incorporation of multiple so-called VWF quanta from the TGN 24, we investigated TGN morphology in Sec22b-depleted cells. TGN46 immunostaining showed that while in control cells the TGN had a compact morphology, shSec22b-treated cells exhibited a dispersed TGN morphology, consistent with an unlinked Golgi ribbon (Fig. 2A-B). Quantification of the area that encompasses the entire TGN46 immunoreactivity in shSec22b and shCTRL cells revealed that, due to their fragmentation, TGNs in Sec22b depleted cells extended to a significantly larger intracellular area than the compact TGNs in control cells (Fig. 2C). The crucial role for Sec22b in maintaining Golgi integrity is not limited to endothelial cells, illustrated by a similar effect on Golgi morphology in Sec22b-depleted HEK293T cells (Fig. S4). It has previously been described that unlinking Golgi stacks using depletion of Golgi matrix proteins or nocodazole gives rise to shorter WPBs 24. When evaluating WPB length in shSec22b cells with compact vs. dispersed TGN we also observed that in those cells in which the Golgi was dispersed, WPBs were on average shorter than in those with the Golgi intact (Fig. 2D), which suggests that the reduction in WPB length after Sec22b depletion is (at least partly) a consequence of Golgi disintegration.

Sec22b silencing results in decreased VWF trafficking to the Golgi and VWF retention in the ER

As Sec22b has been associated with membrane fusion events during anterograde and retrograde trafficking between ER and Golgi 31, we evaluated VWF trafficking in the endothelial early secretory pathway. A key step during VWF biosynthesis is the proteolytic cleavage of proVWF into VWF propeptide and mature VWF, which takes place upon its arrival in the Golgi 37. We used the intracellular ratio between the two distinct forms of VWF, proVWF (ER) and mature VWF (Golgi & post-Golgi), as a measure for ER and Golgi transport by estimating the amount of proVWF and VWF in shCTRL and shSec22b ECs (Fig. 3A). While the total amount of mature VWF was markedly reduced in shSec22b cells,
the proportion of VWF in the unprocessed proVWF form was increased. Therefore, the proVWF:VWF ratio was significantly increased in the Sec22b KD ECs (Fig. 3B). This suggests that proteolytic processing of proVWF is reduced in the absence of Sec22b, possibly due to a reduced flux of VWF from the ER. Consistent with this we observed increased VWF immunoreactivity in reticular perinuclear structures resembling the ER after Sec22b silencing (Fig. 3C). Simultaneous with - but independent of - proteolytic processing, VWF dimers oligomerize into long VWF multimers in the Golgi. VWF multimer analysis using SDS-agarose gel electrophoresis showed that Sec22b silencing did not affect multimerization per se, as evidenced by high molecular weight VWF multimers in lysates of shCTRL and shSec22b ECs. However, the increased proportion of VWF dimers in the Sec22b KD cells indicates that VWF is retained at the ER (Fig. 3D-E). Together this points to a reduction of anterograde ER-Golgi trafficking of VWF in the absence of Sec22b.

**Sec22b silencing results in accumulation of VWF in diluted rough ER.**

Since VWF was retained in the ER, potentially along with other proteins, we used electron microscopy (EM) to examine the impact of reduced anterograde trafficking on the ER (Fig. 4A). When Sec22b was silenced the rough ER (rER) appeared enlarged and ribosome-studded membrane-limited rounded structures developed with electron dense content. These rER structures represent severely diluted ER cisternae as they often retained a membranous connection to the rER. The dilated rER phenotype was observed in the majority of Sec22b depleted cells (72.9 %) (Fig. 4B). Closer examination of the rER morphology revealed that apart from the round rER structures (asterisks), the luminal width of ER sheets (arrowheads) was significantly increased in Sec22b KD cells (0.29 μm +/- 0.18 μm) when compared to control cells (0.10 μm +/- 0.01 μm) (Fig. 4C). This suggests that upon removal of Sec22b the rER expands its size dramatically, possibly to facilitate the accumulation of secretory proteins like VWF. Indeed, immunogold staining for VWF in Sec22b KD ECs localized within diluted rER and was prominently found in the round dense
rER structures (Fig. 4D). Taken together this shows that VWF exits the ER in a Sec22b dependent manner and upon Sec22b silencing it is retained in rER derived structures.

**ER VWF retention results in reduced VWF secretion in Sec22b depleted cells**

The lack of mature VWF, as well as the shorter WPB size, prompted us to investigate how much VWF is stored and secreted in absence of Sec22b. We observed that in the Sec22b-depleted cells, intracellular VWF levels were slightly increased when compared to control cells (Fig. 5A), potentially due to VWF entrapment in the ER. On the other hand, basal secretion was significantly decreased in Sec22b silenced cells (Fig. 5B). Basal secretion primarily originates from unstimulated WPB release \(^{38,39}\), suggesting this compartment is smaller upon Sec22b depletion. In line with this, VWF release through histamine-induced WPB exocytosis was also significantly reduced (Fig. 5C). WPBs acquire secretion competence during maturation by recruiting Rab GTPases and Rab-effectors \(^7\), so potentially this decrease could be attributed to defects in WPB maturation in Sec22b-depleted cells. We assessed whether two maturation-dependent components of the exocytotic machinery, Rab27A and Slp4-a \(^{40,41}\), were recruited to WPBs but found no difference between shSec22b and shCTRL cells (Fig. S5). Thus, the simplest explanation for reduction in (stimulated) VWF secretion is failure of sufficient VWF to progress to a stimulus-sensitive compartment, i.e. the WPBs, because it is retained in the ER.
Discussion

SNARE proteins are key drivers of membrane fusion that initiate and regulate specificity of membrane docking and bilayer mixing \(^{30}\). There is a subcategory of SNAREs, known as longin-SNAREs (YKT6, VAMP7 and Sec22b), that generally participate in fusion events during biogenesis, maturation and exocytosis of secretory organelles in eukaryotic cells \(^{42-46}\). In this study, we used a short hairpin RNA screen targeting longin-SNAREs to identify Sec22b as a novel regulator of WPB formation and VWF trafficking, its depletion leading to short and “stubby” WPBs that were accompanied with Golgi disintegration and retention of VWF in the endoplasmic reticulum.

Sec22b is a known participant in anterograde transport of cargo in the early secretory pathway \(^{47,48}\). Overexpression of the Sec22b-ΔSNARE variant led to a dominant negative effect resulting in smaller WPBs, corroborating experiments that used RNAi and CRISPR strategies to deplete Sec22b expression. This is most probably a consequence of Sec22b-ΔSNARE outcompeting endogenous Sec22b while failing to properly bind with its cognate SNAREs due to the lack of the SNARE helix \(^{49}\), which precludes formation of a complete trans-SNARE complex and subsequent membrane fusion. The importance of a functional, fusion competent Sec22b has also been highlighted by mutations in the SNARE domain of the *Drosophila* homologue of Sec22b, which caused disruption of ER-Golgi transport and resulted in cargo retention in the ER and abnormal ER morphology \(^{50}\); similar ER cargo retention is likely at play in Sec22b-depleted ECs in our study (Fig. 3&4). Congestion of anterograde flux from the ER limits the supply of VWF to the Golgi and as WPB formation is driven by condensation of multimeric VWF in the TGN, we assume this reduction is (at least partly) responsible for the smaller size of WPBs.

The smaller WPB phenotype is likely further exacerbated by the fragmentation of the TGN (Fig. 2), which was previously proposed to limit the possibility of adjacent VWF quanta copackaging into a single, extended WPB \(^{24}\). Interestingly, that study also demonstrated that experimental reduction of VWF trafficking by siRNA silencing of VWF synthesis led to
reduction of WPB length without affecting overall Golgi morphology. The data we present here suggest that a Sec22b-dependent trafficking pathway is used both by VWF and by components that establish or maintain Golgi ribbon integrity. This is in line with previous studies of Sec22b in other models, such as an Arabidopsis mutant deficient for the Sec22b homolog, which displayed comparable TGN disruption. The observed phenotype may be a consequence of defective trafficking of direct regulators of Golgi morphology, such as Golgi reassembly stacking proteins (GRASPs) or Golgins. Indeed, Golgi fragmentation and the concomitant length reduction of WPBs has also been observed after depletion of Golgi tethering proteins (GM130, GRASP55 and Giantin). TGN fragmentation may also merely result from the induced imbalance in trafficking, as the phenotype was also reported after disruption of retrograde trafficking from early endosomes or within the Golgi. While further investigation is required to decipher the precise role of Sec22b in the maintenance of TGN morphology, the resulting consequences highlight its indispensable function in ensuring adequate trafficking of VWF and WPB biogenesis.

Upon Sec22b silencing, dilated ER cisternae were observed accompanied by electron dense ribosome-studded rER structures that contain VWF aggregates. There is a striking resemblance with the dilated ER morphology that is observed in response to VWD causing mutations in VWF that affect its ability to dimerize and leave the ER. Sec22b is recruited onto ER-derived COPII vesicles that transfer proteins from the ER to the Golgi, through interactions of its longin domain with Sec23/Sec24 of the COPII coating complex. Similar dilated ER phenotypes and ER retention of secretory proteins have been previously described in chondrocytes from sec24d deficient zebrafish and in pancreatic acinar cells from Sec23bgt/gt mice that additionally display lack of zymogen granules. This suggests that the rate of COPII-mediated anterograde ER-Golgi traffic underpins the ability of ECs to shape WPBs to their typical elongated morphology.

A recent study has identified GBF1 as a dynamic regulator of anterograde VWF trafficking and WPB morphology that, dependent on external/environmental cues, controls the flux of proteins (including VWF) from ER to Golgi. Similar to what we observed after Sec22b
silencing, depletion of GBF1 led to accumulation of VWF in the ER and a reduction in the overall state of VWF proteolytic processing. However, a number of important phenotypic differences suggest that GBF1 and Sec22b operate through different mechanisms. Unlike Sec22b, GBF1 depletion did not affect Golgi morphology and, unexpectedly, resulted in unusually large WPBs that remain associated with or in close vicinity of the Golgi. Despite their length reduction, WPBs in Sec22b depleted cells normally recruited exocytotic components such as Rab27A and Slp4-a (Fig. S5), contrary to the enlarged WPBs in GBF1 ablated cells which failed to acquire post-Golgi cargo and Rab27A and which were secretion incompetent. Although their short WPBs were still agonist responsive, Sec22b-depleted ECs secreted reduced levels of VWF through the regulated and basal secretory pathway (Fig. 4B-C), which we presume is due to a reduction in WPB pool size. These discrepancies emphasize that future studies are needed to clarify how such opposing effects on WPB formation and secretion can arise from defects in anterograde ER-Golgi transport.

In sum, our study has identified Sec22b as a new regulatory component of the endothelial secretory pathway that controls VWF trafficking and the morphology of its carrier organelle the WPB. We propose a model (Fig. 5D) in which secretory proteins such as VWF and components that control Golgi morphology utilize a Sec22b-dependent pathway to arrive at the Golgi, where VWF is packaged in elongated WPBs with dimensions that are proportional to the size of the Golgi. The reduction in WPB length in the absence of Sec22b is explained by a combination of retention of VWF in the ER and disintegration of the Golgi. Reduced flux of VWF through the secretory pathway ultimately decreases the amount of VWF that can be secreted by ECs that lack Sec22b function. This highlights the importance of efficient transport of VWF through the secretory pathway prior to its packaging in WPBs and identifies Sec22b as a potential determinant of plasma VWF levels. Future studies should address the impact of components of this protein complex on VWF plasma levels in patients with bleeding and thrombotic disorders.
Authorship

EK, PB, JO, and AAM performed research and analyzed data; CRJ and DG contributed vital reagents and expertise; EK, JV and RB designed the research; EK, JV and RB wrote the paper.

Acknowledgements

This study was supported by grants from the Landsteiner Stichting voor Bloedtransfusie Research (LSBR-1517 and LSBR-1707), the Netherlands Ministry of Health (PPOC-2015-24P) and the Dutch Thrombosis Foundation (TSN 2017-01).
References


scoring algorithms and integration into the guide RNA selection tool CRISPOR.


45. Matsui T, Jiang P, Nakano S, Sakamaki Y, Yamamoto H, Mizushima N. Autophagosomal YKT6 is required for fusion with lysosomes independently of


Figure Legends

Figure 1. Sec22b depletion and fusogenic function affects WPB elongation. (A) VWF immunostaining in ECs transduced with shCTRL, shVAMP7, shYKT6 or shSec22b (green channel: mEGFP expressing ECs). (B) Quantification of WPB length in shCTRL, shVAMP7, shYKT6 and shSec22b ECs (n=3, 1-way ANOVA with Dunnett’s multiple comparisons test, **** P<0.0001). (C) WPB length in control and CRISPR SEC22B knockout ECs (n=3, 1-way ANOVA with Dunnett’s multiple comparisons test, **** P<0.0001). (D) VWF immunostaining in mEGFP and mEGFP-Sec22b-ΔSNARE expressing ECs (both in green). (E) WPB length in mEGFP and mEGFP-Sec22b-ΔSNARE expressing ECs (n=3, t-test with Welch’s correction, **** P<0.0001).

Figure 2. Sec22b depletion results in TGN fragmentation. (A) Immunofluorescent staining of TGN (TGN46) in control and Sec22b-depleted cells (blue channel: Hoechst nuclear staining). (B) Quantification of TGN dispersal in control and Sec22b KD ECs. (C) Quantification of TGN area coverage in shCTRL and shSec22b ECs (n=5, t-test with Welch’s correction, **** P<0.001). (D) WPB length in Sec22b KD ECs with compact versus dispersed TGN (n=3, t-test with Welch’s correction, **** P<0.0001).

Figure 3. Sec22b depletion results in VWF ER retention (A) Western blot analysis of monomeric VWF under reducing conditions in control and Sec22b KD ECs. Uncleaved (proVWF) and cleaved (VWF) forms are indicated by arrows. α-tubulin is used as a loading control. Molecular weight standards are indicated on the left (kDa). (B) ProVWF:VWF ratio in control and shSec22b ECs (n=8, paired t-test, * P<0.05). (C) Immunofluorescent staining of VWF in shCTRL and shSec22b HUVECs (boxed areas are shown magnified on the right, size bar corresponds to 10 μm for images or 5 μm for boxed areas). (D) VWF multimer blot (4 samples from 2 independent experiments) in control and Sec22b KD ECs. (E) Line graph of the densitometry of VWF multimer bands.
Figure 4. VWF accumulation in dilated rough ER in Sec22b depleted ECs. (A) EM of control and Sec22b KD ECs (dilated rough ER shown by white arrowheads, ribosome-studded dilated ER by white asterisks, connection of ER structures to rER sheets in yellow arrowhead, scale bar set at 2 μm). (B) Quantification of healthy versus dilated ER in control and Sec22b KD cells. (C) Quantification of ER width in control and Sec22b KD cells (t-test with Welch correction, **** P<0.0001). (D) VWF immunogold staining (10 nm gold particles) in control and Sec22b KD ECs (boxed regions are magnified on the right side with the corresponding color, scale bar set at 1 μm).

Figure 5. Sec22b silencing results in reduced basal and stimulated secretion. (A) Intracellular VWF content in control and Sec22b-silenced ECs measured by ELISA (n=5, paired t-test, * P<0.05). (B) Basal VWF release presented as percentage of intracellular VWF content (n=5, paired t-test, * P<0.05) (C) Histamine-stimulated VWF release presented as percentage of intracellular VWF content (n=3 independent experiments, paired t-test, ** P<0.01). (D) Proposed model of Sec22b-dependent VWF trafficking and WPB size control. Abbreviations: endoplasmic reticulum (ER), rough ER (rER), trans-Golgi network (TGN), Weibel-Palade body (WPB), immature WPB (iWPB), mature WPB (mWPB), sorting endosome (SE).
Methods

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 pH 7.4, 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 S1). 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.

Fluorescence microscopy

Immunostaining and fluorescence imaging of fixed cells was performed as previously described 1. Immunostained cells were mounted in MOWIOL mounting medium and images were acquired using Leica SP5 or SP8 confocal microscopes (Leica, Wetzlar, Germany). Images were processed and analyzed using ImageJ (https://imagej.nih.gov/ij/). WPB length (major axis of cigar-shaped VWF positive structures) and TGN area (periphery of TGN46 staining) were measured as pixels and automatically converted in µm scale in ImageJ; box graphs were plotted in GraphPad Prism 8.

Secretion assay

Endothelial cells were grown in 6-well plates and cultured for 7 days prior to the experiment with regular medium replacement. Basal VWF release was determined as unstimulated secretion over 24 hours in EGM-18 medium. For histamine-induced secretion cells were pre-
incubated in release medium [RM: serum-free M199 (Thermofisher, 22340) supplemented with 0.2% (w/v) bovine serum albumin (BSA) (Merck, 112018)] for 15-30 minutes prior to stimulation. Cells were stimulated in RM medium supplemented with 100 μM histamine (Sigma-Aldrich, H7125). Lysates were obtained in NP-40 based lysis buffer supplemented with Complete protease inhibitor cocktail. VWF levels were determined by ELISA as described previously ². Secretion is expressed as relative proportion of intracellular VWF in lysates of unstimulated cells.

**VWF multimer analysis**

Endothelial cell lysates, produced as described in Immunoblotting, were appropriately diluted to a final concentration of 1 nM. Samples were loaded onto freshly prepared (10×10 cm, 1.5 mm) agarose gels (SeaKem® HGT(P) Agarose, Lonza, 50050) (stacking gel: 0.75% Agarose, running gel: 1.8% Agarose) and separated for approximately 3 hours at 100V and 35 mA. VWF multimers were transferred to a PVDF membrane (BIO-RAD, 162-0177) overnight. Membranes were stained with an anti-VWF-HRP antibody (see Supplemental table S1). Chromogenic visualization of HRP was achieved with DAB peroxidase substrate kit (Vector Laboratories, SK-4100). Images were analyzed using ImageJ (https://imagej.nih.gov/ij/) and densitometry profiles were plotted in GraphPad Prism 8.

**Electron microscopy**

Cells were fixed by adding double concentrated fixative to the culture dish (end concentration of fixative: 1,5% glutaraldehyde (GA) with 0.1M cacodylate buffer) and incubating for 2 hours at room temperature. After rinsing the cells 3 time with 0,1M cacodylate buffer, the cells were postfixed with 1% OsO₄/0.1M cacodylatebuffer on ice for 1 hour. Dehydration followed with a series of ethanol solutions and after that mixtures with EPON (LX112, Leadd) and ethanol 100%, and finally pure EPON. Beem capsules filled with EPON were placed on the dishes with the open face down. After EPON polymerization at 40° at the first night, followed by a day at 70°C, the beem capsules could be snapped off. 80 μm sections parallel to the surface of the Beem capsules containing the cultured cells were
contrasted with uranylacetate and leadcitrate. Examination of the sections was done on a FEI Tecnai Twin transmission electron microscope (FEI, Eindhoven, Netherlands). Overlapping images were collected and stitched together into separate images, as previously described 3.

**Immunoelectron microscopy**

Cells were fixed in 2% PFA/0.2%GA/0.1M PHEM buffer at room temperature for 2 hours. Cells were scraped using a single use plastic scraper and collected in a pellet in 12% gelatin. Pellets were cut into smaller pieces of ~1 mm³ and were impregnated with 2.3M sucrose in PBS (60 min), mounted on a stub and snap frozen in liquid nitrogen. 90 µm sections were made with a Leica EM ultracryotome and were collected on grids. Grids were labeled with rabbit anti-VWF (1:1000) (see Supplemental Table S1) and stained with 10 nm PAAu gold in 1%BSA/PBS following a protocol described previously 4. After rinsing the sections, the grids were mounted in 0.3% uranylacetate / 2% methylcellulose solution and were examined as described above.

**Data and statistical analysis**

Statistical analysis was by student’s t-test and using GraphPad Prism 8 (Graphpad, La Jolla, CA, USA). Significance values are shown in the Figures and in Figure legends. Data are shown as box graphs (min. to max.), as mean ± SEM or as contingency stacked bar graphs.
Supplemental References


<table>
<thead>
<tr>
<th>target</th>
<th>Species (isotype)</th>
<th>Label</th>
<th>Supplier</th>
<th>Cat.nr /clone</th>
<th>Application [concentration/dilution]</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF</td>
<td>mouse (IgG&lt;sub&gt;2b&lt;/sub&gt;)</td>
<td>-</td>
<td>described in&lt;sup&gt;5&lt;/sup&gt;</td>
<td>CLB-RAg20</td>
<td>IF [1:1000]</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>mouse (IgG&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>-</td>
<td>Sigma-Aldrich</td>
<td>T9026</td>
<td>WB [1:1000]</td>
</tr>
<tr>
<td>VWF</td>
<td>rabbit</td>
<td>-</td>
<td>DAKO</td>
<td>A0082</td>
<td>ELISA [6 µg/ml]</td>
</tr>
<tr>
<td>VWF</td>
<td>rabbit</td>
<td>HRP</td>
<td>DAKO</td>
<td>A0082</td>
<td>WB [3 µg/ml], ELISA [2 µg/ml]</td>
</tr>
<tr>
<td>Sec22b</td>
<td>rabbit</td>
<td>-</td>
<td>Synaptic Systems</td>
<td>186 003</td>
<td>IF [2 µg/ml], WB [1 µg/ml]</td>
</tr>
<tr>
<td>TGN46</td>
<td>sheep</td>
<td>-</td>
<td>Bio-Rad</td>
<td>AHP500GT</td>
<td>IF [1:1000]</td>
</tr>
<tr>
<td>Rab27A</td>
<td>rabbit</td>
<td>-</td>
<td>described in&lt;sup&gt;1&lt;/sup&gt;</td>
<td>B2423</td>
<td>IF [1:100]</td>
</tr>
<tr>
<td>Slp4-a</td>
<td>rabbit</td>
<td>-</td>
<td>Atlas Antibodies</td>
<td>HPA001475</td>
<td>IF [1:500]</td>
</tr>
<tr>
<td>CD63</td>
<td>mouse (IgG&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>488</td>
<td>Sanquin</td>
<td>CLB-gran/12</td>
<td>IF [0.4 µg/ml]</td>
</tr>
<tr>
<td>Acti-stain 670</td>
<td>-</td>
<td></td>
<td>Cytoskeleton</td>
<td>PHDN1</td>
<td>IF [1:400]</td>
</tr>
<tr>
<td>Hoechst</td>
<td>-</td>
<td></td>
<td>Life Technologies</td>
<td>H-1399</td>
<td>IF [1:50]</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td>donkey</td>
<td>680LT</td>
<td>Li-Cor</td>
<td>925-68023</td>
<td>WB [0.1 µg/ml]</td>
</tr>
<tr>
<td>mouse IgG</td>
<td>donkey</td>
<td>800CW</td>
<td>Li-Cor</td>
<td>925-32212</td>
<td>WB [0.1 µg/ml]</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td>goat</td>
<td>AF633</td>
<td>ThermoFisher</td>
<td>A11011</td>
<td>IF [2 µg/ml]</td>
</tr>
<tr>
<td>mouse IgG</td>
<td>goat</td>
<td>AF568</td>
<td>ThermoFisher</td>
<td>A11004</td>
<td>IF [2 µg/ml]</td>
</tr>
<tr>
<td>mouse IgG</td>
<td>goat</td>
<td>AF488</td>
<td>ThermoFisher</td>
<td>A11004</td>
<td>IF [2 µg/ml]</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td>chicken</td>
<td>AF647</td>
<td>ThermoFisher</td>
<td>A21443</td>
<td>IF [2 µg/ml]</td>
</tr>
</tbody>
</table>
## Supplemental Table S2. MISSION® Library shRNAs used in this study

<table>
<thead>
<tr>
<th>target</th>
<th>shRNA clone MISSION® library</th>
<th>shRNA target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec22b</td>
<td>TRCN00000159152</td>
<td>GCCATCAATGAGATTTAACTT</td>
</tr>
<tr>
<td></td>
<td>TRCN00000159288</td>
<td>GCCACAATTTGCTAACATTTA</td>
</tr>
<tr>
<td></td>
<td>TRCN00000059888</td>
<td>GCGAGGAAGAAAGATGGAATT</td>
</tr>
<tr>
<td></td>
<td>TRCN00000059889</td>
<td>GCTCACTATTATCATCATCAT</td>
</tr>
<tr>
<td>VAMP7</td>
<td>TRCN00000059890</td>
<td>GAGCAGATTCTGCTAGATA</td>
</tr>
<tr>
<td></td>
<td>TRCN00000059891</td>
<td>GCATTTCCATATGCATGAAT</td>
</tr>
<tr>
<td></td>
<td>TRCN00000059892</td>
<td>CGTACTCACATGGAATTATT</td>
</tr>
<tr>
<td>YKT6</td>
<td>TRCN0000059763</td>
<td>GCGGAACTAGATGAGACCAA</td>
</tr>
<tr>
<td></td>
<td>TRCN0000059764</td>
<td>GCGCATACGTGTTCCTTCTT</td>
</tr>
<tr>
<td></td>
<td>TRCN0000059765</td>
<td>GAGAAGCTGATCCCCATGACTA</td>
</tr>
<tr>
<td></td>
<td>TRCN0000059766</td>
<td>CGGAATGATAGTCTGAGGT</td>
</tr>
<tr>
<td></td>
<td>TRCN0000059767</td>
<td>ACAGTCTAAAGCCTTCTATAA</td>
</tr>
<tr>
<td></td>
<td>non-targeting control (shCTRL)</td>
<td>-</td>
</tr>
</tbody>
</table>

## Supplemental Table S3. SEC22B gRNAs used in this study

<table>
<thead>
<tr>
<th>gRNA</th>
<th>target sequence (+PAM)</th>
<th>oligo</th>
<th>oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA1</td>
<td>GCTAACAATGATCGCCCGAGTGG</td>
<td>RBNL411</td>
<td>5’-caccgGCTAACAATGATCGCCCGAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RBNL412</td>
<td>5’-aaaccTCGGGCGATCATTTGTTAGCc-3’</td>
</tr>
<tr>
<td>gRNA2</td>
<td>AACAATGATCGCCCGAGTGGCGG</td>
<td>RBNL413</td>
<td>5’-caccgAACAATGATCGCCCGAGTGG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RBNL414</td>
<td>5’-aaaccCCACTCGGCGATCATTTGTTC-3’</td>
</tr>
<tr>
<td>gRNA3</td>
<td>TTCGTCCTCCTGATCGAGGCGG</td>
<td>RBNL415</td>
<td>5’-caccgTTCGTCTCCTGATCGAGG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RBNL416</td>
<td>5’-aaaccCTCGATGAGGAGGACGA-ac-3’</td>
</tr>
</tbody>
</table>
Supplemental Figure S1.

A

B

C

D

Sec22b expression (%)

shCTRL
shSec22b

Sec22b

α-tubulin

CTRL

Sec22b

shCTRL

shSec22b

CTCF (×10^4)

shCTRL
shSec22b

****
Supplemental Figure S3.
Supplemental Figure S5.

A

shCTRL

VWF

Rab27A

shSec22b

VWF

Rab27A

B

shCTRL

VWF

Slp4-a

shSec22b

VWF

Slp4-a
Supplemental Figure Legends

Supplemental Figure S1. shRNA silencing of Sec22b in endothelial cells. (A) Sec22b expression in HUVEC lysates after Sec22b silencing determined using immunoblotting. α-tubulin was used as a loading control. Molecular weight indicators are shown on the right in kDa. (B) Quantification of Sec22b expression in Sec22b silenced endothelial cells normalized to shCTRL treated cells. (C) Immunostaining of Sec22b in shCTRL- and shSec22b-treated HUVECs. (D) Quantification of Sec22b immunoreactivity (corrected total cell fluorescence, CTCF) in shCTRL- and shSec22b-treated cells.

Supplemental Figure S2. Analysis of bulk populations of Sec22b CRISPR-engineered HUVECs (A) Graphic representation of gRNA design at the exon 1 of SEC22B for CRISPR/Cas9 knock out generation. gRNAs and PAMs are indicated underneath the sequence and translated region. (B) WB analysis of Sec22b expression in control and gRNA1, 2 and 3 samples (α-tubulin as loading control). (C) Bar graph of Sec22b expression (normalized to α-tubulin) in control and CRISPR knock out cells. (D) Immunofluorescent staining of Sec22b in CTRL, gRNA1, gRNA2 and gRNA3 HUVECs (blue channel: nucleus staining). (E) Immunofluorescent staining of VWF in CTRL, gRNA1, gRNA2 and gRNA3 HUVECs (blue channel: nucleus staining).

Supplemental Figure S3. Morphology and abundance of endolysosomal organelles are not dependent on Sec22b. Immunofluorescent staining of VWF (red) and CD63 (green) in shCTRL and shSec22b HUVECs (scale bar set at 10 µm, merge of channels on the left; magnifications of the cropped regions are shown on the right).

Supplemental Figure S4. Golgi disintegration after Sec22b silencing in HEK293T cells. Immunofluorescent staining of actin (green), Sec22b (cyan) and TGN46 (magenta) in shCTRL and shSec22b treated HEK293T cells (scale bar set at 10 µm, merge of channels on the top left; magnifications of TGN46 positive structures in the cropped regions are
shown on the right). Note that in the few cells in which some Sec22b expression remains an intact Golgi persists, an example of which is shown in the far right magnification

**Supplemental Figure S5. Maturation of WPBs is not in affected shSec22b-silenced endothelial cells.** (A) Immunofluorescent staining of VWF (red) and Rab27A (green) in shCTRL and shSec22b HUVECs (scale bar set at 10 µm, merge of channels on the left). B) Immunofluorescent staining of VWF (red) and Slp4-a (green) in shCTRL and shSec22b HUVECs (scale bar set at 10 µm, merge of channels on the left).