Ferrata Storti Foundation

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

Ellie Karampini,[1] Petra E. Bürgisser,[2] Jenny Olins,[1] Aat A. Mulder,[3] Carolina R. Jost,[3] Dirk Geerts,[4] Jan Voorberg[1,5] and Ruben Bierings[1,2]

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

Haematologica 2021
Volume 106(4):1138-1147

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 called Weibel-Palade bodies (WPB). The hemostatic activity of VWF is strongly related to the length of these bodies, but how endothelial cells control the dimensions of their WPB is unclear. In this study, using a targeted short hairpin RNA screen, we identified 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 together with disintegration of the Golgi and dilation of rough endoplasmic reticulum cisternae. This was accompanied by reduced proteolytic processing of VWF, accumulation of VWF in the dilated rough endoplasmic reticulum and reduced basal and stimulated VWF secretion. Our data demonstrate that the elongation of WPB, and thus adhesive activity of their cargo VWF, is determined by the rate of anterograde transport between endoplasmic reticulum and Golgi, which depends on Sec22b-containing SNARE complexes.

Correspondence:

RUBEN BIERINGS
r.bierings@erasmusmc.nl

Received: November 11, 2019.

Accepted: March 24, 2020.

Pre-published: March 26, 2020.

https://doi.org/10.3324/haematol.2019.242727

Introduction

Endoplasmic reticulum (ER)-to-Golgi transport is the first step in the secretory pathway.[1] As eukaryotic cells are extremely compartmentalized, ER is the first stop in protein production as well as the initial quality check point of whether proteins are correctly folded.[2] 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.[3] At the *trans*-Golgi network (TGN), proteins will either enter the "constant" constitutive pathway for unimpeded release, or will be temporarily stored in secretory vesicles, often of the family of lysosome-related organelles, for regulated secretion. Storage and regulated secretion allow the immediate discharge of larger quantities of protein in a correct physiological setting.[4]

The biogenesis of lysosome-related organelles is crucial for the proper function of a wide variety of cells, their importance being well-highlighted by the fact that defective formation of these organelles results in the manifestation of a large number of clinical abnormalities including bleeding, immunodeficiency, hypopigmentation and neurological defects.[5] Within the family of lysosome-related organelles, Weibel-Palade bodies (WPB) are the storage organelles of endothelial cells.[6,7] WPB primarily contain von Willebrand factor (VWF), a large multimeric hemostatic protein that serves a critical role in platelet adhesion and as a chaperone for coagulation factor VIII.[8] The biogenesis of WPB is directly dependent on the synthesis and correct post-translational processing of VWF.[9-12] WPB 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.[13-15] Quantitative or qualitative defects of VWF, for instance due to mutations in *VWF*, cause von Willebrand disease (VWD), the most common inherited bleeding disorder.[16] *VWF* mutations that affect the synthesis or processing of the protein often result in altered WPB morphology, with WPB being either round or short.[17] Upon regulated, explosive release from WPB, VWF unfurls into strings up to 1 mm long that are anchored on the apical side of the endothelium.[18-20] VWF strings create an adhesive platform for platelets to initiate the formation of the initial platelet plug at the site of vascular damage.[21,22] 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 endothelial cells control WPB size and thus hemostatic activity of VWF is largely unknown.

Due to the distinctive shape of their WPB, endothelial cells are an excellent model system for elucidating how cells manage formation and morphology of lysosome-related organelles. As WPB formation is driven by VWF, monitoring intracellular VWF trafficking can be used as a tool to study the complex mechanisms involved. VWF undergoes extensive post-translational modification during its journey through the endothelial secretory pathway.[25] VWF enters the ER as a single pre-pro-polypeptide chain that 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 exit from the ER, 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 WPB that emerge from the TGN.[28,29]

Trafficking of proteins during formation and maturation of subcellular organelles, such as WPB, 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-SNARE on the acceptor membrane which together form a four-helix bundle that allows the membranes to fuse. Although several SNARE have been associated with WPB exocytosis,[7] the SNARE that take part in the biogenesis of lysosome-related organelles and WPB are not known. The subfamily of longin-SNARE (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-SNARE in the formation of WPB. Using a targeted short hairpin (sh)RNA screen of longin-SNARE in primary endothelial cells we identified Sec22b as a novel determinant of WPB morphology. Sec22b silencing resulted in short WPB, disintegration of the Golgi complex, reduced proVWF processing and retention of proVWF in a dilated ER. Our data suggest that the distinctive morphology of WPB and thus the adhesive activity of their 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

The antibodies used in this study are listed in *Online Supplementary Table S1*.

Cell culture, lentiviral transfection and transduction

Pooled, cryopreserved primary human umbilical vein endothelial cells (HUVEC) were obtained from Promocell (Heidelberg, Germany). HUVEC were cultured in EGM-18 medium, i.e., EGM-2 medium (CC-3162, Lonza, Basel, Switzerland) supplemented with 18% fetal calf serum (Bodinco, Alkmaar, the Netherlands). Human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Cell Culture (Wessel, Germany) and were grown in Dulbecco modified Eagle medium containing D-glucose and L-glutamine (Lonza, Basel, Switzeland) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. HEK293T cells were seeded on collagen-coated plates or flasks and were transfected with third-generation lentiviral packaging plasmids pMD2.G, pRSV-REV and pMDLg/pRRE (Addgene, Cambridge, MA, USA) using transit-LT1 (Mirus Bio LLC, Madison, WI, USA) following the supplier's protocol. After incubation for 6-8 h, the medium was exchanged for EGM-18. Virus particles were collected 24 and 48 h following transfection and were filtered through 0.45 µm pore filters in EGM-18. Two batches of virus were used to transduce HUVEC, cord blood outgrowth endothelial cells or HEK293T cells for a period of 48 h. Transduced endothelial cells were selected by puromycin (0.5 µg/mL), which was added to the medium for 72 h after the second virus installment.

DNA constructs for shRNA silencing of longin-SNARE, CRISPR editing and mEGFP-Sec22b-ΔSNARE

The LKO.1-puro-CMV-mEGFP-U6-shC002 vector, which simultaneously expresses monomeric enhanced green fluorescent protein (mEGFP) and a non-targeting control shRNA from the cytomegalovirus (CMV) and U6 promoter, respectively, was described previously.[32] shRNA 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 (*Online 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 *Sph*I-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 (g)RNA has been described previously.[34] gRNA 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) (*Online Supplementary Figure 2A*). gRNA sequences that have a high predicted efficiency with limited off-target effects were selected. The gRNA used in this study are shown in *Online Supplementary Table S3* and were cloned as hybridized complementary oligos (with *Bsm*BI restriction site-compatible overhangs on either side) into *Bsm*BI-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 was flanked by *Bsr*GI and *Not*I sites. The resulting Sec22b-ΔSNARE fragment was cloned in frame behind

mEGFP in LVX-mEGFP-LIC by subcloning between the *BsrGI* and *NotI* sites. All constructs were sequence verified. Lentiviral plasmids were produced in Stbl3 bacteria.

Further details on materials and methods are provided in the *Online Supplementary Data*.

Results

Weibel-Palade body length is significantly reduced upon Sec22b silencing

To determine the role of longin-SNARE in WPB biogenesis we performed a targeted shRNA screen against VAMP7, YKT6 and Sec22b in HUVEC 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 WPB. However, upon Sec22b silencing (shSec22b) (*Online Supplementary Figure S1*), WPB 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 (Figure 1B). To further substantiate the role of Sec22b in WPB formation we used CRISPR-mediated *SEC22B*-editing of HUVEC (*Online Supplementary Figure S2A-C*). Cells depleted of Sec22b were identified by Sec22b staining (*Online Supplementary*

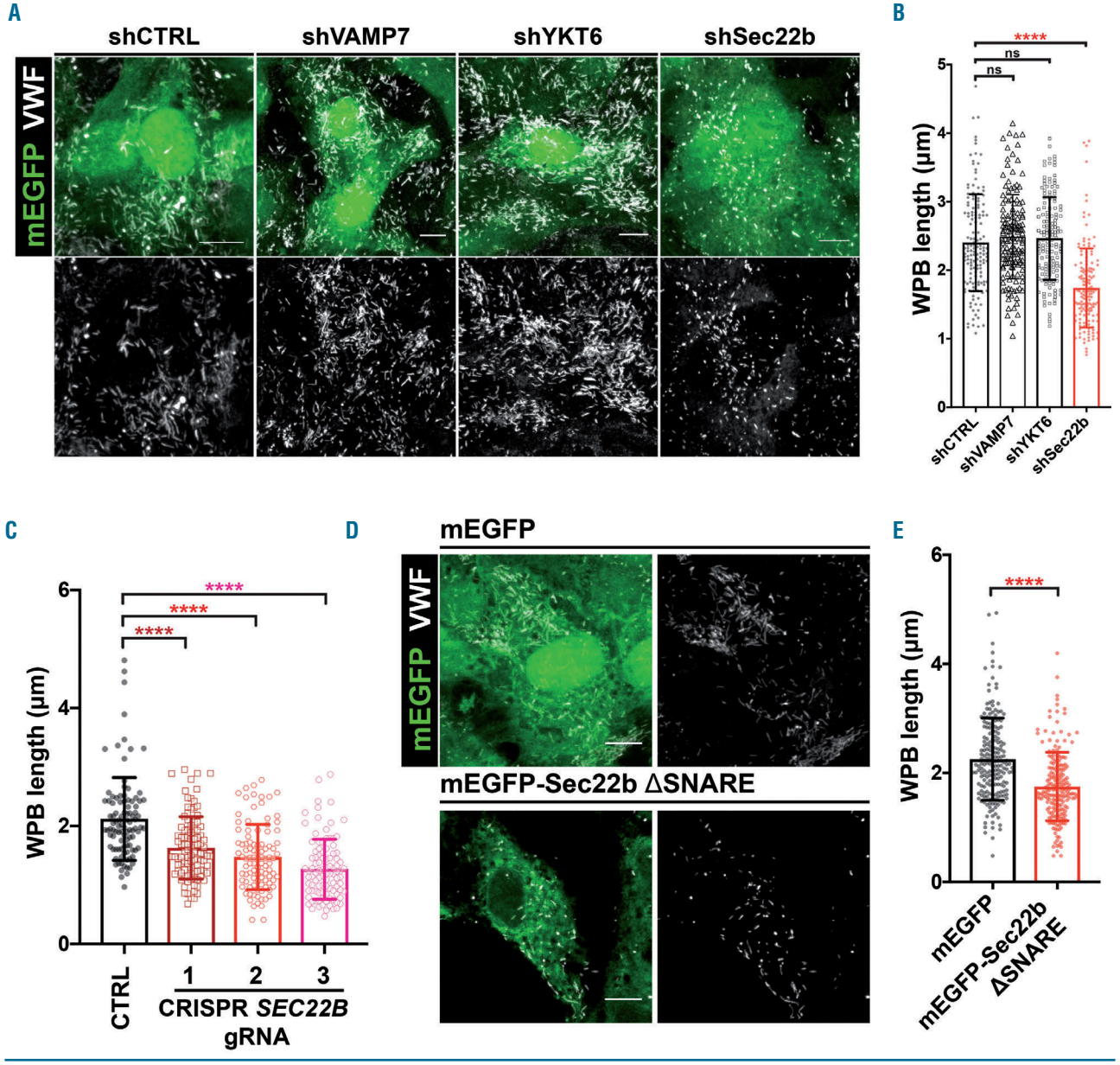

Figure 1. Sec22b depletion and fusogenic function affects Weibel-Palade body elongation. (A) von Willebrand factor (VWF) immunostaining in endothelial cells (EC) transduced with shCTRL, shVAMP7, shYKT6 or shSec22b (green channel: mEGFP-expressing EC). (B) Quantification of Weibel-Palade body (WPB) length in shCTRL, shVAMP7, shYKT6 and shSec22b EC (n=3, one-way analysis of variance [ANOVA] with the Dunnett multiple comparisons test, ****P<0.0001). (C) WPB length in control and CRISPR *SEC22B* knockout EC (n=3, one-way ANOVA with the Dunnett multiple comparisons test, ****P<0.0001). (D) VWF immunostaining in mEGFP and mEGFP-Sec22b-ΔSNARE expressing EC (both in green). (E) WPB length in mEGFP and mEGFP-Sec22b-ΔSNARE expressing EC (n=3, t-test with Welch correction, ****P<0.0001).

Figure S2D). A similar reduction of WPB length was observed in endothelial cells targeted with three separate gRNA directed to exon 1 of Sec22b (Figure 1B, *Online Supplementary Figure 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 (Figure 1D). We found that the ΔSNARE construct had a dominant-negative effect on WPB size, with these WPB being significantly shorter than the mEGFP control (Figure 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, endolysosomal organelles and WPB in an AP-3-dependent manner.[37] 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 WPB (*Online Supplementary Figure S3*). Together these results identify Sec22b as a determinant of secretory organelle size in endothelial cells.

Sec22b silencing results in unlinked Golgi ribbon

Since the size of nascent WPB 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 the TGN in control cells had a compact morphology, shSec22b-treated cells exhibited a dispersed TGN morphology, consistent with an unlinked Golgi ribbon (Figure 2A and B). Quantification of the area that encompassed the entire TGN46 immunoreactivity in shSec22b and shCTRL cells revealed that, due to their fragmentation, TGN in Sec22b-depleted cells extended to a significantly larger intracellular area than the compact TGN in control cells (Figure 2C). The crucial role for Sec22b in maintaining Golgi integrity is not limited to endothelial cells, as illustrated by a similar effect on Golgi morphology in Sec22b-depleted HEK293T cells (*Online Supplementary Figure S4*). It has previously been described that unlinking Golgi stacks using depletion of Golgi matrix proteins or nocodazole gives rise to shorter WPB.[24] When evaluating WPB length in shSec22b cells with compact *versus* dispersed TGN we also observed that in those cells in which the Golgi was dispersed, WPB were on average shorter than in those with intact Golgi (Figure 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 von Willebrand factor trafficking to the Golgi and retention of von Willebrand factor in the endoplasmic reticulum

As Sec22b has been associated with membrane fusion events during anterograde and retrograde trafficking between the ER and Golgi,[31] we evaluated VWF traffick-

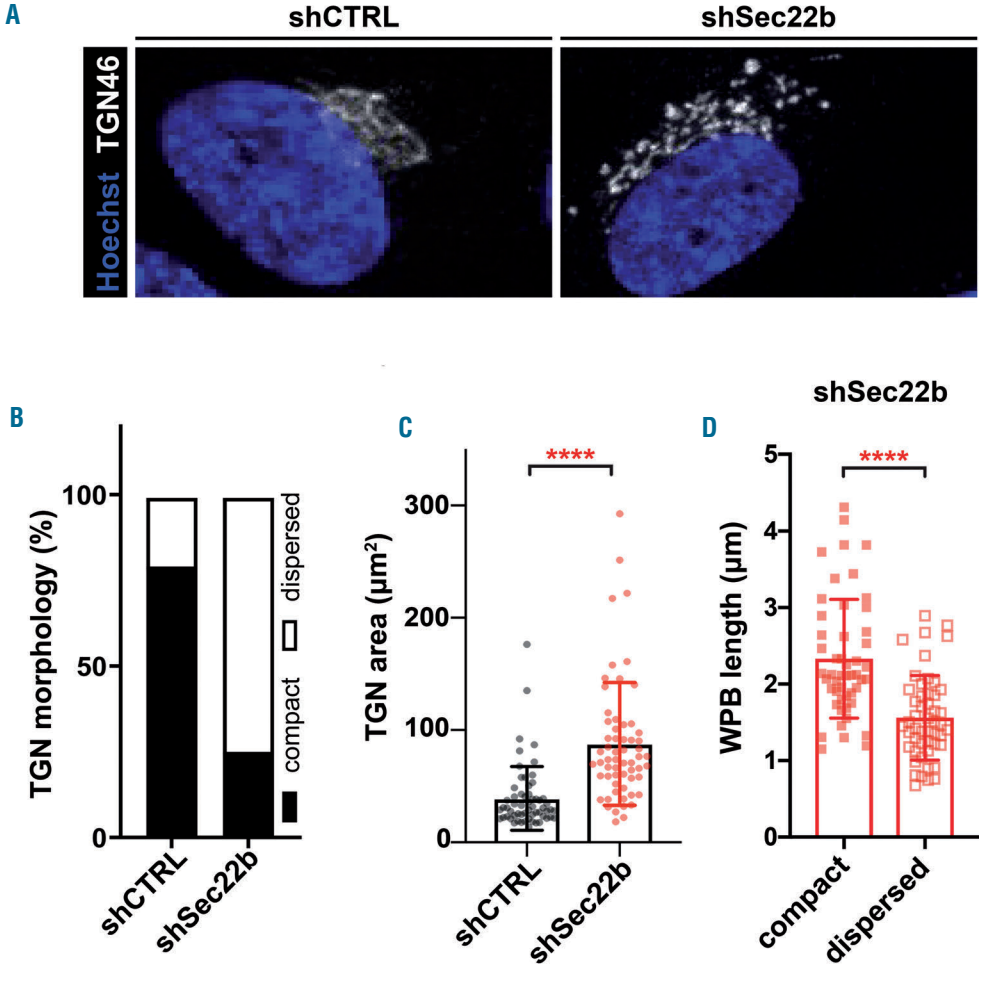

Figure 2. Sec22b depletion results in *trans*-Golgi network fragmentation. (A) Immunofluorescent staining of *trans*-Golgi network (TGN46) in control and Sec22b-depleted cells (blue channel: Hoechst nuclear staining). (B) Quantification of TGN dispersal in control and Sec22b knockdown (KD) endothelial cells (EC). (C) Quantification of TGN area coverage in shCTRL and shSec22b EC (n=5, *t*-test with Welch correction, ****P<0.001). (D) Weibel-Palade body (WPB) length in Sec22b KD EC with compact *versus* dispersed TGN (n=3, *t*-test with Welch correction, ****P<0.0001).

ing 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.[38] We used the intracellular ratio between the two distinct forms of VWF, proVWF (ER) and mature VWF (Golgi and post-Golgi), as a measure of ER and Golgi transport by estimating the amount of proVWF and VWF in shCTRL and shSec22b endothelial cells (Figure 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 endothelial cells (Figure 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 (Figure 3C). Simultaneous with - but independent of - proteolytic processing, VWF dimers oligomerize into long VWF multimers in the Golgi.[10,38] VWF multimer analysis using sodium dodecylsulfate-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 endothelial cells. However, the increased proportion of VWF dimers in the Sec22b KD cells indicates that VWF is retained in the ER (Figure 3D and E). Together this points to a reduction of anterograde ER-Golgi trafficking of VWF in the absence of Sec22b.

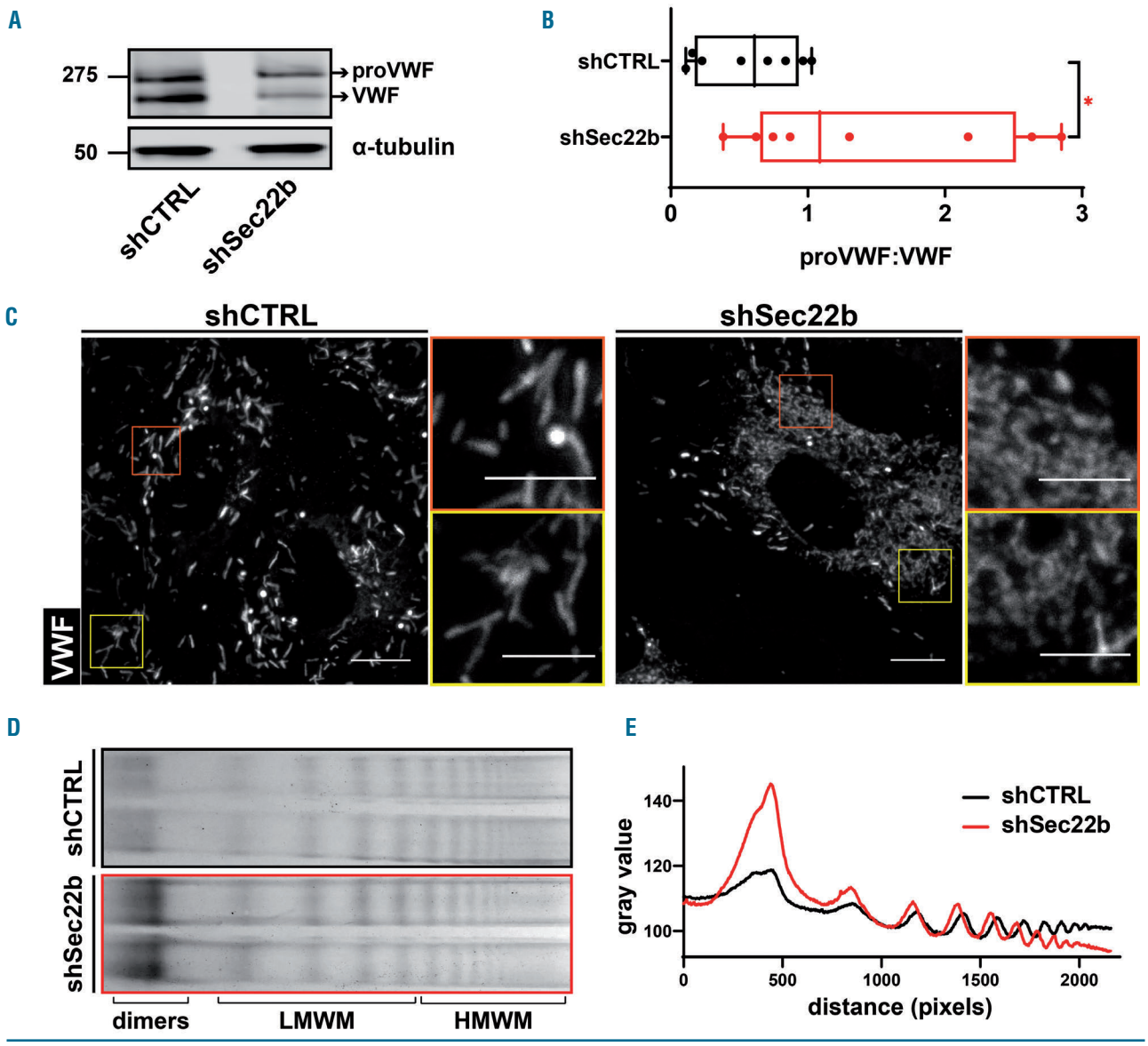

Figure 3. Sec22b depletion results in retention of von Willebrand factor in the endoplasmic reticulum. (A) Western blot analysis of monomeric von Willebrand factor (VWF) under reducing conditions in control and Sec22b knockdown (KD) endothelial cells (EC). Uncleaved (proVWF) and cleaved (VWF) forms are indicated by arrows. α-tubulin was used as a loading control. Molecular weight standards are indicated on the left (kDa). (B) ProVWF:VWF ratio in control and shSec22b EC (n=8, paired *t*-test, *P<0.05). (C) Immunofluorescent staining of VWF in shCTRL and shSec22b human umbilical vein EC (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 EC. (E) Line graph of the densitometry of VWF multimer bands. LMWM: low molecular weight multimers; HMWM: high molecular weight multimers

Sec22b silencing results in accumulation of von Willebrand factor in dilated rough endoplasmic reticulum

Since VWF was retained in the ER, potentially along with other proteins, we used electron microscopy to examine the impact of reduced anterograde trafficking on the ER (Figure 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 dilated 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%) (Figure 4B). Closer examination of the rER morphology revealed that apart from the round rER structures, the luminal width of ER sheets was significantly increased in Sec22b KD cells (0.29 μm ± 0.18 μm) when compared to control cells (0.10 μm ± 0.01 μm) (Figure 4C). This suggests that upon removal of Sec22b the rER expands in size dramatically, possibly to facilitate the accumulation of secretory proteins such as VWF. Indeed, immunogold staining for VWF in Sec22b KD endothelial cells localized within dilated rER and was prominently found in the round, dense rER structures (Figure 4D). Taken together this shows that VWF exits the ER in a Sec22b-dependent manner and upon Sec22b silencing is retained in rER-derived structures.

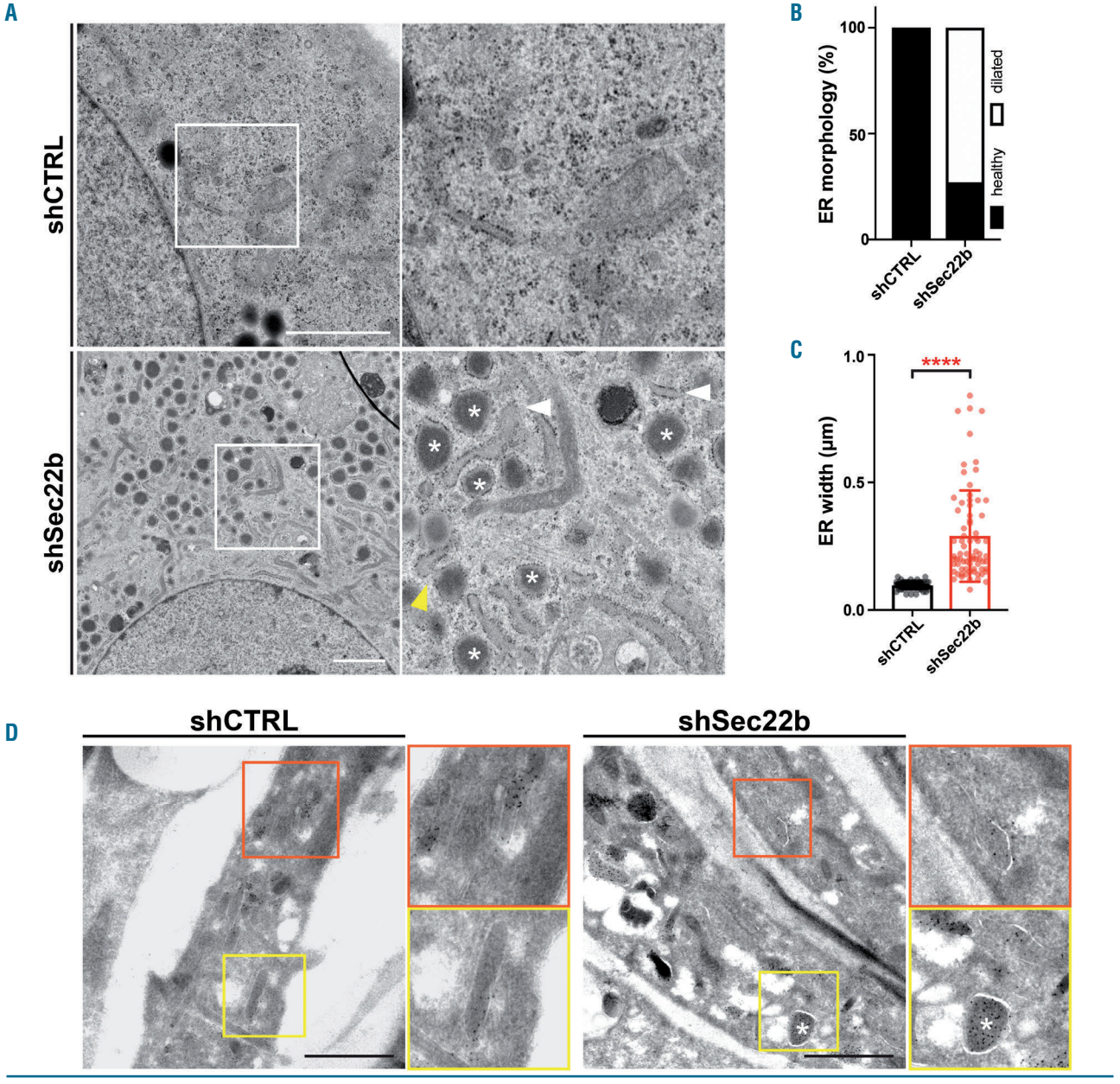

Figure 4. von Willebrand factor accumulation in dilated rough endoplasmic reticulum in Sec22b-depleted endothelial cells. (A) Electron microscopy of control and Sec22b knockdown (KD) endothelium cells (EC) (dilated rough endoplasmic reticulum [ER] shown by white arrowheads, ribosome-studded dilated ER by white asterisks, and the connection of ER structures to rough ER sheets by a yellow arrowhead; scale bar corresponds to 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) von Willebrand factor immunogold staining (10 nm gold particles) in control and Sec22b KD EC (boxed regions are magnified on the right side with the corresponding color; scale bar represents 1 μm).

Retention of von Willebrand factor in the endoplasmic reticulum results in reduced von Willebrand factor secretion in Sec22b-depleted cells

The lack of mature VWF, as well as the shorter WPB, prompted us to investigate how much VWF is stored and secreted in the absence of Sec22b. We observed that in the Sec22b-depleted cells, intracellular VWF levels were slightly increased when compared to the levels in control cells (Figure 5A), potentially because of VWF entrapment in the ER. On the other hand, basal secretion was significantly decreased in Sec22b-silenced cells (Figure 5B). Basal secretion primarily originates from unstimulated WPB release,[39,40] suggesting that this compartment is smaller upon Sec22b depletion. In line with this, VWF release through histamine-induced WPB exocytosis was also significantly reduced (Figure 5C). WPB acquire secretion competence during maturation by recruiting Rab GTPases and Rab-effectors,[7] so this decrease could potentially 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,[41,42] were recruited to WPB but found no difference between shSec22b and shCTRL cells (*Online Supplementary Figure 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 WPB, because it is retained in the ER.

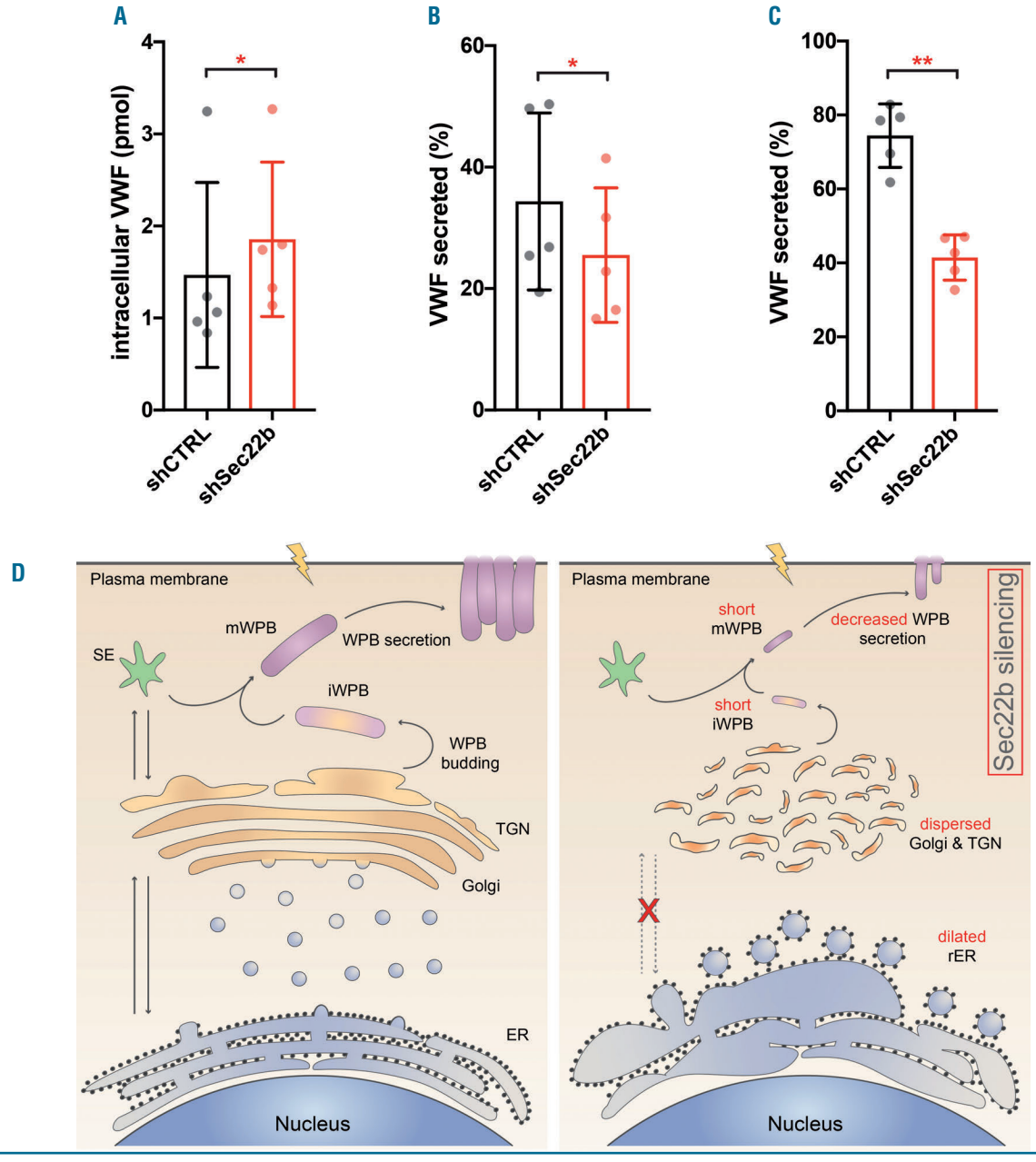

Figure 5. Sec22b silencing results in reduced basal and stimulated secretion. (A) Intracellular von Willebrand factor (VWF) content in control and Sec22b-silenced endothelial cells measured by enzyme-linked immunosorbent assay (n=5, paired *t*-test, **P*<0.05). (B) Basal VWF release presented as percentage 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 Weibel-Palade body size control. SE: sorting endosome; WPB: Weibel-Palade body; iWPB: immature WPB; mWPB: mature WPB; ER: endoplasmic reticulum; rER: rough ER; TGN: *trans*-Golgi network.

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 SNARE, known as longin-SNARE (YKT6, VAMP7 and Sec22b), which generally participate in fusion events during biogenesis, maturation and exocytosis of secretory organelles in eukaryotic cells.[43-47] In this study, using a shRNA screen targeting longin-SNARE we identified Sec22b as a novel regulator of WPB formation and VWF trafficking, its depletion leading to short and "stubby" WPB that were accompanied by Golgi disintegration and retention of VWF in the ER.

Sec22b is a known participant in anterograde transport of cargo in the early secretory pathway.[48,49] Overexpression of the Sec22b-ΔSNARE variant led to a dominant-negative effect resulting in smaller WPB, corroborating experiments that used RNA interference 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 SNARE due to the lack of the SNARE helix,[50] 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* homolog of Sec22b, which caused disruption of ER-Golgi transport and resulted in cargo retention in the ER and abnormal ER morphology.[51] Similar ER cargo retention is likely at play in Sec22b-depleted EC in our study (Figures 3 and 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 WPB.

The smaller WPB phenotype is likely further exacerbated by fragmentation of the TGN (Figure 2), which was previously proposed to limit the possibility of adjacent VWF quanta co-packaging into a single, extended WPB.[24] Interestingly, that study also demonstrated that experimental reduction of VWF trafficking by small interfering RNA 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.[52] The observed phenotype may be a consequence of defective trafficking of direct regulators of Golgi morphology, such as Golgi reassembly stacking proteins (GRASP)[53] or golgins.[54] Indeed, Golgi fragmentation and the concomitant reduction in the length of WPB has also been observed after depletion of Golgi tethering proteins (GM130, GRASP55 and giantin).[24] 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.[55,56] 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 contained VWF aggregates. There was a striking resemblance with the dilated ER morphology that is observed in response to VWD-causing mutations in VWF that affect the protein's ability to dimerize and leave the ER.[57,58] 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.[59,60] Similar dilated ER phenotypes and ER retention of secretory proteins have been described in chondrocytes from *sec24d*-deficient zebrafish[61] and in pancreatic acinar cells from Sec23$^{lgt/lgt}$ mice that additionally display lack of zymogen granules.[62] This suggests that the rate of COPII-mediated, anterograde ER-Golgi traffic underpins the ability of endothelial cells to shape WPB to their typical elongated morphology.

A recent study 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 the ER to Golgi.[63] 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 WPB that remained associated with or in close vicinity of the Golgi. Despite their reduction in length, WPB in Sec22b-depleted cells normally recruited exocytotic components such as Rab27A and Slp4-a (*Online Supplementary Figure S5*), contrary to the enlarged WPB in GBF1-ablated cells which failed to acquire post-Golgi cargo and Rab27A and which were secretion incompetent.[63] Although their short WPB were still responsive to agonists, Sec22b-depleted EC secreted reduced amounts of VWF through the regulated and basal secretory pathway (Figure 4B and 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 conclusion, we 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 (Figure 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 WPB 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 EC that lack Sec22b function. This highlights the importance of efficient transport of VWF through the secretory pathway prior to its packaging in WPB 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.

Disclosures

No conflicts of interest to disclose.

Contributions

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.

Funding

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

1. Gomez-Navarro N, Miller E. Protein sorting at the ER-Golgi interface. J Cell Biol. 2016;215(6):769-778.
2. Araki K, Nagata K. Protein folding and quality control in the ER. Cold Spring Harb Perspect Biol. 2011;3(11):1-25.
3. Kienzle C, von Blume J. Secretory cargo sorting at the trans-Golgi network. Trends Cell Biol. 2014;24(10):584-593.
4. Halban PA, Irminger JC. Sorting and processing of secretory proteins. Biochem J. 1994;299(1):1-18.
5. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet. 2008;9:359-386.
6. Weibel ER, Palade GE. New cytoplasmic components in arterial endothelia. J Cell Biol. 1964;23(1):101-112.
7. Schillemans M, Karampini E, Kat M, Bierings R. Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit. J Thromb Haemost. 2019;17(1):6-18.
8. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67:395-424.
9. Wagner DD, Saffaripour S, Bonfanti R, et al. Induction of specific storage organelles by von Willebrand factor propolypeptide. Cell. 1991;64(2):403-413.
10. Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H. Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. EMBO J. 1993;12(2):749-758.
11. Groeneveld DJ, van Bekkum T, Dirven RJ, et al. Angiogenic characteristics of blood outgrowth endothelial cells from patients with von Willebrand disease. J Thromb Haemost. 2015;13(10):1854-1866.
12. Schillemans M, Kat M, Westeneng J, et al. Alternative trafficking of Weibel-Palade body proteins in CRISPR/Cas9-engineered von Willebrand factor-deficient blood outgrowth endothelial cells. Res Pract Thromb Haemost. 2019;3(4):718-732.
13. Michaux G, Abbitt KB, Collinson LM, Haberichter SL, Norman KE, Cutler DF. The physiological function of von Willebrand's factor depends on its tubular storage in endothelial Weibel-Palade bodies. Dev Cell. 2006;10(2):223-232.
14. Huang R-H, Wang Y, Roth R, et al. Assembly of Weibel Palade body-like tubules from N-terminal domains of von Willebrand factor. Proc Natl Acad Sci U S A. 2008;105(2):482-487.
15. Berriman JA, Li S, Hewlett LJ, et al. Structural organization of Weibel-Palade bodies revealed by cryo-EM of vitrified endothelial cells. Proc Natl Acad Sci U S A. 2009;106(41):17407-17412.
16. Leebeek FWG, Eikenboom JCJ. Von Willebrand's disease. N Engl J Med. 2016;375(21):2067-2080.
17. Valentijn KM, Eikenboom J. Weibel-Palade bodies: a window to von Willebrand disease. J Thromb Haemost. 2013;11(4):581-592.
18. Conte IL, Cookson E, Hellen N, Bierings R, Mashanov G, Carter T. Is there more than one way to unpack a Weibel-Palade body? Blood. 2015;126(18):2165-2167.
19. Dong JF, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100(12):4033-4039.
20. Zheng Y, Chen J, López JA. Flow-driven assembly of VWF fibres and webs in in vitro microvessels. Nat Commun. 2015;6(1):7858.
21. De Ceunynck K, De Meyer SF, Vanhoorelbeke K. Unwinding the von Willebrand factor strings puzzle. Blood. 2013;121(2):270-277.
22. Sadler JE. von Willebrand factor assembly and secretion. J Thromb Haemost. 2009;7(Suppl 1):24-27.
23. Ferraro F, da Silva ML, Grimes W, et al. Weibel-Palade body size modulates the adhesive activity of its von Willebrand factor cargo in cultured endothelial cells. Sci Rep. 2016;6(1):32473.
24. Ferraro F, Kriston-Vizi J, Metcalf DJ, et al. A two-tier Golgi-based control of organelle size underpins the functional plasticity of endothelial cells. Dev Cell. 2014;29(3):292-304.
25. Springer TA. von Willebrand factor, Jedi knight of the bloodstream. Blood. 2014;124(9):1412-1426.
26. Zhou Y-F, Eng ET, Nishida N, Lu C, Walz T, Springer TA. A pH-regulated dimeric bouquet in the structure of von Willebrand factor. EMBO J. 2011;30(19):4098-4111.
27. Purvis AR, Gross J, Dang LT, et al. Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. Proc Natl Acad Sci U S A. 2007;104(40):15647-15652.
28. Zenner HL, Collinson LM, Michaux G, Cutler DF. High-pressure freezing provides insights into Weibel-Palade body biogenesis. J Cell Sci. 2007;120(Pt 12):2117-2125.
29. Mourik MJ, Faas FGA, Zimmermann H, Voorberg J, Koster AJ, Eikenboom J. Content delivery to newly forming Weibel-Palade bodies is facilitated by multiple connections with the Golgi apparatus. Blood. 2015;125(22):3509-3516.
30. Jahn R, Scheller RH. SNAREs - engines for membrane fusion. Nat Rev Mol Cell Biol 2006;7(9):631-643.
31. Daste F, Galli T, Tareste D. Structure and function of longin SNAREs. J Cell Sci. 2015;128(23):4263-4272.
32. Lenzi C, Stevens J, Osborn D, Hannah MJ, Bierings R, Carter T. Synaptotagmin 5 regulates Ca 2+ -dependent Weibel–Palade body exocytosis in human endothelial cells. J Cell Sci. 2019;132(5):jcs221952.
33. Schillemans M, Karampini E, van den Eshof BL, et al. Weibel-palade body localized syntaxin-3 modulates von Willebrand factor secretion from endothelial cells. Arterioscler Thromb Vasc Biol. 2018;38(7):1549-1561.
34. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014;11(8):783-784.
35. Haeussler M, Schönig K, Eckert H, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016;17(1):148.
36. van Breevoort D, Snijders AP, Hellen N, et al. STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. Blood. 2014;123(20):3185-3194.
37. Karampini E, Schillemans M, Hofman M, et al. Defective AP-3-dependent VAMP8 trafficking impairs Weibel-Palade body exocytosis in Hermansky-Pudlak syndrome type 2 blood outgrowth endothelial cells. Haematologica. 2019;104(10):2091-2099.
38. Vischer U, Wagner D. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. Blood. 1994;83(12):3536-3544.
39. Giblin JP, Hewlett LJ, Hannah MJ. Basal secretion of von Willebrand factor from human endothelial cells. Blood. 2008;112(4):957-964.
40. Lopes da Silva M, Cutler DF. von Willebrand factor multimerization and the polarity of secretory pathways in endothelial cells. Blood. 2016;128(2):277-285.
41. Hannah MJ, Hume AN, Arribas M, et al. Weibel-Palade bodies recruit Rab27 by a content-driven, maturation-dependent mechanism that is independent of cell type. J Cell Sci. 2003;116(Pt 19):3939-3948.
42. Bierings R, Hellen N, Kiskin N, et al. The interplay between the Rab27A effectors Slp4-a and MyRIP controls hormone-evoked Weibel-Palade body exocytosis. Blood. 2012;120(13):2757-2767.
43. Jani RA, Purushothaman LK, Rani S, Bergam P, Setty SRG. STX13 regulates cargo delivery from recycling endosomes during melanosome biogenesis. J Cell Sci. 2015;128(17):3263-3276.
44. Koseoglu S, Peters CG, Fitch-Tewfik JL, et al. VAMP-7 links granule exocytosis to actin reorganization during platelet activation. Blood. 2015;126(5):651-660.
45. Kweon Y, Rothe A, Conibear E, Stevens TH. Ykt6p is a multifunctional yeast R-SNARE that is required for multiple membrane transport pathways to the vacuole. Mol Biol Cell. 2003;14(5):1868-1881.
46. Matsui T, Jiang P, Nakano S, Sakamaki Y, Yamamoto H, Mizushima N. Autophagosomal YKT6 is required for fusion with lysosomes independently of syntaxin 17. J Cell Biol. 2018;217(8):2633-2645.
47. Dai J, Lu Y, Wang C, et al. Vps33b regulates Vwf-positive vesicular trafficking in

megakaryocytes. J Pathol. 2016;240(1):108-119.
48. Zhang T, Wong SH, Tang BL, Xu Y, Hong W. Morphological and functional association of Sec22b/ERS-24 with the pre-Golgi intermediate compartment. Mol Biol Cell. 1999;10(2):435-453.
49. Liu Y, Barlowe C. Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. Mol Biol Cell. 2002;13(9):3314-3324.
50. Liu Y, Flanagan JJ, Barlowe C. Sec22p export from the endoplasmic reticulum Is independent of SNARE pairing. J Biol Chem. 2004;279(26):27225-27232.
51. Zhao X, Yang H, Liu W, et al. Sec22 regulates endoplasmic reticulum morphology but not autophagy and is required for eye development in Drosophila. J Biol Chem. 2015;290(12):7943-7951.
52. El-Kasmi F, Pacher T, Strompen G, et al. Arabidopsis SNARE protein SEC22 is essential for gametophyte development and maintenance of Golgi-stack integrity. Plant J. 2011;66(2):268-279.
53. Xiang Y, Wang Y. GRASP55 and GRASP65 play complementary and essential roles in Golgi cisternal stacking. J Cell Biol. 2010;188(2):237-251.
54. Wang T, Grabski R, Sztul E, Hay JC. p115-SNARE interactions: a dynamic cycle of p115 binding monomeric SNARE motifs and releasing assembled bundles. Traffic. 2015;16(2):148-171.
55. Shitara A, Shibui T, Okayama M, et al. VAMP4 is required to maintain the ribbon structure of the Golgi apparatus. Mol Cell Biochem. 2013;380(1-2):11-21.
56. Zolov SN, Lupashin V V. Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. J Cell Biol. 2005;168(5):747-759.
57. Wang J-W, Valentijn KM, de Boer HC, et al. Intracellular storage and regulated secretion of von Willebrand factor in quantitative von Willebrand disease. J Biol Chem. 2011;286(27):24180-24188.
58. Wang JW, Groeneveld DJ, Cosemans G, et al. Biogenesis of Weibel-Palade bodies in von Willebrand's disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation. Haematologica. 2012;97(6):859-866.
59. Mancias JD, Goldberg J. The transport signal on Sec22 for packaging into COPII-coated vesicles Is a conformational epitope. Mol Cell. 2007;26(3):403-414.
60. Adolf F, Rhiel M, Hessling B, et al. Proteomic profiling of mammalian COPII and COPI vesicles. Cell Rep. 2019;26(1):250-265.
61. Ohisa S, Inohaya K, Takano Y, Kudo A. sec24d encoding a component of COPII is essential for vertebra formation, revealed by the analysis of the medaka mutant, vbi. Dev Biol. 2010;342(1):85-95.
62. Tao J, Zhu M, Wang H, et al. SEC23B is required for the maintenance of murine professional secretory tissues. Proc Natl Acad Sci U S A. 2012;109(29):E2001-2009.
63. Lopes-da-Silva M, McCormack JJ, Burden JJ, Harrison-Lavoie KJ, Ferraro F, Cutler DF. A GBF1-dependent mechanism for environmentally responsive regulation of ER-Golgi transport. Dev Cell. 2019;49(5):786-801.e6.