# Chapter 4

## Biogenesis of Weibel-Palade bodies in von Willebrand disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation

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## Summary

*Background:* Mutations of cysteine residues in von Willebrand factor (VWF) are known to reduce its storage and secretion, thus leading to reduced antigen levels. However, one cysteine mutation, p.Cys2773Ser, has been found in type 2A(IID) von Willebrand disease (VWD) patients who have normal plasma levels of VWF. We hypothesize that disruption of either intra- or interchain disulfide bonds by cysteine mutations in VWF has different effects on the biogenesis of Weibel-Palade bodies (WPB).

*Methods:* The effect of specific cysteine mutations that either disrupt intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disulfide bonds on storage and secretion of VWF was studied by transient transfection in HEK 293 cells. Upon expression of VWF these cells formed endothelial WPB-like organelles called pseudo-WPB. Storage of VWF was analyzed with both confocal immunofluorescence and electron microscopy. Regulated secretion of VWF was induced with phorbol 12-myristate 13-acetate.

*Results:* p.Cys1130Phe and p.Cys2671Tyr reduced the storage of VWF into pseudo-WPB with notable retention of VWF in the endoplasmic reticulum (ER), whereas p.Cys2773Ser was stored normally. As expected, wild type VWF formed proteinaceous tubules that displayed as longitudinal striations in pseudo-WPB under electron microscopy. p.Cys2773Ser caused severe defects in VWF multimerization but it formed normal tubules. Furthermore, the basal and regulated secretion of VWF was drastically impaired by p.Cys1130Phe and p.Cys2671Tyr, but not by p.Cys2773Ser.

*Conclusions:* We postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect the storage in WPB and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds will lead to reduced VWF storage and secretion due to ER retention.

## Introduction

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that facilitates platelet adhesion to an injured vessel wall and carries coagulation factor VIII (FVIII) in the circulation [1]. Aberration in the function or concentration of VWF may lead to the most common human inherited bleeding disorder von Willebrand disease (VWD).

The main source of plasma VWF is the endothelial cell which stores VWF in a unique organelle called Weibel-Palade body (WPB). VWF is released from WPB by basal and regulated mechanisms [2,3]. During its biosynthesis, VWF undergoes extensive posttranslational modifications that include formation of intrachain disulfide bonds and interchain disulfide bonds. The interchain disulfide bonds bridge, respectively, the cysteine knot (CK) domains ("tail-to-tail") and D3 domains ("head-to-head") of VWF to form dimers and multimers of up to 80 monomeric subunits [1,4,5]. Such interchain disulfide bond formation has been recently shown to be facilitated by the low pH seen in the *trans* Golgi and WPB [6,7]. At the *trans* Golgi, VWF is organized into proteinaceous tubules (tubulation) that are stored into WPB. Under an electron microscope VWF tubules appear as striations (if viewed longitudinally) or as hollow rings (if viewed in cross-section) [4,5].

VWF contains numerous cysteines [8] and a large number of mutations of cysteines have been identified in VWD patients with either quantitative (VWD types 1 and 3) or qualitative (VWD type 2) defects of VWF (ISTH-SSC VWF mutation database www.vwf.group.shef.ac.uk/). The mechanisms by which specific cysteine mutations lead to the different clinical phenotypes of VWD are largely unknown. Based on the expression data of cysteine mutations in VWF, we and others have shown that intracellular retention of VWF is a common mechanism underlying VWD with quantitative deficiencies of VWF [9-12]. Recently we showed that impaired WPB formation and reduced regulated secretion of VWF contributes to this intracellular retention [13]. However, cysteines p.Cys2771 and p.Cys2773 that are involved in the interchain-disulfide bond formation at the CK domain [14] have been implicated in type 2A(IID) VWD patients with normal plasma VWF antigen level [15-17] suggesting normal storage and secretion. Therefore, we hypothesize that disruption of interchain disulfide bonds may not hamper VWF induced biogenesis of WPB and secretion into the circulation whereas disruption of intrachain disulfide bonds does.

To address this hypothesis, the intracellular storage and secretion of VWF mutant p.Cys2773Ser was compared with that of two other mutants, p.Cys1130Phe and

p.Cys2671Tyr, that disrupt intrachain disulfide bonds in VWF. We found that disruption of intrachain disulfide bonds in VWF by mutations p.Cys1130Phe and p.Cys2671Tyr impairs WPB biogenesis whereas disruption of an interchain disulfide bond by p.Cys2773Ser does not affect WPB formation. Our findings also confirmed that VWF tubulation and storage in WPB is not dependent on C-terminal dimerization.

## Materials and methods

*Patients and mutations*

The mutations investigated in this study were all identified earlier in VWD patients. p.Cys1130Phe (c.3389G>T) was identified in heterozygous state in patients originally diagnosed with type 1 VWD with moderately severe bleeding tendencies [18,19]. The phenotype of patients carrying p.Cys1130Phe has more recently also been classified as 2A(IIE) [20,21]. p.Cys2671Tyr (c.8012G>A) was described in a compound heterozygous type 3 VWD patient with a deletion of the second allele [22]. p.Cys2773Ser (c.8318G>C) was identified in heterozygous type 2A(IID) patients and described before [17].

*Plasmid constructs*

Recombinant pSVH expression plasmids containing full length cDNAs encoding either the wild-type human VWF (WT-VWF) or the p.Cys2671Tyr and p.Cys2773Ser VWF variants have been described before [9]. The full-length VWF cDNA fragments, obtained by EcoRI restriction of these pSVH-VWF plasmids, were cloned into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA). Mutation p.Cys1130Phe was introduced into pCI-neo WT-VWF plasmid with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified by sequencing.

*Cell culture and transfection*

HEK293 cells were purchased from the ATCC (Rockville, USA) and cultured in Minimum Essential Medium eagle alpha modification (MEM-α, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μg/mL gentamicin (Invitrogen, Carlsbad, CA, USA). Using FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany), 0.66 μg/mL plasmid constructs (final concentration in the medium; 0.33 μg/mL WT-VWF and mutant VWF plasmids, respectively, for co-transfections) were transiently

transfected into HEK293 cells according to the manufacturer's instruction.

*Immunofluorescent staining and antibodies*

Transfected cells were stained with immunofluorescent antibodies and analyzed with Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective, as previously described [13]. Monoclonal antibody CLB-RAg35 and polyclonal rabbit anti-human Protein Disulfide Isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used as primary antibodies against VWF and the ER marker PDI, respectively [23,24]. Alexa 488- and Alexa 594-conjugated secondary antibodies were purchased from Invitrogen. To quantify the extent of retention of VWF in the ER, we determined the percentage of cells expressing VWF that also showed retention of VWF in the ER by co-staining of VWF with PDI.

*Transmission electron microscopy*

Transfected cells grown on 35-mm petri dishes were fixed and prepared for transmission electron microscopy (TEM) as previously described [13,25]. Briefly, osmium tetroxide and tannic acid treated cells were serially dehydrated with ethanol and embedded in Epon. The samples cut into thin sections of 70-100 nm were stained with uranyl acetate and lead citrate, and then analyzed with a Tecnai 12 at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, The Netherlands).

*Structural analysis of VWF*

Seventy-two hours post transfection cells were incubated with release medium (OPTIMEM1 medium, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.2% bovine serum albumin, pH7.4) for 24 hours. The conditioned media were electrophoresed through 1.6% agarose gel containing 1% sodium dodecyl sulphate for multimer analysis. VWF multimers were visualized by Western blot as described [9]. Electrophoresis as well as blotting was performed under non-reducing conditions. To analyze the processing of VWF, reducing SDS-PAGE and Western blot was performed for the conditioned media and cell lysates. Cell lysates were prepared with Passive Lysis Buffer (Promega) supplemented with 100µM phenylmethylsulfonyl fluoride (Roche) and the protease inhibitor cocktail Complete™ with EDTA (Roche). Samples were reduced using 20 mM dithiothreitol, separated by Novex 6% Tris-Glycine gel electrophoresis (Invitrogen),

immunostained with polyclonal rabbit anti-human VWF conjugated to horseradish peroxidase (DAKO) and visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

*Basal and regulated secretion of VWF*

In the absence of stimulation, VWF is mainly secreted via the basal secretory pathway from WPB [3]. HEK293 cells are known to form pseudo-WPB that are similar to the WBP in endothelial cells [13]. Basal and regulated secretion was determined as previously described [13]. Briefly, 24-hours post transfection, cells were incubated with fresh culture medium for 48 hours and then the conditioned media and cell lysates were collected to determine basal secretion of VWF. To analyze the regulated secretion of VWF, 72-hours post transfection cells were incubated for 60 minutes with release medium containing 160 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or vehicle (DMSO). VWF:Ag in the media and cell lysates was determined by ELISA assay. For the calculation of the regulated secretion, secreted VWF was expressed as a fraction of total VWF (medium plus lysate). Statistical analyses were performed by the Student's *t*-test using GraphPad Prism (version 4.02).

## Results

*Retention of VWF in the ER*

As shown in Figure 1, WT-VWF formed numerous elongated pseudo-WPB organelles. Each of the VWF mutants was able to form pseudo-WPB although those organelles were relatively short or more granular compared with those formed by WT-VWF. As indicated by the co-localization of VWF and the ER marker PDI, both p.Cys1130Phe and p.Cys2671Tyr led to considerable retention of synthesized VWF in the ER. In contrast, most of VWF p.Cys2773Ser was stored in pseudo-WPB similar to those observed for WT-VWF. Quantification of VWF storage showed that 60~80% of cells expressing p.Cys1130Phe or p.Cys2671Tyr displayed retention of VWF in the ER, whereas this was less than 10% for WT-VWF or p.Cys2773Ser (Figure 1). The retention of p.Cys1130Phe in the ER was reduced by co-transfection with WT-VWF. The proportion of cells with ER retention decreased from 83% to 26%. There was no reduction in ER retention upon co-transfection with WT-VWF for p.Cys2671Tyr. The differential efficiencies in storage of VWF variants were also reflected by the PMA-induced pseudo-WPB exocytosis (see section on "Secretion of VWF").

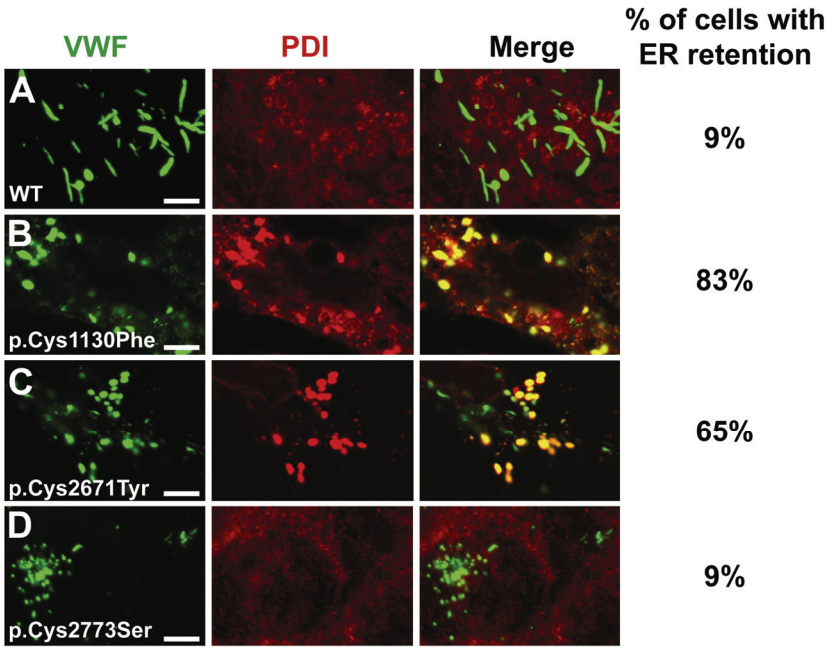


**Figure 1. Intracellular localization of VWF variants in transfected HEK293 cells.** HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. Fixed cells were stained for VWF (left panel, green) and for PDI (the ER marker, middle panel, red). In the right panel (merge of green and red channels), the pseudo-WPB show up in green (VWF staining only), and the ER containing VWF shows up in yellow as a result of double staining for VWF and PDI. Scale bar = 5 μm. 175~370 cells expressing each of VWF variants from two independent experiments were analyzed and the percentage of cells that showed retention of VWF in the ER (cells displaying yellow) is indicated on the right.

Analysis of the cell ultrastructure with TEM revealed pseudo-WPB containing VWF tubules in cells expressing WT-VWF as well as each of the mutants (Figure 2A-E). The tubules formed by the three VWF mutants appeared normal compared to those formed by WT-VWF (Figure 2A) [25]. This indicates that all three mutants were able to form pseudo-WPB though it was rare for p.Cys1130Phe. Furthermore, grossly dilated ER was frequently observed in cells expressing p.Cys1130Phe (Figure 2F) or p.Cys2671Tyr (Figure 2G). The ER morphology in cells expressing p.Cys2773Ser was normal (Figure 2H).

The retention of VWF mutants in the ER was further confirmed, using Western blotting under reducing condition, by evaluating the extend to which VWF was processed into its mature form. Secreted VWF consisted mainly of fully processed

mature VWF subunits (Figure 3A). In the cell lysates, both mature subunits and uncleaved proVWF were detectable for all VWF variants (Figure 3B). However, p.Cys1130Phe and p.Cys2671Tyr showed relatively much more uncleaved proVWF compared to WT-VWF and p.Cys2773Ser (Figure 3C), indicating that VWF p.Cys1130Phe and p.Cys2671Tyr were retained in the ER, and did not reach the Golgi apparatus where furin cleaves proVWF [26-28].

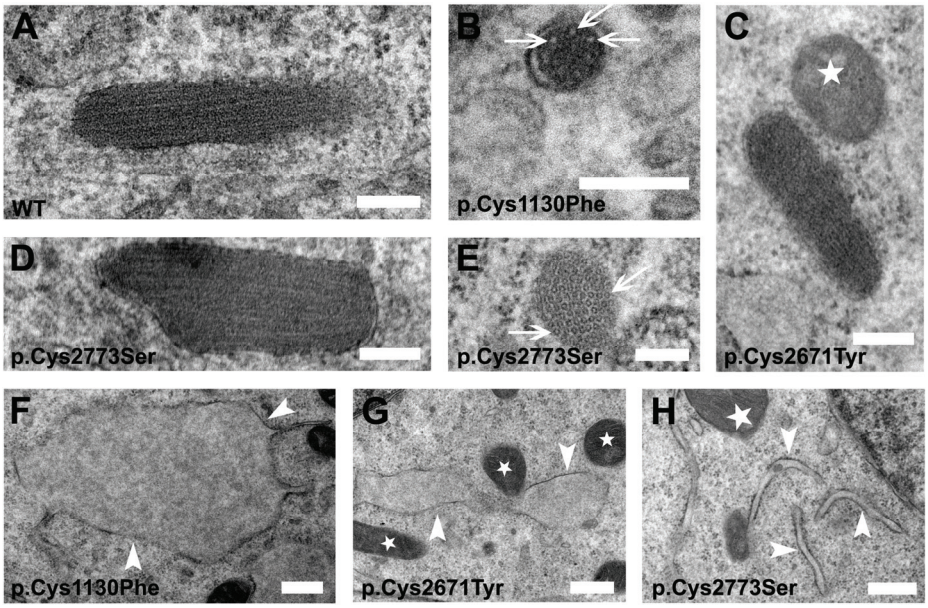


**Figure 2. Ultrastructure of transfected HEK293 cells visualized by TEM.** HEK293 cells were transiently transfected as indicated. Pseudo-WPB displaying VWF tubules were formed by WT-VWF (A) and the three mutants (B-E). Dilated ER was observed in cells expressing p.Cys1130Phe (F) or p.Cys2671Tyr (G) but not in cells expressing p.Cys2773Ser (H) or WT-VWF (not shown). Note that the VWF tubules were clearly visualized in the cross sections of pseudo-WPB (indicated by the arrows in panels B and E). The ER (indicated by the arrowheads) shown in panels F, G and H were imaged respectively in the same cells shown in panels B, C and D. The stars in panels C, G and H indicate mitochondria. In panel A-E, scale bar = 200 nm; in panels F-H, scale bar = 500 nm.

*Secretion of VWF*

WPB contributes to both the basal and regulated secretion of VWF [3]. To determine the effect of pseudo-WPB formation on the secretion of VWF mutants, both secretory pathways were examined. Basal secretion of VWF was defined as the secreted VWF:Ag level in the conditioned medium over 48 hours (supplemental Table S1). Compared to WT-VWF, both p.Cys1130Phe and p.Cys2671Tyr significantly reduced the basal secretion of VWF, which was apparent from a decrease in VWF level in the medium as well as from the decreased ratio of VWF:Ag in medium over lysate. In contrast, p.Cys2773Ser displayed a normal basal secretion of VWF, and a similar ratio medium/lysate as WT-VWF.

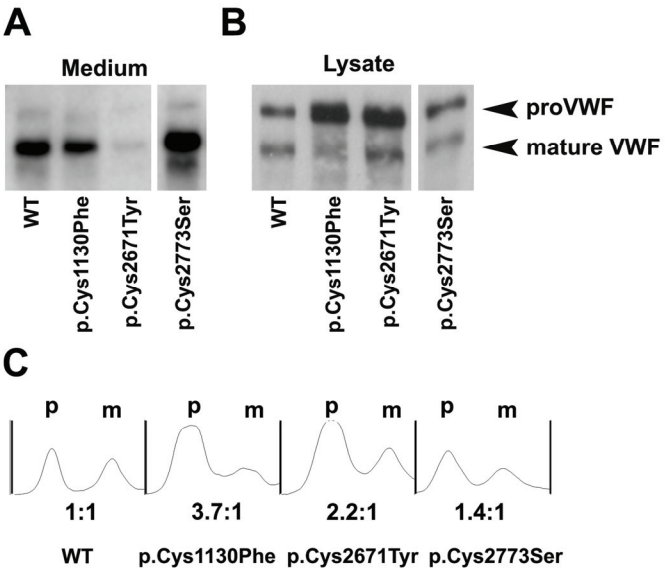


**Figure 3. Subunit composition of VWF under reducing conditions.** HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. VWF secreted into the medium (panel A: Medium) or VWF retained in lysate (panel B: Lysate) was reduced with DTT and analyzed with SDS-PAGE and Western blot. In panel C, the ratio of proVWF (p) and mature VWF subunit (m) in the cell lysate was analyzed with Image J (NIH software, version 1.44P). Note that in panels A and B the parts separated by the space between p.Cys2671Tyr and p.Cys2773Ser are from the same Western blot.

To mimic the heterozygous state of the mutations in carriers, co-transfections of VWF mutants with WT-VWF at a 1:1 ratio were done. After co-transfection with

WT-VWF, all three mutants showed similar basal secretion of VWF:Ag into medium. However, the decreased ratio of VWF:Ag in medium over lysate observed for p.Cys1130Phe and p.Cys2671Tyr indicates that the secretion of these VWF variants remained compromised in the heterozygous state (supplemental Table S1). Furthermore, the absolute amount of VWF protein produced by WT-VWF or the three variants was similar (total VWF:Ag in the medium and lysates; about 100 mU VWF per $7\times10^5$ cells) in both single and co-transfections, confirming that the effects on WPB formation and ER retention of VWF (Figure 1) could not be explained by the expression level of those mutants. The secretion and total amount of VWF (medium plus lysate) of p.Cys2773Ser was higher than WT-VWF, although this was not statistically significant (supplemental Table S1; 126.5 mU in single transfections and 117.7 mU in co-transfections versus 97.9 mU for WT-VWF per $7\times10^5$ cells), consistent with the normal to high plasma levels of VWF in the patients [17]. For p.Cys1130Phe and p.Cys2671Tyr, rapid clearance *in vivo* was shown to account for more severe phenotypes of the patients [29].

The regulated secretion of VWF was analyzed with PMA that efficiently induces exocytosis of pseudo-WPB in HEK293 cells [13]. Incubation of HEK293 cells for 60 minutes with PMA increased the secretion of WT-VWF by 2.7 fold (12.2% of total VWF was secreted by PMA stimulated cells versus 4.5% by control cells, Figure 4). Both p.Cys1130Phe and p.Cys2773Ser transfected cells showed good response to PMA stimulation albeit that the total secretion of VWF p.Cys1130Phe was much lower. Only a minimal increase in the secretion of VWF p.Cys2671Tyr was observed after stimulation. Co-transfections of p.Cys2773Ser or p.Cys1130Phe showed a regulated secretion of VWF comparable to that of WT-VWF if judged by the folds increase, but to a much lower absolute level in the case of p.Cys1130Phe. Co-transfection of p.Cys2671Tyr only slightly increased the secretion of VWF during stimulation. These in vitro data for p.Cys1130Phe (co-transfection mimicking the heterozygous state) and p.Cys2671Tyr (single transfection mimicking the compound heterozygous state) are consistent with the response to DDAVP infusion in the patients with these mutations [29,30]. One may speculate that patients with p.Cys2773Ser will show a normal response to DDAVP, however, no patient was available for testing.

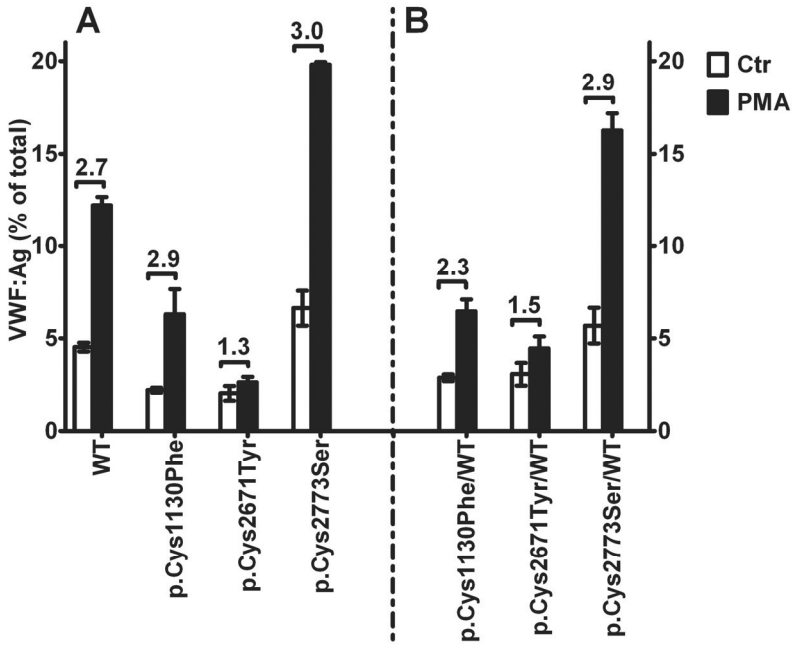


**Figure 4. Regulated secretion of WT-VWF or VWF variants.** HEK293 cells were transiently transfected with WT-VWF, p.Cys1130Phe, p.Cys2671Tyr and p.Cys2773Ser, respectively (Panel A). In panel B, p.Cys1130Phe, p.Cys2671Tyr or p.Cys2773Ser were co-transfected with WT-VWF at 1:1 ratio. Seventy-two hours post transfection HEK293 cells were rinsed twice with the release medium and incubated at 37°C for 60 minutes in the release medium without (Ctr) or with 160 nM PMA (PMA). Each bar represents VWF secreted into the release medium as a fraction of total VWF (medium plus lysate) times 100%. Mean and SEM are based on three independent experiments in duplicate. The numbers above the bars indicate the fold increase of secreted VWF comparing the stimulated (PMA) and control (Ctr) samples.

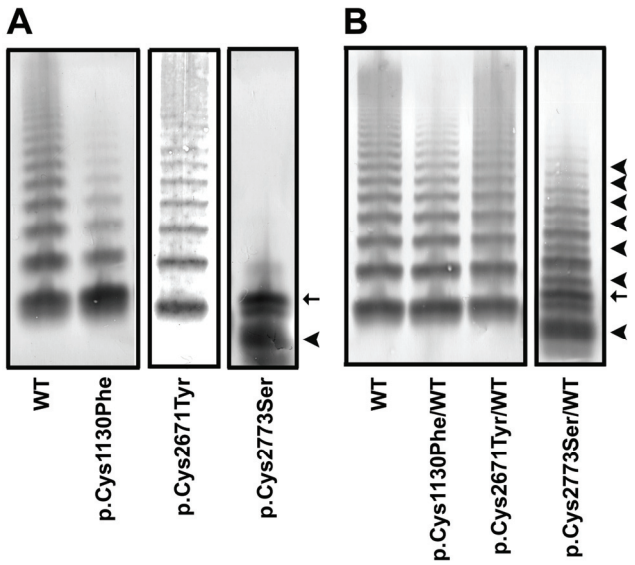


**Figure 5. Multimer analysis of secreted VWF.** Multimers are shown for single transfections (A) and co-transfections with WT-VWF at 1:1 ratio (B). The N-terminal dimers and odd-numbered multimers formed by VWF p.Cys2773Ser are indicated by the arrow and arrowheads, respectively. In panel A, the multimers of VWF p.Cys2671Tyr are from a different gel, the other separated parts are from one same gel. In panel B, the separated parts are from the same gel. The multimer patterns of secreted VWF were analyzed by SDS-agarose gel electrophoresis and Western blot under non-reducing conditions.

*Multimeric analysis of secreted VWF*

We performed multimer analysis of the conditioned media (Figure 5). A full range of VWF multimers is present in the conditioned medium for WT-VWF and VWF p.Cys2671Tyr, while VWF p.Cys1130Phe showed relative loss of large multimers indicating that mutations close to the interchain disulfide bonds may have a minor but significant effect on VWF multimerization. VWF p.Cys2773Ser mainly displayed monomers and dimers. The appearance of double bands for the dimers was caused by the different mobility between C-terminal dimers and N-terminal dimers formed by p.Cys2773Ser [17]. The multimeric defects caused by p.Cys1130Phe and p.Cys2773Ser were only partly restored by co-transfections with WT-VWF. This is consistent with the multimer patterns in the patients [17,19,20]. Furthermore, the odd-numbered VWF multimers produced by co-transfection of p.Cys2773Ser with WT-VWF confirm co-expression of both constructs in individual cells.

## Discussion

Chemical modification and mutagenesis studies have demonstrated that cysteines p.Cys2771 and p.Cys2773 are involved in interchain disulfide bond formation and are equally essential for VWF dimerization [14]. Natural mutations in the two cysteines led to essentially the same clinical phenotype [15-17]. In addition, extensive studies have indicated that p.Cys1130 at the N-terminal and p.Cys2671 at the C-terminal are not involved in the interchain disulfide bonds but rather involved in the intrachain disulfide bonds [14,31,32]. We therefore selected the three mutations p.Cys2773Ser, p.Cys1130Phe and p.Cys2671Tyr that were identified in our center [17,18,22] as exemplary mutants to examine the differential effects of interchain versus intrachain disulfide bonds on WPB biogenesis. p.Cys2773Ser was normally stored in and secreted from transfected HEK293 cells despite strongly reduced dimerization and multimerization. In contrast, p.Cys1130Phe and p.Cys2671Tyr resulted in strong retention of VWF in the ER and less VWF stored in WPB, which is consistent with previous reports for other cysteine mutations that disrupt intrachain disulfide bonds in VWF [13,33]. Therefore, our data suggest that VWF mutations that disrupt interchain disulfide bonds differ from mutations that disrupt intrachain disulfide bonds in the effect on WPB biogenesis.

The mutations that were analyzed in this study are located in different domains in VWF: p.Cys1130Phe in the D3 domain, p.Cys2671Tyr in the region immediately amino-terminal to the CK domain and p.Cys2773Ser in the CK domain itself. The different phenotypes of these mutations may be ascribed to the fact that they disrupt different domains of VWF but the differences in phenotypes may also be related to interchain versus intrachain defects. Several cysteine mutations, all involved in intrachain disulfide bonds, located in the D' (p.Cys788Arg), D3 (p.Cys1060Tyr, p.Cys1149Arg and p.Cys1225Gly) and CK (p.Cys2739Tyr and p.Cys2754Trp) domains showed very similar disruptive effects on the storage and secretion of VWF as p.Cys1130Phe and p.Cys2671Tyr [13,33]. This suggests that the effects are not domain specific. Furthermore, p.Cys2739Tyr and p.Cys2754Trp in the CK domain are not stored properly, whereas p.Cys2773Ser in the same domain but involved in interchain disulfide bonds showed normal storage [13]. In addition, another mutation p.Cys1099Tyr in the D3 domain that disrupts interchain disulfide bonds between N-termini of VWF was identified in VWD type II C Miami patients characterized by normal plasma level of VWF [31,34,35]. Even stronger support for our hypothesis comes from the 2010 version of the ISTH-SSC VWF

mutation database (www.vwf.group.shef.ac.uk/). It lists 72 different mutations involving cysteine residues, 50 loss of cysteines, 18 gain of cysteines and 4 premature stop codons (Supplemental Table S2). Among these mutations, 42 that predict the loss of intrachain disulfide cysteines are associated with low plasma levels of VWF, whereas 5 (p.Cys1099Tyr, p.Cys2771Ser/Tyr and p.Cys2773Arg/Ser) that predict the loss of interchain disulfide cysteines are associated with normal to relatively high plasma levels of VWF [14-17,31,35]. Therefore, we believe the differences are related to differential effects of mutations involved in intrachain versus interchain disulfide bonds.

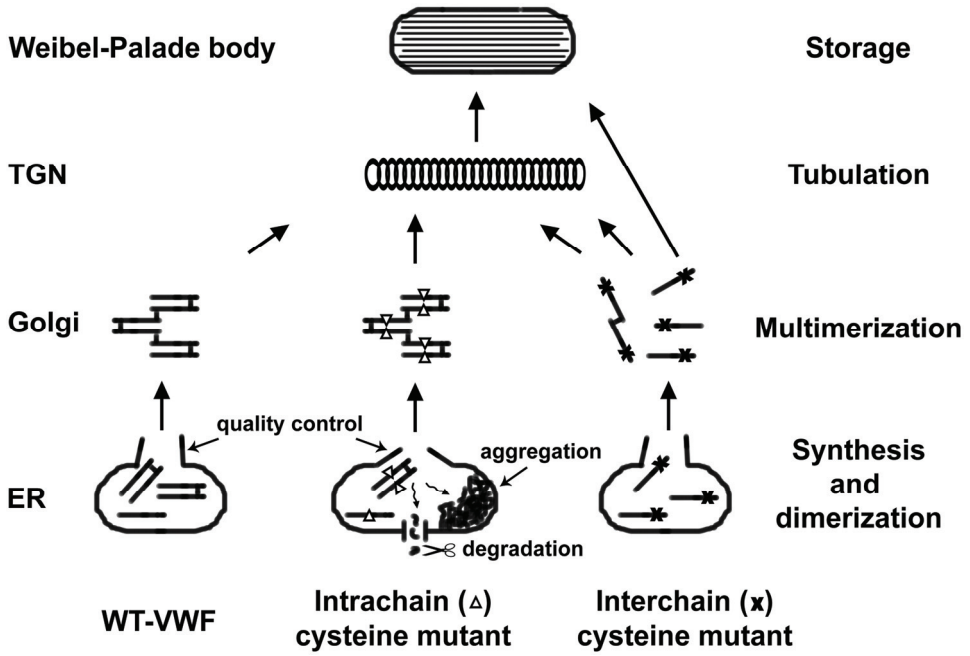


**Figure 6. Schematic illustration of VWF intracellular trafficking.** WT-VWF (left) is synthesized and dimerized in the ER, and passes the quality control to Golgi apparatus to multimerize and tubulize therein, and is eventually packed into WPB. Part of VWF mutants with impaired intrachain disulfide bond formation (middle) escape the quality control and are stored in WPB, the other part are retained in the ER undergoing aggregation or intracellular degradation. VWF mutants with impaired C-terminal interchain disulfide bond formation (right) fail to form C-terminal dimers in the ER. The monomers can get through the ER to the Golgi and partly form N-terminal dimers which are tubulized and stored into WPB. The remaining monomers are presumably stored into WPB, however, whether they are incorporated into VWF tubules is unknown. This hypothesis may also apply to the mutants lacking N-terminal interchain disulfide bonds.

We propose a model to explain the differential effects of intrachain versus interchain disulfide bonds on WPB formation (Figure 6). Disruption of intrachain disulfide bonds in VWF may mainly disturb the conformation of VWF such that the ER quality control system prevents trafficking to the Golgi apparatus [36]. The retained VWF molecules either aggregate in the ER to lead to ER dilation (Figure 2F-G) [13,33] or undergo intracellular degradation [13,37]. A fraction of VWF mutant molecules appear to escape the quality control system and are able to reach the Golgi apparatus. The extent to which this escape takes place appears to depend on the nature of defects. Once the mutants get to the *trans* Golgi where further processes of VWF are highly regulated by pH [6,7,38], their effects are minimal unless the defects cause severe changes in VWF conformation. In contrast, disruption of interchain disulfide bonds such as that in VWF C-terminal does not hamper trafficking to the Golgi apparatus and WPB thereafter. Therefore, C-terminal dimerization of VWF is not required for WPB formation. According to the notion that N-terminal dimerization (ie. multimerization) of VWF is not required for WPB formation [39], the mutations that disrupt interchain disulfides in VWF N-terminal may not disrupt the biogenesis of WPB as well. It is thus temping to speculate that unpairing all the five interchain disulfide bonds (two at N-terminal and three at C-terminal) should have minimal, if any, effect on the storage and secretion of VWF.

Tubulation is a key step to store VWF into WPB. Recent studies provide emerging evidence for a hypothesis that VWF tubulation is regulated by the low pH in *trans* Golgi and WPB [7,38,40,41]. In a cell-free system with low pH as seen in *trans* Golgi, Huang and colleagues showed that truncated N-terminal dimers of VWF D'D3 domains together with VWF propeptide formed tubular structures by self-assembly [38]. The present data confirmed this finding for full-length VWF in a cell system. The VWF p.Cys2773Ser that forms N-terminal dimers [17] and partly remains as monomers showed normal tubulation (Figure 2E). The persistence of monomers raises two questions: 1) whether (and how) are the monomers stored into pseudo-WPB, and 2) are those monomers able to form VWF tubules? As VWF is mainly secreted from WPB via basal and regulated pathways, the large amount of monomers that were detected in the medium of cells expressing p.Cys2773Ser (Figure 5A) suggest that VWF monomers are probably stored in the pseudo-WPB. Furthermore, immunofluorescent staining showed that very little VWF p.Cys2773Ser was localized in the ER (Figure 1D), indicating that the excessive monomers found in the lysate (data not shown) are indeed stored in the pseudo-

WPB. It is unknown whether those monomers are able to form tubules in the *trans* Golgi network as it has been suggested that N-terminal dimerization is required for the tubulation of VWF in the cell-free system [38]. Strikingly, Zhou and colleagues observed a dimeric bouquet-like VWF complex formed at the acidic pH of the *trans* Golgi and WPB [7]. C-terminal dimer of VWF was zipped up by the acidic pH from CK to B1 domain (six domains) into a dimeric stem. This process was proposed to facilitate VWF tubulation [7]. As such, unpairing the interchain disulfide bonds at the CK domain, such as by mutation p.Cys2773Ser, will unlock the start point of the zip. It is unknown whether the stability and/or conformation of the dimeric complex will be affected. As formation of the dimeric complex is highly pH dependent, the p.Cys2773Ser monomers are likely to form the bouquet-like complex and therefore to incorporate into VWF tubules. In contrast, VWF mutations p.Cys2739Tyr and p.Cys2754Trp that cause severe conformational changes by defects in intrachain disulfide bonds led to disorganized tubular storage of VWF [13]. This suggests that the conformational changes in VWF may be more disruptive to VWF tubulation than no interchain pairing.

In conclusion, we postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds, p.Cys2771, p.Cys2773 and p.Cys2811 in the CK domain and p.Cys1099 and p.Cys1142 in the D3 domain, do not affect the storage and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds will lead to reduced VWF storage and secretion due to ER retention.

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## Table S1. Expression of VWF variants in transfected HEK293 cells

| Variant | Transfection | VWF:Ag* Medium | | VWF:Ag* Lysate | | Ratio† |
|---|---|---|---|---|---|---|
| | | mU | % | mU | % | |
| **WT-VWF** | | 74.1 ± 4.7 | 100 | 23.8 ± 3.2 | 100 | 3.2 ± 0.2 |
| **p.Cys1130Phe** | Single transfection | 41.3 ± 2.7 | 56.4 ± 6.4 | 59.2 ± 11.1 | 255.9 ± 56.7 | 0.7 ± 0.1‡ |
| | Co-transfection | 63.7 ± 4.8 | 86.3 ± 4.1 | 44.0 ± 5.8 | 186.3 ± 9.9 | 1.5 ± 0.1‡ |
| **p.Cys2671Tyr** | Single transfection | 55.2 ± 5.8 | 76.0 ± 12.7 | 45.5 ± 4.8 | 194.3 ± 18.2 | 1.2 ± 0.2‡ |
| | Co-transfection | 65.8 ± 4.4 | 89.0 ± 4.1 | 32.2 ± 3.2 | 136.5 ± 5.9 | 2.1 ± 0.1‡ |
| **p.Cys2773Ser** | Single transfection | 90.8 ± 10.7 | 124.0 ± 18.2 | 35.7 ± 2.1 | 154.1 ± 16.1 | 2.5 ± 0.2§ |
| | Co-transfection | 84.1 ± 11.6 | 114.7 ± 17.7 | 33.6 ± 4.1 | 142.1 ± 5.9 | 2.5 ± 0.4§ |

*VWF:Ag (mU) was produced by about $7\times10^5$ cells in the medium or lysate. In parallel, VWF:Ag is expressed as percentage relative to the amount of VWF:Ag in the medium and lysate of cells expressing WT-VWF. Each value represents the mean ± SEM of three independent experiments in duplicate.

†Ratio of the absolute amount of VWF:Ag in Medium to VWF:Ag in Lysate.

‡Compared with WT-VWF $p<0.01$.

§Compared with WT-VWF $p>0.05$.

## Table S2. VWF cysteine mutations identified in VWD*

| *Loss of cysteine* | | | |
|---|---|---|---|
| **Domain** | **Mutation** | **VWD type** | **VWF level** |
| D1 | p.Cys57* | 3 | ↓ |
| D1 | p.Cys263_E270del | 3 | ↓ |
| D1 | p.Cys275(Ser/Arg)† | 1 or 3 | ↓ |
| D2 | p.Cys570Ser | 2A | ↓ |
| D2 | p.Cys623Trp | 2A | ↓ |
| D' | p.Cys709Leufs*3 | 2A | ↓ |
| D' | p.Cys788(Arg/Tyr) | 2N | ↓ |
| D' | p.Cys804Phe | 2N | ↓ |
| D' | p.Cys858Phe | 2N | ↓ |
| D3 | p.Cys996Glu | 1 | ↓ |
| D3 | p.Cys1060(Arg/Tyr) | 1 or 2N | ↓ |
| D3 | p.Cys1071Phe | 3 | ↓ |
| D3 | p.Cys1099Tyr‡§ | IIC Miami | =/↑ |
| D3 | p.Cys1101(Arg/Trp) | Unclassified | N.A. |
| D3 | p.Cys1111Tyr | 1 | ↓ |
| D3 | p.Cys1130(Phe/Gly/Arg) | 1 | ↓ |
| D3 | p.Cys1149Arg | 1 | ↓ |
| D3 | p.Cys1157Phe | Unclassified | ↓ |
| D3 | p.Cys1190Arg | 1 | ↓ |
| D3 | p.Cys1196Arg | 2M or 3 | ↓ |
| D3 | p.Cys1225Gly | 2N | ↓ |
| D3 | p.Cys1227Arg | 1 | ↓ |
| D3 | p.Cys1234Trp | Unclassified | ↓ |
| A1 | p.Cys1272(Phe/Gly/Arg/Ser) | 2A | ↓ |
| A1 | p.Cys1458Tyr | 2A | N.A. |
| D4 | p.Cys2174Gly | 3 | ↓ |
| D4 | p.Cys2257Ser | 1 | ↓ |
| B1-B3 | p.Cys2304Tyr | 1 | ↓ |
| B1-B3 | p.Cys2340Arg | 1 | ↓ |
| B1-B3 | p.Cys2362Phe | 1 or 3 | ↓ |
| C2 | p.Cys2477(Ser/Tyr) | 1 | ↓ |
| C2 | p.Cys2533* | Unclassified | ↓ |
| C2 | p.Cys2557Serfs*8 | 3 | ↓ |
| C2-CK¶ | p.Cys2671Tyr | 3 | ↓ |
| C2-CK¶ | p.Cys2693Tyr | 1 | ↓ |
| CK | p.Cys2739Tyr | 3 | ↓ |
| CK | p.Cys2754Trp | 3 | ↓ |
| CK | p.Cys2771(Ser/Tyr)‡ | 2A(IID) | =/↑ |
| CK | p.Cys2773(Arg/Ser)‡ | 2A(IID) | =/↑ |
| CK | p.Cys2804(Tyr/fs) | 1 or 3 | ↓ |
| CK | p.Cys2806Arg | Unclassified | ↓ |

## Table S2. VWF cysteine mutations identified in VWD* (continued)

*Gain of cysteine*

| Domain | Mutation | VWD type | VWF level |
|---|---|---|---|
| D1 | p.Trp377Cys | 3 | ↓ |
| D2 | p.Arg760Cys | 2N | ↓ |
| D' | p.Tyr795Cys | 2N | ↓ |
| D3 | p.Tyr1107Cys | Unclassified | ↓ |
| D3 | p.Tyr1146Cys | 1 | ↓ |
| A1 | p.Arg1308Cys | 2B | N.A. |
| A1 | p.Trp1313Cys | 2B | N.A. |
| A1 | p.Arg1315Cys | 1 or 3 | ↓ |
| A1 | p.Arg1342Cys | 1 | ↓ |
| A1 | p.Arg1374Cys | 1 or 2A or 2M | ↓ |
| A1 | p.Arg1379Cys | 1 or unclassified | ↓ |
| A1 | p.Arg1399Cys | Unclassified | N.A. |
| A2 | p.Phe1514Cys | 2A | N.A. |
| A2 | p.Tyr1584Cys | 1 | ↓ |
| A3 | p.Trp1745Cys | 2M | ↓ |
| B1-B3 | p.Arg2379Cys | 1 | ↓ |
| C2 | p.Gly2441Cys | 1 | ↓ |
| C2 | p.Arg2464Cys | 1 or unclassified | ↓ |

*Summarized from the ISTH-SSC VWF mutation database up to 2010 (www.vwf.group.shef.ac.uk/); †Multiple mutations reported at a position; ‡Cysteines involved in the interchain disulfide bonds in VWF; N.A., data not available; §Not yet in the ISTH-SSC VWF mutation database;[35] ¶The connecting area between C2 and CK domain.