Dear Editor,

Von Willebrand factor (VWF) assembly begins in the endoplasmic reticulum of endothelial cells and megakaryocytes where VWF is synthesized as a precursor with multiple domains (Fig. 1a). The polypeptide including the propeptide and mature VWF chain (proVWF) subsequently forms “tail-to-tail” homodimers through their C-terminal cystine knot (CK) domains. These proVWF dimers are then transported to the Golgi where they assemble into large multimers ‘head-to-head’ through interchain disulfide bonds between D3 domains of two proVWF dimers. The Sadler group firstly identified a peptide containing the Cys1142–Cys1142 disulfide bond from plasma multimeric VWF. Subsequently, through differential alkylation, proteolytic digestion, and mass spectrometry, they identified two free cysteines Cys1099 and Cys1142 from D3 monomer and proposed their involvement in D3 dimer formation. However, the crystal structure of a monomeric D3 mutant (C1099A/C1142A) reported by Dong and Springer revealed that residue Cys1099 is largely buried inside of the D3 domain and it has to undergo a dramatic conformational change to allow forming a disulfide bond with the same cysteine from the other D3 molecule. Moreover, the structures of dimeric D3 domains of Mucin 2 (MUC2), a homologous multimeric protein of VWF, showed Cys1142–Cys1142 and Cys1097–Cys1097′ disulfide linkages (using VWF residue numbering). Cys1091 formed an intramolecular disulfide bond with Cys1099 in MUC2 instead of the Cys1091–Cys1097 seen in the crystal structure of the VWF D3 mutant. Therefore, the disulfide linkages and the cysteines involved in the VWF D3 dimer interface remain to be confirmed with the discrepancy mainly on Cys1097 and Cys1099 (Fig. 1a). Here we identified the disulfide linkages from the D3 dimer interface through chemical digestion and mass spectrometry and solved the cryo-electron microscopy (cryo-EM) structure of VWF tubules with each repeating unit containing one D3 dimer and one D1D2 dimer.

As the disulfide linkage of Cys1099 or Cys1097 from the VWF D3 dimer interface has never been identified and no suitable enzymic cleavage sites near the dimeric interface could be utilized to distinguish the cysteines of our interest, namely Cys1091, Cys1097, and Cys1099, here we designed a series of D3 mutants with residues flanking Cys1097, Cys1099, or Cys1142 mutated to methionines (Fig. 1b), and identified the intermolecular disulfide linkages directly by CNBr cleavage and mass spectrometry. For the D3 dimer variant with Glu1092 and Ala1098 flanking Cys1097 mutated to methionines (D3′-E1092M-A1098M), a doubly charged parent ion with m/z = 576.223 corresponding to the predicted Cys1097–Cys1097′ disulfide-linked peptide (1093SIGDC (Hsl)1098)2 where M was converted to homoserine lactone (Hsl) could be readily identified. The identity of this peptide was further confirmed by the MS/MS spectrum of this ion with all the expected ions of peptide fragments detected such as y4 (m/z = 951.321, z = 1) and b2 (m/z = 201.124, z = 1) (Fig. 1b). Similarly, a doubly charged ion with m/z = 796.270 was identified from the dimer of the D3 variant (D3′-R1136M-E1143M), which corresponded to the Cys1142–Cys1142′ disulfide-linked peptide (1137ENGYEC (Hsl)1143)2 (Supplementary Fig. S1a). Notably, the disulfide-linked peptides where one or two methionines were converted to homoserines could also be readily identified (Supplementary Fig. S1b, c). Therefore, these results...
Propeptide  Furin  Mature VWF chain

---C--S--C--M--S--I--G--D--C--M--C--F--C---

---C--S--C--M--S--I--G--D--C--M--C--F--C---

a)

b)

D1

D2

D'                  D3

A1-3

D4

C1-6

CK

---C--S--C--E--S--I--G--D--C--A--C--F--C---

---C--S--C--E--S--I--G--D--C--A--C--F--C---

1091                   1097 1099                     1136                     1142        1145

---C--S--C--M--S--I--G--D--C--M--C--F--C---

---C--S--C--M--S--I--G--D--C--M--C--F--C---

C1142

C1101

C1126

C1097

C1099

C1071

C1111

Fig. 1 (See legend on next page.)
The peptides (1137ENGYEC(NEM)EWR1145) with dithiothreitol (DTT) and a mechanism proposed by Dong and Springer6,7. Notably, the replacement of either Cys1097 or Cys1142 alone would disulfide bond the two minor peaks (II, III) were identified previously where D1D2 derived from the expression medium was complexed with D1D2 domains at mild acidic pH, only D3 monomers with Cys1097–Cys1142 bond could readily bind each other complementarily and allow intermolecular Cys1097–Cys1142 disulfide bond formation.

We then tried to verify the free cysteines in the D3 monomer using the same differential alkylation procedure reported previously where D3 monomers were treated with N-ethylmaleimide (NEM) to block free thiols and those with Cys1097 modified by NEM could be readily detected from the tryptic digestion (data not shown). Interestingly, four peptide peaks were identified from the Asp-N digestion by liquid chromatography with identical doubly charged ions with $m/z = 498.675$, which corresponded to peptide 1096DC(4-VP)AC(NEM)FC(4-VP)1101 modified by one NEM and two 4-VP molecules (Fig. 1c). The peptides in the two major peaks (I and IV) were confirmed by the MS/MS spectrum to be 1096DC(4-VP)AC(NEM)FC(4-VP)1101 with two peaks representing two diastereomers from the NEM derivative. This is consistent with previous findings that Cys1099 in D3 monomer could be modified by NEM8. The peptides in the two minor peaks (II, III) were identified to be 1096DC(NEM)AC(4-VP)FC(4-VP)1101, corresponding to those with Cys1097 modified by NEM (Fig. 1c). These results indicate that Cys1099 is the free cysteine in most D3 monomers, but Cys1097 is free in a small amount of D3 monomers. Thus, it is highly plausible that there is an equilibrium of Cys1099 and Cys1097 forming an alternative disulfide bond with the same cysteine, most likely Cys1097, which may be consistent with the disulfide exchange mechanism proposed by Dong and Springer6,7. Notably, the replacement of either Cys1097 or Cys1142 alone would not prevent D3 dimerization, consistent with a similar mutagenesis study of MUC28 (Supplementary Fig. S2).

In order to gain further structural information of the D3 dimer interface, we purified D3 dimers complexed with D1D2 derived from the expression medium of HEK293 cells transfected with expression plasmid of D1D2/D3-wt or D1D2/D3-R1136M-E1143M. The tube-like oligomers of these complexes were analyzed by single-particle cryo-EM (Supplementary Figs. S3, S4). This yielded an electron density map covering two repeating units for D3-R1136M-E1143M dimer complexed with D1D2 at 3.3 Å resolution and an electron density map covering one repeating unit for D3-wt dimer complexed with D1D2 at 3.4 Å resolution respectively (Supplementary Table S1). Each repeating unit contains a D3 dimer and a D1D2 dimer. As the overall configuration of the refined D3-wt complex is essentially the same as that of the D3-R1136M-E1143M mutant, the structure of the mutant was selected for presentation due to its slightly better resolution. The shape of the repeating unit largely resembles that of MUC2 where a D3 dimer is docked in the central hole of the donut-shaped D1D2 dimer8 (Fig. 1d, e). Since the crystal structure of a monomeric D3 variant has been solved previously8, this allowed us to build the D3 dimer structure with confidence (Fig. 1e; Supplementary Table S1). The electron density near the center covering the D3 dimer is unambiguous and a clear density covering the disulfide bonds near the dimeric interface can be observed (Fig. 1f).

The structure (Fig. 1e) showed that D3 retained the overall conformation seen in the crystal structure of D3 monomer (rmsd of 1.7 Å) but with local conformational rearrangements near the dimeric interface (Fig. 1g, h and Supplementary Figs. S5, S6). The connecting loop linking two helices in the D3 monomer restrained by the Cys1091–Cys1142 disulfide is anchored in a hydrophobic pocket formed by Phe1100, Met1105, and Val1106 through Ile1194, but in the dimer, it is more flexible with the intramolecular Cys1091–Cys1099 bond and flips over to dock in the same hydrophobic surface pocket of the other D3 molecule in a domain swapping-like fashion (Supplementary Fig. S6). This brings two Cys1097 residues in proximity and the subsequent formation of an intermolecular disulfide bond. As Cys1142 in a double-stranded hairpin was shielded from solvent exposure in the D3 monomer structure6, but rotated and shifted ~6 Å to form...
an intermolecular disulfide bond with its counterpart in the other D′D3 molecule in the dimer (Supplementary Figs. S5, S6), it seems that VWF has adopted an allosteric mechanism for the intermolecular disulfide formation with the free thiols in the monomers protected from surface exposure avoiding unwanted oxidation during the biosynthesis.

Overall, our studies here indicate that D′D3 monomer equilibriums between two configurations where Cys1091 forms an intramolecular disulfide bond with either Cys1097 or Cys1099 (Fig. 1i). However, only the minor D′D3 confirmation containing a longer connecting loop with the Cys1091–Cys1099 bond and free Cys1097 could allow domain swapping-like complementary binding of the two loops in the dimer interface and the formation of intermolecular Cys1097–Cys1099 disulfide bond in the presence of VWF domain D1D2s (Fig. 1i). As there are very limited noncovalent interactions in the D′D3 dimeric interface (Supplementary Fig. S6), D1D2 is indispensable for its dimerization by aligning two D3 monomers in optimal positions for their intermolecular disulfide formation (Fig. 1d, i). Although it remains unclear how the disulfides in the D′D3 dimer interface would reshuffle or form at pH 5.8 in Golgi and various mechanisms have been proposed7,9, our cryo-EM structure of a D′D3 dimer complexed with D1D2s shows that the two Cys1097 are largely buried in the center of the dimeric interface and there is limited space to allow the participation of any bulky redox-regulating enzymes (Supplementary Figs. S7, S8). Therefore, it is plausible that only small redox molecules are directly involved in this process, which would be consistent with the previously observed formation of VWF oligomers in vitro10.

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A.Z. conceived the project. Z.S., J.Z., L.X., and H.C. performed the experiments. All authors analyzed the data and contributed to the paper preparation. A.Z. and Z.S. wrote the paper.

Conflict of interest

The authors declare no competing interests.

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Supplementary information

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