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Structure-function and regulation of ADAMTS13 protease

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Summary

ADAMTS13, a plasma reprolysin-like metalloprotease, cleaves von Willebrand factor (VWF). Severe deficiency of plasma ADAMTS13 activity results in thrombotic thrombocytopenic purpura (TTP), while mild to moderate deficiencies of plasma ADAMTS13 activity are emerging risk factors for developing myocardial and cerebral infarction, preeclampsia, and malignant malaria. Moreover, *Adamts13*$^{-/-}$ mice develop more severe inflammatory responses, leading to increased ischaemia/perfusion injury and formation of atherosclerosis. Structure-function studies demonstrate that the N-terminal portion of ADAMTS13 (MDTCS) is necessary and sufficient for proteolytic cleavage of VWF under various conditions and attenuation of arterial/venous thrombosis after oxidative injury. The more distal portion of ADAMTS13 (TSP1 2–8 repeats and CUB domains) may function as a disulphide bond reductase to prevent an elongation of ultra large VWF strings on activated endothelial cells and inhibit platelet adhesion/aggregation on collagen surface under flow. Remarkably, the proteolytic cleavage of VWF by ADAMTS13 is accelerated by FVIII and platelets under fluid shear stress. A disruption of the interactions between FVIII (or platelet glycoprotein 1bα) and VWF dramatically impairs ADAMTS13-dependent proteolysis of VWF *in vitro* and *in vivo*. These results suggest that FVIII and platelets may be physiological cofactors regulating VWF proteolysis. Finally, the structure-function and autoantibody mapping studies allow us to identify an ADAMTS13 variant with increased specific activity but reduced inhibition by autoantibodies in patients with acquired TTP. Together, these findings provide novel insight into the mechanism of VWF proteolysis and tools for the therapy of acquired TTP and perhaps other arterial thrombotic disorders.

ADAMTS13 and potential human diseases

ADAMTS13, first identified and cloned in 2001, is a member of the ADAMTS (A Disintegrin And Metalloprotease with ThromboSpondin type 1 repeats) family (1,2). It cleaves a large polymeric adhesion protein von Willebrand factor (VWF). VWF is synthesised in vascular endothelial cells and megakaryocytes (3,4). The newly synthesised VWF is stored in intracellular organelles: Weibel-Palade bodies in endothelial cells and α-granules in megakaryocytes and platelets (3,4). VWF is released upon physiological or pathological stimulation and forms an ultra-long or ultra-large (UL) “string-like” structure on the endothelial surface (5–7). These UL-VWF “string-like” structures are hyperactive in recruiting circulating platelets to the site of endothelial activation and/or injury. Plasma ADAMTS13, which is primarily synthesised and released from hepatic stellate cells (8–10) and endothelial cells (11,12), binds and cleaves cell bound UL-VWF strings at the Tyr^{1605}-Met^{1606} bond, thereby eliminating the UL-VWF from the endothelial surface and resulting

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in fragmentation of the VWF strings (5–7). In addition, ADAMTS13 cleaves UL or large VWF in solution after being exposed to high fluid shear stress as seen in microcirculation or at the site of narrow vessels and thrombus formation after injury (13–15) (Fig. 1). Arterial shear stress induces conformational changes in soluble multimeric VWF (16–19) so that it becomes accessible by ADAMTS13 for cleavage. The conformational changes can also be induced *in vitro* by an addition of a denaturant such as urea (20,21) or guanidine-HCl (22,23), which is the molecular basis of various biochemical assays for plasma ADAMTS13 activity.

An inability to cleave cell-bound and soluble UL or large VWF in circulation because of severe deficiency of plasma ADAMTS13 activity (less than 5% of normal) results in the persistence of hyperactive UL-VWF on endothelial cells (5,6,24,25) and in circulating blood (26). This leads to excessive platelet aggregation and disseminated VWF/platelet-rich thrombus formation (27–29), the characteristic feature of thrombotic thrombocytopenic purpura (TTP). Patients with TTP manifest by marked thrombocytopenia (platelet count, usually less than 20×10^9/L) and microangiopathic haemolytic anaemia (with low haematocrit, elevated lactate dehydrogenase, and fragmentation of red blood cells) (30–33). Some patients may exhibit neurological signs and symptoms or renal abnormalities (30–33). Idiopathic TTP in most adult patients is caused by an acquired deficiency of plasma ADAMTS13 activity resulting from the formation of inhibitory autoantibodies against ADAMTS13 (23,34,35). Rarely, TTP is caused by one or several germ line mutations in the *ADAMTS13* gene (1,34,36–38), resulting in severe deficiency of plasma ADAMTS13 activity at birth. Several animal models have been established for testing novel therapies. *Adamts13*$^{-/-}$ mice develop a “TTP-like” syndrome only after a trigger such as shigatoxin (39,40) or recombinant VWF challenge (41). However, in baboon, TTP syndrome occurs subsequently after complete inhibition of plasma ADAMTS13 activity by an intravenous administration of anti-ADAMTS13 monoclonal antibodies against the metalloprotease domain without additional triggers (42).

The importance of the discovery of ADAMTS13 extends beyond its association with the potentially fatal TTP syndrome (Table 1). Studies have demonstrated that reduced plasma ADAMTS13 activity and increased plasma VWF (the only known substrate for ADAMTS13) are risk factors for the development of myocardial infarction (43–45), ischaemic stroke (46–48), preeclampsia (49), and malignant (or cerebral) malaria (50–54). Moreover, *Adamts13*$^{-/-}$ mice demonstrate an increased (~2–5 fold) area of atherosclerotic lesion *en face* and increased macrophage infiltration as compared with those in WT mice in an *ApoE*$^{-/-}$ background (55,56). More recently, studies have demonstrated that *Adamts13*$^{-/-}$ mice exhibit an increase of infarct sizes in the myocardium (57–59) and brain (47,60) after ischaemic/perfusion injury. An infusion of recombinant human ADAMTS13 into *Adamts13*$^{-/-}$ mice significantly attenuates infarct sizes (47,60). These findings indicate that ADAMTS13 offers systemic protection against ischaemic myocardial and cerebral infarctions. Therefore, an investigation of the biosynthesis, structure-function relationship, and cofactor-dependent regulation of ADAMTS13 protease will provide novel tools for diagnosis and treatment of many potentially fatal human diseases.

Biosynthesis and secretion of ADAMTS13

ADAMTS13 is primarily synthesised in the liver of humans, mice, and rats (1,2,8–10). The mRNA encoding the full-length *ADAMTS13* (~4.3 kb) is detected only in the liver by Northern blotting analysis (1,2). However, a truncated form of *ADAMTS13* mRNA (~2.4 kb) is found in other tissues such as placenta and skeletal muscle by the same method (2). Using reverse polymerase chain reaction (PCR), fragments of *ADAMTS13* mRNA are amplified in many tissues including the kidneys, pancreas, spleen, thymus, prostate, testis,

ovary, small intestine, colon, and peripheral blood leukocytes (1,61). Whether it is synthesised in endothelial cells or parenchymal cells remains to be determined.

In the liver, ADAMTS13 is localised to the hepatic stellate cells (HSCs) residing in the interstitial area of human (9), mouse (10), and rat (8) livers. *ADAMTS13* mRNA and protein expression in the rat HSCs are dramatically up-regulated upon activation by mechanics and inflammatory cytokines such as transforming growth factor-β (TGF-β) *in vitro* and *in vivo* (8), suggesting that ADAMTS13 may play a role in tissue remodelling after injury. In addition, ADAMTS13 protein produced in the HSCs may diffuse into capillaries and enters into the blood stream, thereby regulating plasma levels of ADAMTS13 activity. The evidence supporting this hypothesis include: a) reduced plasma ADAMTS13 activity after a partial hepatectomy in humans (62) or after treatment with dimethylnitrosamine, which damages stellate cells in rats (63); and b) increased expression of ADAMTS13 in HSCs and elevated plasma ADAMTS13 activity upon activation in rat models of cholestasis and steatohepatitis (64).

ADAMTS13 mRNA and protein have also been detected in vascular endothelial cells *in vitro* and *in vivo* (11,12,65). It is estimated that unstimulated human umbilical vessel endothelial cells (HUVECs) in culture produce approximately 1 ng ADAMTS13 per millilitre of conditioned medium every 60 minutes. The amount of ADAMTS13 is ~100-fold less than VWF (100 ng/ml) produced by HUVECs under the same conditions (65). Immunohistochemistry demonstrates that ADAMTS13 is not co-localised with VWF in Weibel-Palade bodies (11,12,65), suggesting that ADAMTS13 is constitutively secreted from cells.

The function of ADAMTS13 synthesised in the endothelium is not fully understood. While endothelial cells produce trace amounts of ADAMTS13 in culture, their massive surface coverage suggests a potentially substantial contribution of the endothelium-derived ADAMTS13 to plasma ADAMTS13 activity. In addition, ADAMTS13 released from endothelial cells may cleave newly formed UL-VWF strings on the cell surface, providing an additional mechanism to maintain a VWF-free surface (65–68). Moreover, Lee *et al.* have demonstrated that ADAMTS13 has either pro-angiogenic or anti-angiogenic effects depending on the cellular environments (69). On one hand, treatment of HUVECs with recombinant ADAMTS13 results in dramatically increased tube formation and cell migration, suggesting enhanced angiogenesis. On the other hand, when vascular endothelial growth factor (VEGF) is present in the culture medium ADAMTS13 inhibits VEGF-induced angiogenic activity. This anti-angiogenic effect is reversed by pre-incubation of ADAMTS13 with a polyclonal antibody against the C-terminal TSP1 5–7 repeats of ADAMTS13 (69), suggesting a role of TSP1 repeats in mediating the pro- and anti-angiogenic effects.

A small quantity of *ADAMTS13* mRNA and protein is detectable in human megakaryocytes and platelets (70,71). One study shows that platelets contain less than 160 ng ADAMTS13 per 1×10^8 platelets (70). The amount of ADAMTS13 produced in platelets may be overestimated. The biological function of platelet-derived ADAMTS13 remains unknown. Preliminary study from our laboratory, presented in the 54th Annual Meeting of the American Society of Hematology, demonstrated that transgenically overexpressed ADAMTS13 in the platelets of *Adamts13*$^{-/-}$ mice is releasable upon activation by thrombin and collagen, as well as during the thrombus formation after injury with 10% ferric chloride (72). The secreted human ADAMTS13 was able to dramatically inhibit thrombus growth in mesenteric arterioles after oxidative injury and protects *Adamts13*$^{-/-}$ mice from VWF- and shigatoxin-induced “TTP-like” syndrome (72). These results suggest that platelet-derived ADAMTS13 may be biologically important. The release of ADAMTS13 at the site of

thrombus formation may offer a novel treatment for acquired TTP with inhibitors. A similar strategy has been reported for the treatment of haemophiliacs with inhibitors with success in murine models (73–75).

Structure-function relationship of ADAMTS13

ADAMTS13 shares similar domain structure as compared with other ADAMTS family proteases, comprising a signal peptide, a propeptide, a metalloprotease, a disintegrin-like domain, first thrombospondin type 1 repeat (TSP1), Cys-rich and spacer domains. The more distal C-terminus contains seven additional TSP1 repeats and two CUB domains (Fig. 2). The function of each domain of ADAMTS13 in its biosynthesis, secretion, and proteolytic activity has been extensively studied in recent years.

Unlike the propeptides of other ADAMTS or ADAM family proteases (typically ~200 amino acid residues) (76,77), the ADAMTS13 propeptide is exceptionally short (only ~41 residues). While the propeptides of other ADAMTS proteases function as molecular chaperones to assist protein folding and maintain the latency of the protease by a mechanism of “cysteine-switch”, the ADAMTS13 propeptide is not required for its secretion and activation. Recombinant human ADAMTS13 expressed in cells with or without a propeptide secrets normally and is able to cleave VWF substrates with similar efficacy (78).

The metalloprotease domain of ADAMTS13 has the expected hallmarks of the reprolysin or adamalysin family of metalloproteases, which include three histidine residues that coordinate the essential Zn^{2+} ion in the sequence **H**E*XX***H***_*XX*G*XX***H**D (Fig. 3) (2). In addition, three putative Ca^{2+}-binding sites have been postulated based on modelling the metalloprotease domain of ADAMTS4 and ADAMTS5 (79) (Fig. 3). The first putative Ca^{2+} binding site comprises amino acid residues Glu83, Asp173, Cys281, and Asp284 that are broadly conserved among ADAMTS and other metalloproteases and appear to mediate low affinity Ca^{2+} binding. The second putative Ca^{2+} binding site consists of residues Glu164 and Asp166 in conjunction with one or more of residues Asn162, Asp165, and Asp168. Mutations at the second putative site have no effect on the Ca^{2+}-dependent ADAMTS13 activity. The third site is predicted to include residues Asp187 and Glu212 in conjunction with Asp182 or Glu184 (Fig. 3). Mutations at this site dramatically reduce Ca^{2+}-induced ADAMTS13 activity, suggesting that the residues at the third site play an important role in high-affinity Ca^{2+} binding and proteolytic activity (79).

Like many clotting factors, exosite interaction appears to govern enzymatic activity and substrate specificity. The ADAMTS13 metalloprotease domain alone has no or little proteolytic activity toward VWF substrates. The proteolytic activity increases as more and more non-catalytic domains are sequentially added to the metalloprotease domain (80–83). As shown in the model of MDTCS fragments based on the crystal structure of ADAMTS13-DTCS (84) and the metalloprotease domains of ADAMTS1 (85), ADAMTS4, and ADAMTS5 (86) (Fig. 2), the metalloprotease and disintegrin-like domain (MD) appear to be an inseparable functional unit (80,81,87,88). Experimental data also show that the addition of the disintegrin-like domain to the metalloprotease domain significantly increases cleavage efficiency and specificity (80,87). ADAMTS13 variants lacking the disintegrin-like domain (80,81,87) or carrying point mutations in the variable regions of disintegrin-like domain (Arg349Ala and Leu350Gly) (87) have dramatically reduced proteolytic activity toward both peptide substrates and multimeric VWF, suggesting the importance of the disintegrin-like domain in substrate recognition. Further studies demonstrate that residues Arg349 and Leu350 of the ADAMTS13 disintegrin domain may interact with residues Asp1614 and Ala1612 in the central A2 domain of VWF. Such interactions appear to assist in positioning the Tyr1605-Met1606 bond into the active-site cleft, thereby markedly affecting the rate

constant (K_m) and catalytic efficiency (k_{cat}) for substrate proteolysis (89,90). More recently, Xiang *et al* demonstrated that VWF Leu1603 (P3) Tyr1605, and Asp1614 appear to make direct contact with Leu198, Val195, and Arg349 in ADAMTS13, respectively (90). Therefore, the disintegrin domain, working in concert with other non-catalytic domains, ensures that the scissile bond is brought into position over the active centre for cleavage to occur.

Much of our attention has been focused on the role of the Cys-rich and spacer domains of ADAMTS13 in substrate recognition (80–83,88,91). ADAMTS13 variants lacking both the Cys-rich and spacer domains or the spacer domain alone exhibit only minimal activity toward peptidyl substrates but have nearly no activity toward cell bound UL-VWF (66,92) and soluble VWF under various conditions (18,80,82,83). We and others have shown that proteolytic cleavage of a peptidyl substrate increases as function of each of the non-catalytic domains (DTCS) is added to the metalloprotease domain (80,81,83). However, further addition of the TSP1 2–8 and CUB domains does not increase proteolytic activity. These results suggest that the exosite interaction between the ADAMTS13-DTCS domains and VWF-A2 domain is critical for proteolysis of VWF. These non-catalytic (DTCS) domains of ADAMTS13 are shown to directly interact in a linear fashion with various segments in the central VWF-A2 between residues Asp1614 and Arg1668 (81,89,93).

A replacement of the TCS domains of ADAMTS5, a closely related member of ADAMTS proteases, with those of ADAMTS13 alters ADAMTS5 substrate specificity (93). ADAMTS5 is not known to cleave VWF, but a chimeric variant that consists of the metalloprotease and disintegrin domains-like of ADAMTS5 (MD5) and three non-catalytic TCS domains of ADAMTS13 (TCS13) (MD5/TCS13) is able to cleave the Glu1615-Ile1616 bond of VWF domain A2 in peptide substrates or VWF multimers that has been sheared (93). However, this cleavage site is no longer at the Tyr1605-Met1606 bond (93), further confirming the critical role of each of non-catalytic domains of ADAMTS13 in substrate specificity.

Further sequence analysis of the spacer domains from ADAMTS13 and other ADAMTS family proteases has allowed us to identify several potential exosites that are highly conserved in human, murine, and zebrafish ADAMTS13 but absent in the other members of the ADAMTS family (Fig. 4B) (94). One of these exosites, also described as exosite 3, comprises amino acid residues Tyr658, Arg659, Arg660, Tyr661, and Tyr665 (in conjunction with two surrounding residues Arg568 and Phe592) (Fig. 4C and 4D) (84,94). A deletion of residues Arg659-Glu664 (Δ6aa) or a substitution of residues Arg659, Arg660, and Tyr661 with alanine dramatically reduces proteolytic cleavage of various VWF substrates (94,95).

Additional evidence to support the physiological role of exosite 3 comes from our mouse models of thrombosis. The ADAMTS13-Δ6aa variant exhibits dramatically reduced anti-thrombotic activity compared with WT-ADAMTS13 in the mesenteric arteriolar occlusion assay in *Adamts13*$^{-/-}$ mice (18). Together, these data suggest the critical role of exosite 3 in the spacer domain in substrate recognition and proteolysis of VWF *in vitro* and *in vivo*.

ADAMTS proteases have a variable number of TSP1 repeats that may play a role in cellular localisation and substrate recognition (76,96,97). All TSP1 repeats contain the sequence W*XX*W, which is often modified by attachment of an α-mannosyl group to the C2- atom of the first Trp (98). Seven of the eight TSP1 repeats also contain the conserved sequence CSX(S/T)CG, in which the hydroxyamino acid at position 4 usually is modified by the disaccharide Glc-Fuc-*O*-Ser/Thr (98). It is not clear whether these posttranslational modifications play a role in ADAMTS13 function *in vivo*.

The TSP1 repeats in other ADAMTS proteases participate in substrate recognition and cell surface binding. The first TSP1 repeat of ADAMTS13 binds directly to VWF73 with a K_d of ~136 nM (80). The TSP1 5–8 repeats of ADAMTS13 appear to bind native VWF through the D4 domain (99). Moreover, the C-terminal TSP1 repeats of ADAMTS13 are shown to interact with the endothelial cell surface receptor CD36 (100,101), and such an interaction may enhance proteolytic cleavage of UL-VWF under flow conditions (102). However, human and murine ADAMTS13 variants lacking the C-terminal 2–8 TSP1 repeats and CUB domains (MDTCS) cleave cell bound UL-VWF (66,92,103) and soluble VWF (18) with similar efficacy as does the full-length ADAMTS13. These different conclusions may be attributed to assay sensitivity and the source of recombinant ADAMTS13 variants. Further studies are necessary to elucidate the biological function of the TSP1 repeats.

More recently, the TSP1 repeats of ADAMTS13 are shown to contain free thiols that may react with the free thiols on the surface of UL-VWF or plasma VWF exposed under shear stress (104,105). Such an interaction may prevent disulphide bond exchange and formation between two apposed VWF multimers under flow, thereby attenuating VWF-mediated platelet adhesion and aggregation (104,105).

The CUB domains are unique to ADAMTS13 and are not found in other ADAMTS and ADAM proteases (76,77,97). The role of the ADAMTS13-CUB domains is not fully understood. Recombinant CUB-1 and CUB-1+2 domains or synthetic peptides derived from the CUB-1 domain of ADAMTS13 partially block proteolytic cleavage of endothelial cell bound UL-VWF by full-length ADAMTS13 under flow (106), suggesting that the CUB domains of ADAMTS13 may interact with UL-VWF on the endothelial cell surface. Consistent with this hypothesis, a murine ADAMTS13 variant lacking the CUB domains (delCUB) appears to be defective in cleaving platelets-decorated UL-VWF strings in the mesenteric arterioles of *Adamts13*$^{-/-}$ mice after ferric chloride injury (92). However, this hypothesis poses some challenges. A human ADAMTS13 variant lacking the CUB domains normally cleaves the newly released UL-VWF strings in the absence of flow (66) or platelet-decorated UL-VWF strings on cultured HUVECs under flow (103). Moreover, a similar human ADAMTS13 variant is significantly able to attenuate the rate of thrombus growth in the mesenteric arteriolar occlusion assay (18), suggesting that the CUB domains are dispensable under these conditions. These conflicting results point to the complexity of assessing ADAMTS13 function under more physiological conditions.

Regulation of ADAMTS13 function

Cofactor-dependent regulation of coagulation enzymes has been well recognised and increases the rate of an enzymatic reaction by several orders of magnitude (107,108). Unlike other clotting factors that are synthesised as inactive zymogens, ADAMTS13 is secreted as a constitutively active protease (10,11,65,83). There has been no inhibitor identified to date. Plasma α2-macroglobulin inhibits many other matrix metalloproteases including ADAMTS-4, 5, 7, and 12 (109–112) but does not seem to bind and affect ADAMTS13 activity toward VWF (83). Therefore, ADAMTS13 function must be regulated at the substrate level.

Newly released UL-VWF anchored on the endothelial cell membrane can be rapidly cleaved by ADAMTS13 in the presence (5–7) or absence of flow (66,67), suggesting that cell bound UL-VWF is in its “open” conformation. The mechanism underlying the “open” conformation is not known. Once released into solution, the UL-VWF rapidly adopts a “closed” conformation that becomes highly resistant to proteolysis by ADAMTS13 in the absence of shear stress or denaturants. The soluble UL-VWF regains its sensitivity to ADAMTS13 when exposed to high fluid shear stress (~20–100 dynes/cm^2) that presumably

unfolds the central A2 domain of VWF (16,17,113). Such high shear conditions can be found *in vivo* in narrowed or branching vessels, small arterioles, and microcirculation. The increased VWF proteolysis by ADAMTS13 and a reduction of the plasma VWF activity to antigen ratio (114) correlates with the severity of aortic stenosis (15,115). Surgical correction normalises plasma VWF multimer distribution (15,116).

Arterial shear stress can be simulated *in vitro* using a cone plate viscometer (117,118), a bench-top mini-vortex (99,113,119), and a microfluidic system (120–122) that generates laminar flow. Under mechanically induced shear stress, proteolytic cleavage of multimeric VWF by recombinant ADAMTS13 increases as a function of increasing shear rate (or shear stress), incubation time, and concentrations of ADAMTS13 enzyme (113). The mechanical force-induced cleavage of an A1A2A3 tri-domain molecule (19) or the A2 domain of VWF (123,124) has also been demonstrated. Together, these findings suggest that fluid shear stress plays a critical role in regulating proteolytic cleavage of soluble VWF by ADAMTS13.

In addition to shear stress, coagulation factor VIII (FVIII), which binds VWF with high affinity (K_D, 0.25–0.5 nM), may alter the domain-domain interaction of the neighbouring A1A2A3 of VWF and regulate proteolytic cleavage of the A2 domain by ADAMTS13. To test this hypothesis, recombinant FVIII at various concentrations (0–20 nM) was incubated for 30 minutes with plasma-derived or recombinant multimeric VWF prior to incubation with recombinant ADAMTS13. After 2 minute incubation under constant vortexing (2,500 rpm, ~28.5 dynes/cm^2), the proteolytic cleavage product (~350 kDa) increases as a function of increasing concentrations of FVIII (119). In the presence of 20 nM FVIII, the maximal increase in the cleavage product formation approaches ~10–12 fold of that with VWF alone. The rate-enhancing effect of FVIII on VWF proteolysis was detected under fluid shear stress but not under static/denaturing conditions (119), suggesting that the binding of FVIII to VWF may facilitate the unfolding processes of the VWF-A2 domain under these conditions.

Structure-function analysis demonstrates that the B-domain deleted FVIII variant (FVIII-SQ) exhibits a similar rate enhancing effect on proteolysis of VWF by ADAMTS13 as does full-length FVIII (119,125). However, a FVIII variant lacking the acidic (a3) region that contains a major VWF binding site (FVIII-2RKR) has no effect under the same conditions (119,125). Interestingly, a light chain of FVIII (FVIII-LC), despite a 10-fold reduction in its binding affinity to VWF, is sufficient for accelerating the cleavage of VWF to a similar extent as are wild-type FVIII and FVIII-SQ(125), suggesting that binding of FVIII to VWF through its light chain mediates this cofactor activity.

These biochemical findings are further corroborated with those obtained *in vivo* in a murine model. Hydrodynamic injection is a commonly used method to instantly transfect hepatocytes with plasmids of interest. This manoeuvre also activates endothelial cells, triggering the release of UL-VWF into plasma in mice. When injected with PBS alone, plasma ratios of high to low molecular weight VWF multimers in *fVIII*$^{-/-}$ mice are higher than those in the *fVIII*$^{-/-}$ mice reconstituted with a plasmid encoding FVIII-SQ or FVIII-LC (125), suggesting that the expression of a functional VWF-binding FVIII variant eliminates the accumulation of UL-VWF multimers under (patho)physiological conditions.

Additional evidence to support the physiological role of FVIII-dependent regulation of VWF proteolysis by ADAMTS13 comes from our recent findings (presented in part at the ASH meeting, 2009) (126). The result demonstrated that type 2N VWF variants which exhibit a moderate to severe defect for FVIII binding were also compromised in accelerating cleavage of VWF by ADAMTS13 in the presence of FVIII under shear stress (126). Together, our findings support the critical role of FVIII as a physiological cofactor regulating proteolytic

cleavage of VWF by ADAMTS13. Such cofactor activity is dependent on the reaction between the light chain of FVIII and the D'D3 domains of VWF.

Platelet glycoprotein 1bα (GP1bα) also binds VWF with high affinity. Studies have demonstrated that an addition of formalin-fixed, lyophilised or fresh platelets, and soluble GP1bα to multimeric VWF increases proteolytic cleavage by ADAMTS13 under static (117,127) or fluid shear conditions (118). Ristocetin, an antibiotic that binds the A1 domain of VWF close to the site that GP1bα binds, also enhances cleavage of multimeric VWF by ADAMTS13 (118,128). These results suggest that the interaction between platelet GP1bα (or ristocetin) and the A1 domain affects the accessibility of the A2 domain by ADAMTS13. Interestingly, ristocetin alleviates the requirement of FVIII to enhance the cleavage of VWF by ADAMTS13 (118), while binding of platelet GP1bα to VWF enhances the effect of FVIII or *vice versa* as previously demonstrated. In the presence of physiological concentrations of platelets (150×10^3/μl), the C_{50} shifts to the left (from 5 nM to 0.5 nM) (118). These results suggest that FVIII and platelet GP1b have synergistic effects that enhance VWF proteolysis by ADAMTS13 under fluid shear stress. It has been postulated that the binding of FVIII to the D'D3 domain of VWF may result in large-scale conformational changes of the VWF multimers, such as pulling away the D'D3 domain from its neighbouring A1 or A2 domain under shear stress. Similarly, binding of platelets or soluble GP1bα to the VWF-A1 domain may expose the A2 domain for cleavage. Two or more platelets bound on each side of the scissile bond may dramatically increase the peak tensile force exerted on the central A2 domain (129), which enhances A2 domain unfolding and proteolysis by ADAMTS13, demonstrated by single molecule experiments (19,123,124).

Mechanism of anti-ADAMTS13 autoantibody inhibition

Idiopathic TTP in adults is caused by a severe deficiency of plasma ADAMTS13 activity due to immunoglobulin G (IgG) type antibodies. Depending on the assays, the inhibitory antibodies are detected in 44% to 100% of acquired idiopathic TTP patients with a severe deficiency of plasma ADAMTS13 activity (130). With more sensitive assays such as an enzyme-linked immunosorbent assay (ELISA) (131,132) or flow cytometry-based technology (133), anti-ADAMTS13 IgGs can be detected in all TTP patients who have a severe deficiency of plasma ADAMTS13 activity (130). Antibody mapping and profiling reveal that anti-ADAMTS13 IgG1 and IgG4 predominate in the plasma of acquired TTP patients (134,135) and nearly all anti-ADAMTS13 IgGs bind the Cys-rich and spacer domains, particularly the spacer domain (35,95,136–140). Other ADAMTS13 domains including the propeptide, metalloprotease domain, disintegrin domain, first TSP1 repeat, more distal TSP1 repeats, and CUB domains are less reactive with autoantibodies (35,136,140). Further analysis demonstrates that the major antigenic epitopes are localised to residues Tyr572-Asn579 (139), Val657-Gly666 (95,139), and Gly662-Val687 (141). A majority of TTP patients (90%) lose reactivity towards ADAMTS13 following the substitution of residues Arg568, Phe592, Arg660, Tyr661, and Tyr665 in exosite 3 of the spacer domain (140). These residues have been demonstrated to play a critical role in substrate recognition and proteolysis of VWF under various conditions (94,95). Therefore, it is conceivable that the binding of anti-ADAMTS13 autoantibodies to this region blocks substrate binding and its proteolytic function.

The mechanism of how patients develop autoantibodies against ADAMTS13 protease is not known. Female predominance (33,35) and autoantibodies observed in identical twin sisters (142) suggest a genetic predisposition. A recent study demonstrates an overrepresentation of the HLA-DRB1*11 allele in acquired TTP patients (143), consistent with the hypothesis. ADAMTS13 is efficiently internalised by immature dendritic cells (antigen-presenting cells)

through the surface macrophage mannose receptor (144,145). Interestingly, dendritic cells from HLA-DRB1*11 donors pulsed with higher concentrations of ADAMTS13 in culture present derivatives of a single CUB-2 derived peptide, suggesting that functional presentation of CUB-2 derived peptides on HLA-DRB1*11 may contribute to the onset of acquired TTP by stimulating low affinity self-reactive CD4+ T cells (144,146). Moreover, bacterial or viral infections often precede the acute epitope of initial or recurrent TTP (147–149). This indicates that infections may act as a trigger boasting the production of autoantibody production or substrate release from activated endothelium. However, one cannot exclude the possibility of a “by-stander” or molecular mimic hypothesis in which antibodies against microbes may cross react with ADAMTS13.

Improving on nature, re-engineering ADAMTS13

Others and we have demonstrated that the spacer domain (35,95,136,138–140), particularly exosite 3 (Fig. 2 and Fig. 4), is the major target of anti-ADAMTS13 autoantibodies in patients with acquired TTP (95,140). Autoantibody binding to this region is expected to block substrate binding and inhibit proteolytic activity of ADAMTS13. Replacement of the residues in exosite 3 with alanine nearly completely abolishes anti-ADAMTS13 IgG binding, but also significantly impairs proteolytic activity of ADAMTS13, rendering these mutants useless for therapy. We sought to subtly modify these residues by changing Arg to Lys or *vice versa* in hopes of eliminating autoantibody binding but retaining ADAMTS13 activity. Through site-directed mutagenesis, we have created and tested a panel of ADAMTS13 mutants for antibody binding and proteolysis. The results show that a substitution of 4–5 residues in exosite 3 generates ADAMTS13 variants (M4: R660K/F592Y/R568K/Y661F and M5: R660K/F592Y/R568K/Y661F/Y665F) with increased specific activity toward FRETS-VWF73 and multimeric VWF by 4- to 5-fold and 10- to12-fold, respectively (Fig. 4) (150). More interestingly, these variants were more resistant than WT and M1-M3 (with one to three residues mutated) to inhibition by anti-ADAMTS13 autoantibodies from acquired TTP patients (150). The reduction M4 and M5 inhibition by autoantibodies is correlated with the impaired binding of anti-ADAMTS13 IgG to the variants (150). Together, our findings indicate that it is possible to re-engineer ADAMTS13 protease to improve specific activity in the presence of autoantibodies, which may offer therapeutic benefits to acquired TTP patients. In summary, tremendous progress has been made in the past decade toward our understandings of the biosynthesis, the structure-function relationship, and the cofactor-dependent regulation of ADAMTS13 function. These advances provide invaluable information concerning the mechanisms of TTP and other atherothrombotic disorders, as well as inflammatory diseases. Therefore, novel diagnostic tools and therapeutics may be developed for managing these potentially fatal diseases.

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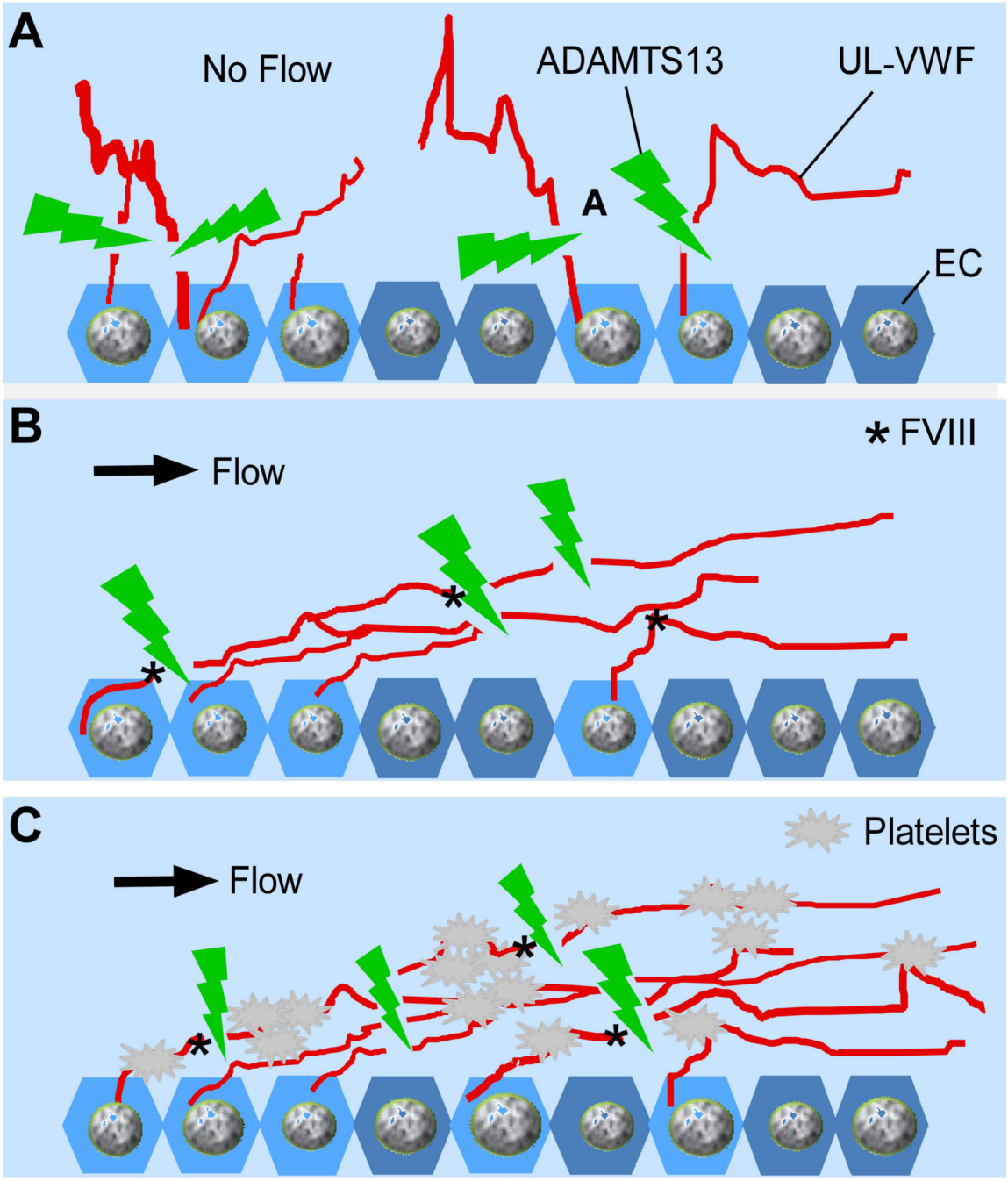

Fig. 1. ADAMTS13 cleaves UL-VWF under various conditions

A. ADAMTS13 rapidly cleaves newly released UL-VWF or large VWF strings/bundles in the absence (**A**) and in the presence (**B**) of flow; ADAMTS13 also cleaves platelet-decorated UL-VWF or large VWF strings/bundles anchored on the endothelial cell surface, in solution, and within growing thrombi under fluid shear stress (**C**).

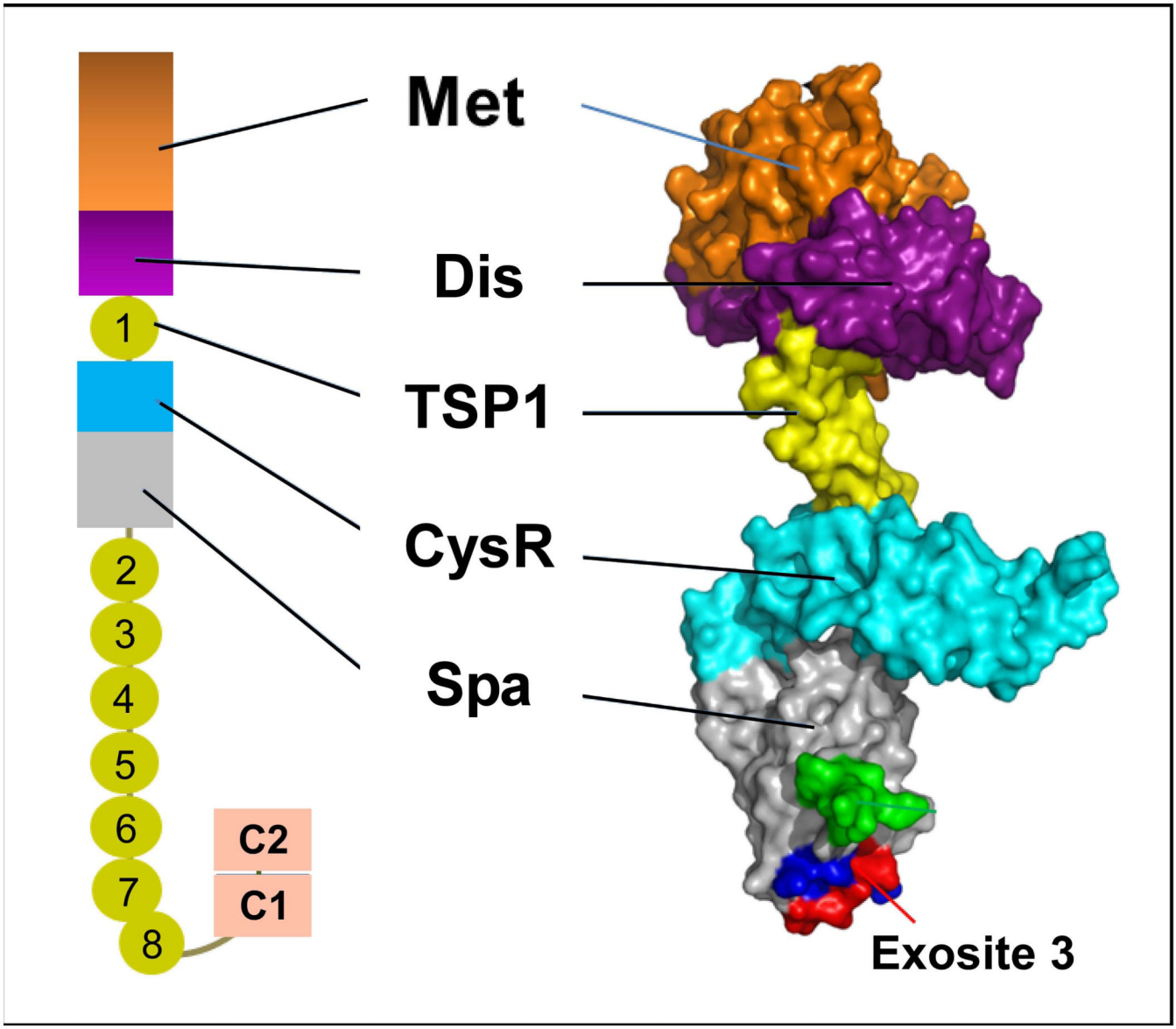

Fig. 2. Domain organisation and partial crystal structure of ADAMTS13

On the left, the domain organization of human mature ADAMTS13 is shown, which consists of a metalloprotease domain (M), a disintegrin-like domain (D), the first TSP1 repeat, a Cys-rich domain (C), and a spacer domain (S). In addition, the C-terminus contains 7 more TSP1 repeats (2–8) and two CUB domains (C1 and C2). On the right, the surface and carton presentation of the crystal structure of ADAMTS13 disintegrin-like domain (Dis), first TSP1 repeat (TSP1), Cys-rich (Cys) and spacer domain (Spa) in addition to a modelled metalloprotease domain (based on the metalloprotease domains of ADAMTS4 and ADAMTS5).

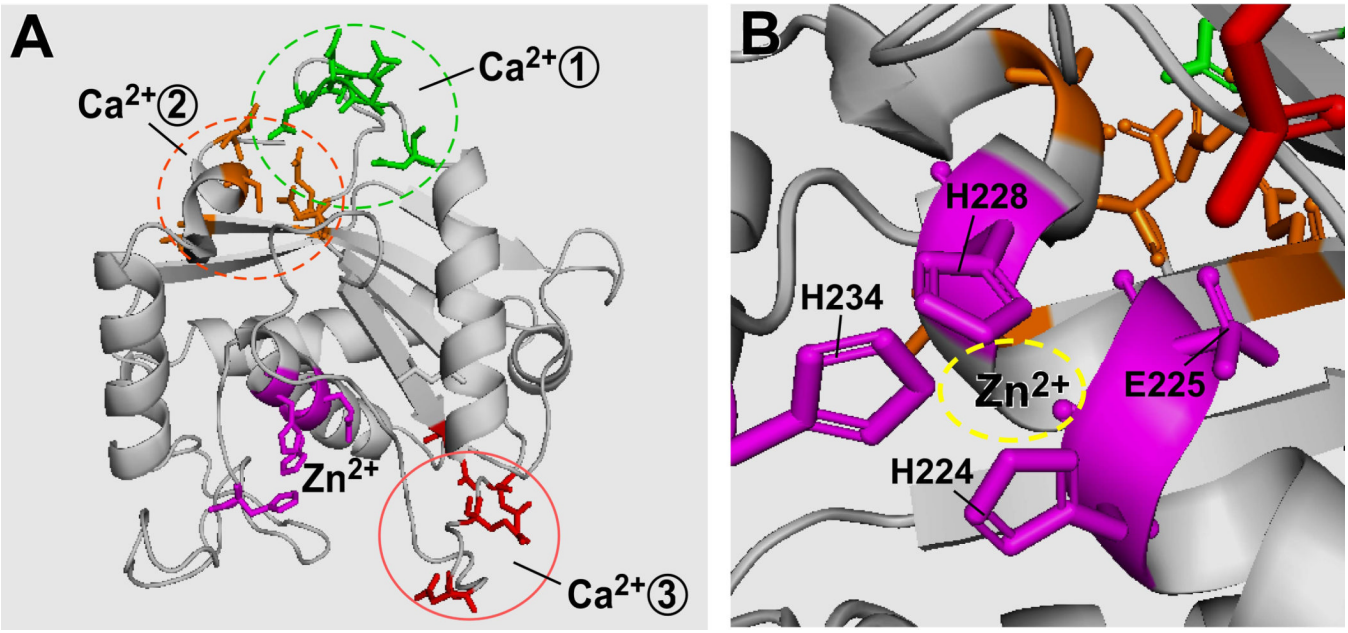

Fig. 3. Zinc and calcium binding sites in the metalloprotease domain of ADAMTS13

A. The ADAMTS13 metalloprotease domain with an active site and three putative calcium-binding sites; **B**. Three histidine residues and one glutamic acid coordinate the zinc binding.

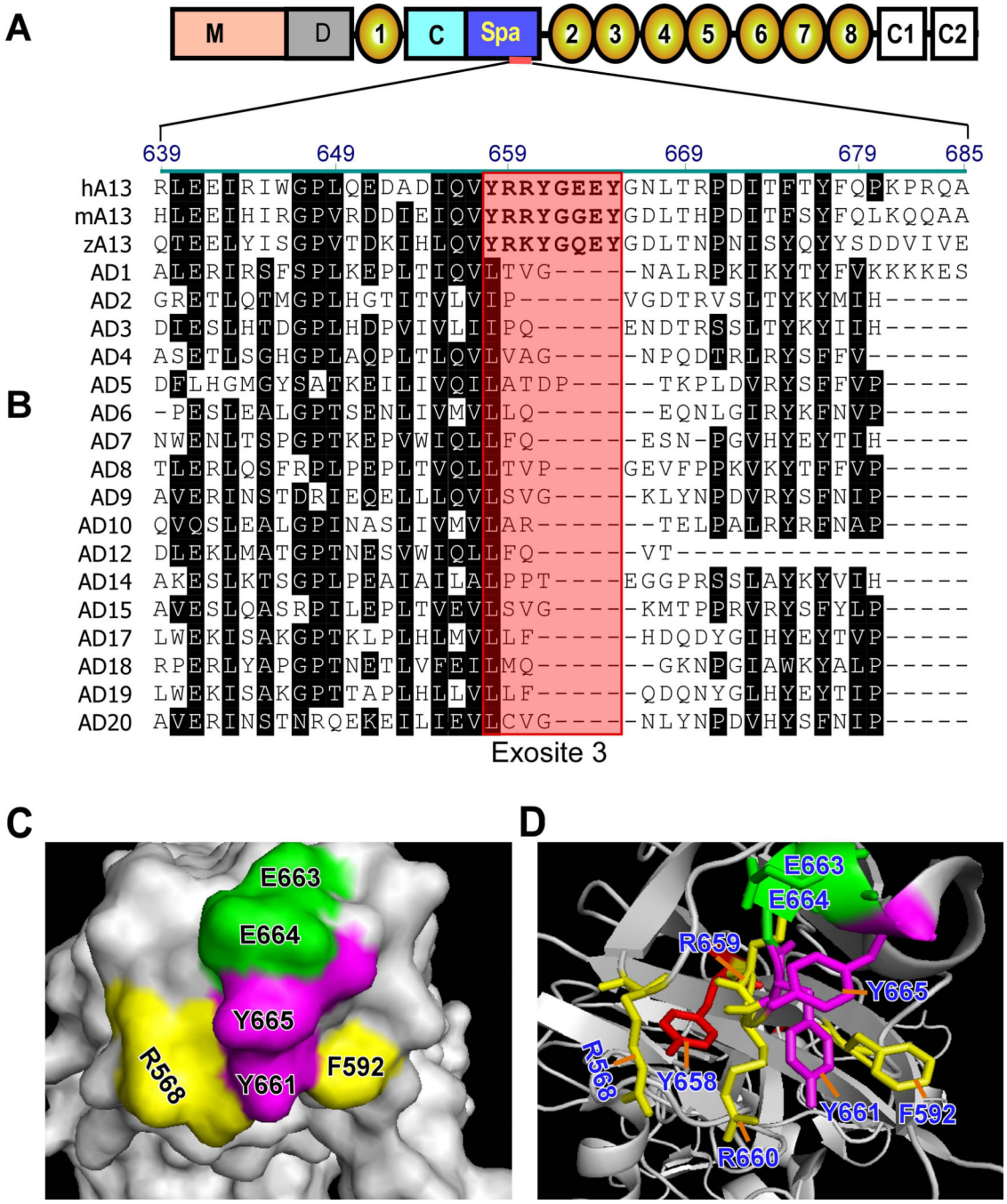

Fig. 4. Sequence alignment of the spacer domains of ADAMTS13 and other ADAMTS proteases

A. Domain organization of ADAMTS13; **B**. Sequence alignment of a partial spacer domain (Arg629-Lys681) from human (h), murine (m), and zebrafish (z) ADAMTS13 (A13), as well as a partial spacer domain of various ADAMTS family proteases (AD1–AD20). Boxed area in pink presents a region (exosite 3) that is highly conserved in the ADAMTS13-spacer domain but absent in the spacer domain in other ADAMTS proteases. **C** and **D** are the surface and ribbon representation of exosite 3, respectively.

Table 1

ADAMTS13 deficiency and potential human diseases

Diseases associated	Species	ADAMTS13 status	References cited
TTP	H	<5%	1, 20, 22, 33
	M	Null	39, 40
	B	<5%	42
Ischemic stroke	H	Reduced	43, 48
	M	Null	46, 47, 60
Myocardial infarction	H	Reduced	43–45
	M	Null	56, 58
Atherosclerosis	H	Reduced	48
	M	Null	55, 56
Cerebral malaria	H	Reduced	50–54
Preeclampsia	H	Reduced	49

TTP, thrombotic thrombocytopenic purpura
H, B, M and R are human, baboon, mouse, and rat, respectively.