Supplemental data

Antibodies that conformationally activate ADAMTS13 allosterically enhance metalloprotease domain function

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Materials and methods

Expression and purification of recombinant ADAMTS13 variants

For immunization, the recombinant MDTCS variant, fused to C-terminal V5- and 6xHis tags ($MDTCS_{(V5\text{-}6xHis)}$), was expressed in stably transfected tetracycline-inducible HEK T-REx 293 cells (Invitrogen) (Supplemental Table 1 and Supplemental Figure 1)[1,2] and purified using a Zn^{2+}-coupled HiTrap HP column (GE Healthcare), as previously described.[2]

For epitope mapping, the following variants were used; MDTCS, MDT, MD, MP and Spacer-CUB2 (Supplemental Figure 1). MDTCS, MDT, MD and MP domain fragments, all fused to a C-terminal FLAG-tag and constructed in pCAGG-neo, were expressed in stably transfected HEK T-REx 293 cells (Invitrogen) ($MDTCS_{(FLAG)}$, $MDT_{(FLAG)}$, $MD_{(FLAG)}$, $MP_{(FLAG)}$, Supplemental Table 1).[3,4] The Spacer-CUB2 variant carrying V5- and 6xHis tags (Spacer-$CUB2_{(V5\text{-}6xHis)}$) (Supplemental Table 1) was stably expressed from a pSecTag/FRT/V5-His TOPO vector (Invitrogen) in Flp-In CHO cells (Invitrogen) and purified using a Ni^{2+}-Sepharose Fast Flow column (GE Healthcare, Waukesha, WI, USA).

For kinetic analyses using the VWF96 assay, recombinant wild-type ADAMTS13 and an MDTCS variant were used (Supplemental Figure 1). ADAMTS13, fused to C-terminal V5- and 6xHis tags ($ADAMTS13_{(V5\text{-}6xHis)}$), was expressed in stably transfected tetracycline-inducible HEK T-REx 293 cells (Invitrogen) and purified as described.[1,2] MDTCS fused to a C-terminal 6xHis tag ($MDTCS_{(6xHis)}$), was expressed from the pMT-BiP-PURO vector (Invitrogen) in stably transfected Drosophila Schneider 2 cells (Supplemental Table 1)[5], and conditioned media were concentrated by tangential flow filtration and dialyzed in Tris-buffered saline containing calcium chloride (20mM Tris pH 7.4, 50mM NaCl, 5mM $CaCl_2$).

Expression and purification of VWF96, VWF87(ΔSpacer) and VWF96-Cys

VWF96 (Gly^{1573}-Arg^{1668}) and its variants, VWF96-Cys (I1642Q/W1644Y/A1647S/I1649Q/L1650Q/I1651Q) and VWF87(ΔSpacer) (Gly^{1573}-Arg^{1659}) (Supplemental Figure 2), all fused to N-terminal HisG-SUMO tags and C-terminal HSV-6xHis tags in pET-25b(+) bacterial expression vectors, were expressed in BL21 (DE3) *E. coli* (Invitrogen). Proteins were extracted from the soluble bacterial fraction and purified by affinity chromatography on Ni^{2+}-charged HiTrap HP chelating columns (GE Healthcare), followed by anion exchange chromatography on CaptoQ ImpRes columns (GE Healthcare), as previously described.[5]

Immunization strategy and generation of anti-MDTCS mAbs

Animal experiments were approved by the Institutional Animal Care and Use Committee of KU Leuven, Belgium (project number P055/2015). Anti-MDTCS mAbs were developed by immunizing BALB/c mice (Janvier Labs) with $MDTCS_{(V5\text{-}6xHis)}$ (Supplemental Table 1 and Supplemental Figure 1).[4] On day 1 mice were injected subcutaneously with 10 µg $MDTCS_{(V5\text{-}6xHis)}$ in complete Freund's adjuvant (Becton Dickinson) and on day 14 intraperitoneally with 10µg $MDTCS_{(V5\text{-}6xHis)}$ in incomplete Freund's adjuvant. The immune response was boosted with additional intraperitoneal $MDTCS_{(V5\text{-}6xHis)}$ injections on days 56 and 58. Mice immunized with vehicle only were used as a negative control. On day 60, mice were sacrificed, the spleen was isolated and antibody-producing hybridoma cells were generated by fusing SP2/0 myeloma with isolated spleen cells following the method of Köhler and Milstein.[6] Hybridoma clones producing anti-MDTCS mAbs were cultured and purified using a Protein G-coupled HiTrap HP column. The purity of the mAbs was assessed by SDS-PAGE and Coomassie staining. mAb concentrations were determined via spectrophotometry.

Epitope mapping of mAbs by ELISA

Antibodies present in mouse sera (collected on day 21 after immunization) and in hybridoma conditioned media (collected on day 14 after fusion), as well as purified mAbs, were initially screened for MDTCS binding. 7nM $MDTCS_{(V5\text{-}6xHis)}$ (Supplemental Table 1 and Supplemental Figure 1) in PBS was coated on 96-well microtiter plates overnight at 4°C. After blocking with PBS containing 3% milk, serial dilutions of the samples were added. Sera collected from mice prior to immunization, complete hybridoma medium or PBS containing 0.3% milk were used as negative controls. The anti-MP domain 3H9 mAb (starting form 1µg)[4] was used as a

reference. Bound anti-MDTCS mAbs were detected with horseradish peroxidase (HRP)-labeled goat anti-mouse antibodies (Jackson ImmunoResearch). Colorimetric development was performed using ortho-phenylenediamine (OPD) and H_2O_2, and stopped with 4M sulfuric acid. Absorbance was measured at 490nm.

Further epitope mapping was carried out using the $MDTCS_{(FLAG)}$, $MDT_{(FLAG)}$, $MD_{(FLAG)}$ and $MP_{(FLAG)}$ variants[3,4] (Supplemental Table 1 and Supplemental Figure 1), as previously described.[4] 96-well microtiter plates were coated with the purified anti-MDTCS mAbs (5 µg/mL in 0.05M Na_2CO_3/$NaHCO_3$, pH 9.6) overnight at 4°C. The plates were blocked with PBS containing 3% milk. Serial dilutions of the conditioned media containing $MDTCS_{(FLAG)}$, $MDT_{(FLAG)}$, $MD_{(FLAG)}$, or $MP_{(FLAG)}$ were added. Bound $MDTCS_{(FLAG)}$, $MD_{(FLAG)}$ and $MP_{(FLAG)}$ were detected using HRP-labeled anti-FLAG antibodies (Sigma Aldrich). Bound $MDT_{(FLAG)}$ was detected using the biotinylated anti-MP domain mAb 3H9 followed by HRP-labeled streptavidin (Roche Diagnostics GmbH). Colorimetric development was performed as described above. Further Spacer mapping of the anti-MDTCS mAbs was performed by using the Spacer-$CUB2_{(V5\text{-}6xHis)}$ variant. 96-well microtiter plates were coated with Spacer-$CUB2_{(V5\text{-}6xHis)}$ (conditioned media 1/10 diluted) overnight at 4°C. After blocking, anti-MDTCS mAbs (10 µg/mL) were added and bound mAbs were detected with HRP-labeled goat anti-mouse antibodies. Colorimetric development was performed as described above.

ADAMTS13 proteolysis of FRETS-VWF73

The effect of the anti-MDTCS mAbs on the activity of plasma ADAMTS13 was measured using the FRETS-VWF73 substrate (Peptides International) [1,7]. Reaction mixtures (200µl final volume) containing purified mAbs (10 µg/mL), 2% normal human plasma (NHP) and 1 mg/mL bovine serum albumin (BSA) were made in HEPES-buffered saline (50mM HEPES, 5mM $CaCl_2.2H_2O$, 1µM $ZnCl_2$, 150mM NaCl, pH 7.4). Reactions were initiated by addition of 2µM FRETS-VWF73 substrate. Proteolysis of FRETS-VWF73 was monitored every 2.5 minutes for 75 minutes by measuring the fluorescence (excitation at 355nm and emission at 460nm) using a FLUOstar OPTIMA reader (BMG Labtech GmbH). Fluorescence intensity was plotted as a function of time, where the gradient corresponds to the rate of proteolysis. Relative ADAMTS13 activities were estimated from reference reactions performed at multiple dilutions of NHP (0.5%, 1%, 2%, 2.5% and 3%) and in the absence of mAbs. Negative control reactions were performed in the presence EDTA (10mM) or the inhibitory anti-MP mAb 3H9 (10 µg/mL).

Kinetics of VWF96, VWF96-Cys and VWF87(ΔSpacer) proteolysis

Kinetic analysis of VWF96, VWF96-Cys and VWF87(ΔSpacer) proteolysis was performed as previously described.[5] Purified ADAMTS13$_{(V5\text{-}6xHis)}$ (0.6-11nM) or MDTCS$_{(6xHis)}$ in concentrated conditioned media (estimated ~1.2nM) (Supplemental Table 1 and Supplemental Figure 1) were incubated with or without the mAbs (final concentration 10 µg/mL) in reaction buffer (20mM Tris pH 7.55, 50mM NaCl, 5mM $CaCl_2$, 1% BSA) at 37°C for 15 minutes. Reactions were initiated by adding VWF96, VWF96-Cys or VWF87(ΔSpacer) (final concentration 0.5µM) (Supplemental Figure 2). Reaction sub-samples were stopped between 0-90 minutes in stopping buffer (20mM Tris pH 7.55, 50mM NaCl, 25mM EDTA, 1% BSA). Stopped reaction sub-samples were analyzed by ELISA. 96-well microtiter plates were coated with either polyclonal chicken anti-SUMO/SUMOstar IgY antibodies (0.5 µg/mL, LifeSensors) or mouse anti-HisG antibodies (1.18 µg/mL, Thermo Fischer Scientific) in 0.05M Na_2CO_3/$NaHCO_3$ pH 9.6, overnight at 4°C. Plates were washed in PBS/0.1% Tween and blocked with blocking buffer (20mM Tris pH7.55, 50mM NaCl, 3% BSA) for 2 hours. Purified VWF96, VWF96-Cys or VWF87(ΔSpacer) were diluted in dilution buffer (20mM Tris pH7.55, 50mM NaCl, 1% BSA) to generate standard curves (0-0.15nM VWF96 variants on anti-SUMO/SUMOstar-coated plates, or 0-1.25nM VWF96 variants on anti-HisG-coated plates) and added to the plates for 1.5 hours. In parallel, stopped reaction sub-samples were diluted in dilution buffer and added to the plates for 1.5 hours (0.09nM VWF96 variants on anti-SUMO/SUMOstar-coated plates, or 0.75nM VWF96 variants on anti-HisG-coated plates). After washing, plates were incubated with HRP-labeled polyclonal anti-HSV antibodies (0.5 µg/mL, Bethyl) in dilution buffer (20mM Tris pH7.55, 50mM NaCl, 1% BSA) for 1 hour, to detect bound uncleaved VWF96 variants. A final washing step was performed, then colorimetric development was carried out using SIGMA*FAST*TM-OPD (Sigma Aldrich) and stopping with 2.5 M sulfuric acid. Absorbance was read at 492 nm.[5]

Percentage proteolysis was plotted as a function of time and the catalytic efficiency of proteolysis was calculated by fitting the data into the equation: $P = 1 - \exp(-1 \times [enzyme] \times t \times k_{cat}/K_m)$ [8] where, P is the fraction of substrate proteolyzed (0 to 1), [enzyme] is the concentration of ADAMTS13$_{(V5\text{-}6xHis)}$ or MDTCS$_{(6xHis)}$ used in the reaction, t is the time in seconds, and k_{cat}/K_m is the catalytic efficiency ($\mu M^{-1}s^{-1}$). For Michaelis Menten kinetic analysis, the initial rate of proteolysis (nMs^{-1}) at the linear part of the progress curve (<15% proteolysis) was normalized per unit (nM) of enzyme used and plotted as a function VWF96 concentration (0-25µM), both in the presence and absence of mAbs. The individual kinetic parameters, k_{cat} and K_m, were assessed by fitting the data into the Michaelis Menten equation:

$Vi = \frac{V_{max} \times [substrate]}{K_m + [substrate]}$, where, V_i is the initial rate of proteolysis per nM of enzyme, V_{max} is the maximum velocity/rate of proteolysis (nMs^{-1}), [substrate] is the concentration of VWF96 (μM) and K_m is the Michaelis constant (the concentration of VWF96 that achieves half V_{max}). k_{cat} (s^{-1}), or turnover number, measures the functionality of the active-site, and is calculated by dividing V_{max} by the enzyme concentration used in the reaction. Since the initial rates of proteolysis were normalized for enzyme concentration prior to plotting/fitting the data, the enzyme concentration in the equation is equal to 1nM, and k_{cat} (s^{-1}) is numerically equal to the V_{max} (nMs^{-1}).[5]

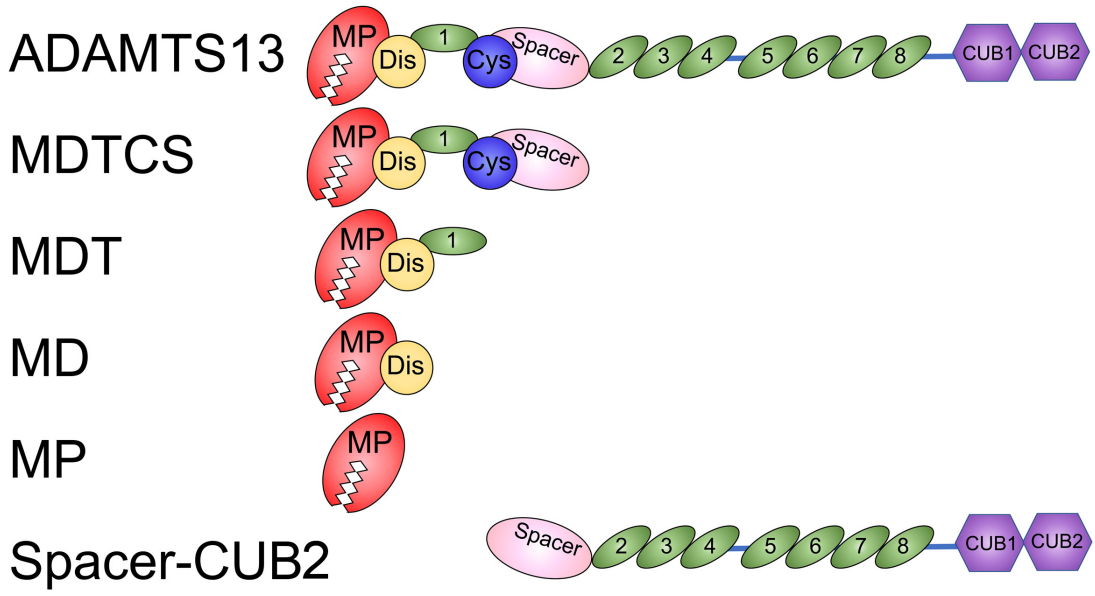
Analysis of conformational changes in the ADAMTS13 MP domain induced by mAb binding

A serial dilution of the mAbs 3E4, 17G2 and 15D1 (0-10 μg/mL) were pre-incubated with NHP (50%, v/v) in a pre-blocked plate at 37°C for 30 minutes. Dilution buffer (PBS with 0.3% milk powder) only (no mAb) was added to NHP as a negative control. The mixture was then transferred to a 96-well microtiter plate that had been coated with the mAb 6A6 (5 μg/mL in 0.05M Na_2CO_3/$NaHCO_3$ pH 9.6) overnight at 4°C and blocked with PBS containing 3% milk. Bound plasma ADAMTS13 was detected using biotinylated anti-TSP8 mAb 19H4[4] (1.5 μg/mL) followed by the addition of HRP-labeled high-sensitivity streptavidin (1/10,000; Thermo Fisher Scientific). Colorimetric development was performed as described above and absorbance at 490nm was measured.

Supplemental Table 1: ADAMTS13 variants used in the study

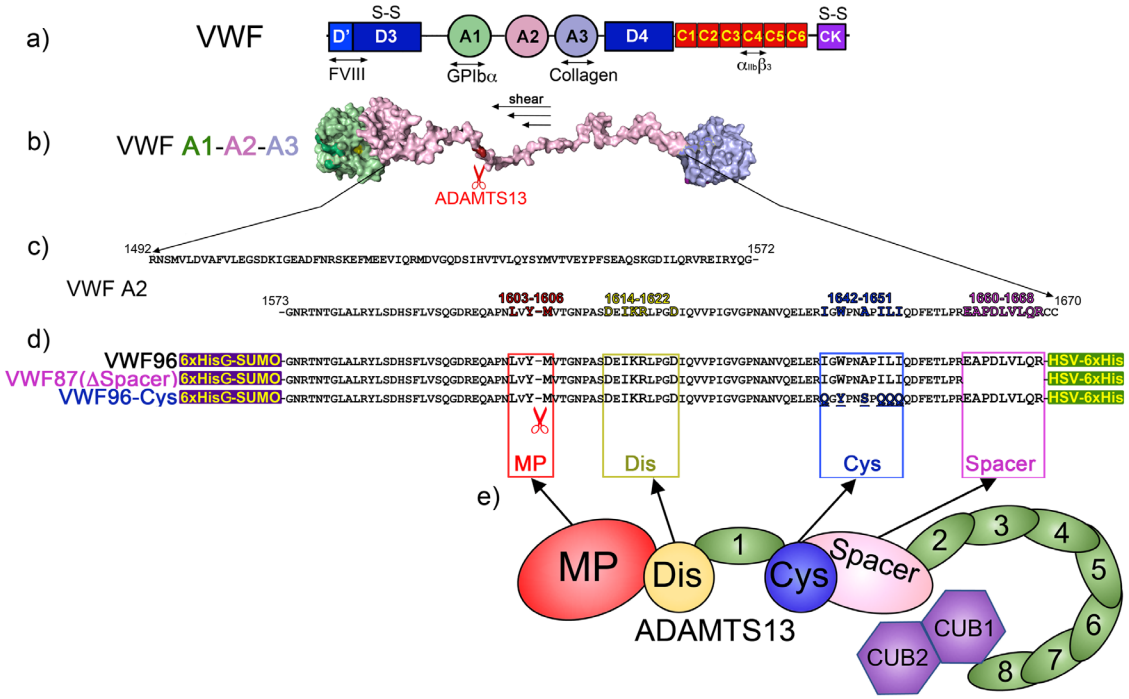
Name	Tag	Cell line	Experiment
$MDTCS_{(V5\text{-}6xHis)}$	V5-6xHis	HEK-T-REx	Immunization
$MDTCS_{(FLAG)}$ $MDT_{(FLAG)}$ $MD_{(FLAG)}$ $MP_{(FLAG)}$	FLAG	HEK-T-REx	Epitope mapping
$Spacer\text{-}CUB2_{(V5\text{-}6xHis)}$	V5-6xHis	Flp-In CHO	Epitope mapping
$MDTCS_{(6xHis)}$	6xHis	Drosophila Schneider 2 cells	Kinetic analysis
$ADAMTS13_{(V5\text{-}6xHis)}$	V5-6xHis	HEK-T-REx	Kinetic analysis

Supplemental Figure 1



Supplemental Figure 1: Full length ADAMTS13 and its variants
Schematic representation of full-length ADAMTS13 and its variants MDTCS, MDT, MD, MP and Spacer-CUB2 used in this study. ADAMTS13 consists of a metalloprotease domain (M or MP), a disintegrin-like domain (D), a first thrombospondin type-1 repeat (T1), a cysteine-rich domain (C), a Spacer domain (S), seven additional thrombospondin type-1 repeats (T2–8) and two CUB domains (CUB1-2). As mentioned in Supplemental Table 1, full length ADAMTS13 used in this study had a C-terminal V5-6xHis tag, the MDTCS fragment used had either a C-terminal V5-6xHis tag, a FLAG tag or a 6xHis tag. The MDT, MD and MP fragments had a C-terminal FLAG tag. The Spacer-CUB2 variant had a V5-6xHis tag.

Supplemental Figure 2



Supplemental Figure 2: VWF96 Substrates.
a) VWF domain organization, disulfide bonds involved in multimerization are marked (S-S) and major ligand binding sites are shown. **b)** Models of the central VWF A domains (A1-A2-A3) are shown following shear-induced unfolding. The Tyr^{1605}-Met^{1606} scissile bond in the VWF A2 domain that is revealed and cleaved by ADAMTS13 is shown in red. **c)** Amino acid sequence of the VWF A2 domain (residues Arg^{1492}-Cys^{1670}). Amino acids involved in ADAMTS13 exosite recognition are highlighted (MP domain – red, Dis domain – yellow, Cys-rich domain – blue, Spacer domain – pink). **d)** VWF A2 domain fragments that were used in this study. VWF96 comprises Gly^{1573}-Arg^{1668} with a HisG and a 13kDa SUMO N-terminal tags and a short HSV and a 6xHis C-terminal tags. The regions deleted or mutated in VWF87(ΔSpacer) and VWF96-Cys are highlighted. **e)** Schematic representation of the domain organization of ADAMTS13. Domains are labeled. The C-terminal tail consisting of the TSP repeats 2-8 and CUB domains is depicted folding back to interact with the central Spacer domain.

References

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