

The Allosteric Activation of ADAMTS13

A thesis submitted to Imperial College London for the degree of Doctor
of Philosophy in the Faculty of Medicine

By

Anastasis Petri BSc/MSc

Centre for Haematology, Department of Medicine, Faculty of Medicine

Imperial College London

Hammersmith Hospital Campus

Commonwealth Building

Du Cane Road, London, W12 0NN

October 2018

ABSTRACT

ADAMTS13 regulates the multimeric size of Von Willebrand Factor (VWF) in plasma by specific, shear-dependent proteolysis. Despite circulating in a seemingly proteolytically competent form, and with a very long active plasma half-life, ADAMTS13 is specific for VWF, and is resistant to plasma inhibitors. This unprecedented specificity has been attributed to exosite interactions between the ADAMTS13 Spacer, Cysteine-rich, Disintegrin-like and Metalloprotease domains and the unraveled VWF A2 domain, which serve to direct proteolysis. I hypothesized that one or more exosite interactions allosterically activate ADAMTS13 by modulating the conformation of its active site, specifically shaping it to accommodate the VWF scissile bond.

My first aim was to characterise the independent contribution of each ADAMTS13 exosite to VWF binding and proteolysis. I generated a new VWF A2 domain fragment (VWF96) as a substrate for ADAMTS13, with/without mutations that ablate each exosite interaction separately. I developed a novel ELISA that specifically detects VWF96, and optimized protocols for monitoring the kinetics of proteolysis. My data show that disruption of the Spacer and Cysteine-rich domain exosite interactions reduces proteolysis by ~15-to-20-fold. These deficits were due to reductions in VWF binding. Intriguingly, a remarkable role in proteolysis was revealed for the Disintegrin-like domain exosite interaction; when ablated, this exosite caused a ~780-fold reduction in proteolysis, due to a ~15-fold reduction in binding (K_m), and a ~56-fold reduction in substrate turnover (k_{cat}), consistent with the existence of an allosteric link between the Disintegrin-like domain exosite and the active site in the Metalloprotease domain.

ADAMTS13 was previously shown to adopt a folded conformation, maintained by inter-domain interactions. My second aim was to characterise the mechanism by which anti-Spacer/anti-CUB domain monoclonal antibodies unfold ADAMTS13, enhancing proteolysis. I showed that the antibody-induced unfolding primarily enhances the substrate turnover (k_{cat}), rather than substrate binding (K_m), which would be consistent with an allosteric link between the non-catalytic domains and the active site.

Taken together, these findings provide insights into the molecular mechanisms by which the non-catalytic domains of ADAMTS13 influence the active site and confer specificity to VWF proteolysis, and highlight the importance of conformational changes in ADAMTS13 to its enzymatic function.

DECLARATION OF ORIGINALITY

I, Anastasis Petri, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr James Crawley. Jim, you have been a true mentor, who understands, cares, motivates, inspires, promotes and leads by example. You very generously offered me a BSc project, a PhD project, a job as research assistant and a post-doc; I will always be hugely grateful for all these opportunities and for believing in me more than I do. Thank you for teaching me how to understand, perform, write, present and enjoy science. You have put up with my bad sleeping habits, time management and made room for my band practices. You have been there for everything I (and everyone else) needed, be it guidance in the lab, moral support, career advice, even when the liquid nitrogen dispenser got stuck! You are a superstar!

I am equally grateful to my supervisor Dr Rens de Groot. Rens, I feel so lucky to have been taught by you. You are the person who introduced me to the lab during my BSc project, who taught me how to hold a pipette, and the person that I still go to every time I need help, and I owe most of my lab skills to you. You have a great talent in teaching people how to think and understand what they are doing, and to critically evaluate everything. You always make time for helping people. If one day I become half the scientist you are, mission accomplished! It has been a pleasure working next to you in the lab, and sitting next to you by the unhealthy corner of treats in the office, and I am happy that I will carry on doing these for the next few years!

Dear colleagues of the haemostasis lab, you have been an amazing team. Each one of you has contributed to making lab work so smooth, pub nights and conferences so much fun, and all these years so enjoyable! Isabelle, thank you for offering me the opportunity to work on the Bambi project. You have been very kind and understanding, and you have compromised your project so that I get to finish my PhD! I will never forget this, and I hope I will manage to pay back over the next few years working as your post-doc. Mary and Frank, sharing the lab bench with you has been a pleasure; thanks for making the time in the lab go so fast. Magda, thanks for knowing everything, and for all the help with thesis preparation! Adrienn, hang in there, you are next! Adela, I have enjoyed organising Christmas dinners, spying on Harvey, and being arrested by the police in Berlin (thank you Mary!) with you. Salvo, no more kinetics, at least for now- the PCR machine is all yours! Patricia, this is officially the end of my PhD, and my duties in topping up liquid nitrogen. You have been the best liquid nitrogen partner one could ask for, and, after four years of shadowing me, I hope you know by now how to do it! Chris, pub-style quizzes, nights out and, mostly, conference dinners with you have been... interesting! Looking

forward to catching up with you at the next one! Josefin, thank you for always being so kind to everyone and ready to offer help and feedback.

I would like to thank Prof Karen Vanhoorelbeke and An-Sofie Schelpe for the nice collaboration opportunity, which constituted part of this PhD thesis.

I am so thankful to have had true friends by my side throughout this journey. 'Friends from the village', despite the distance, I felt you so close. Thanks to the 90% of you who got married during the past four years; your weddings were a nice distraction from the PhD! Petros, Antonia, Machi and Antonia Maria, you are an unforgettable part of my time in London and you are greatly missed, 'goutsi'! YOLO squad, we have shared so many memories in and out of Imperial during these eight years in London. Sorry for the bad attendance over the past year, I am now submitting, which means I'll attend the next BBQ! Stefanos, Marita, Costa, Katerina, Chrys, Angela and Georgia, you are my family in London. Thank you for keeping me sane this past year! Stefanos- I did not feature in your thesis acknowledgements (and I had many reasons to!) but thank you, 'να 'σαι καλά'!

Dad, mum, Florentia, I don't get to thank you too often. Thank you for making every step of my life so easy for me, for your constant support in every form, and for your unconditional love. I would have achieved nothing without you!

TABLE OF CONTENTS

ABSTRACT.....	2
DECLARATION OF ORIGINALITY.....	3
ACKNOWLEDGEMENTS.....	5
TABLE OF CONTENTS	7
LIST OF FIGURES.....	11
LIST OF TABLES.....	12
1 INTRODUCTION	13
1.1 OVERVIEW OF HAEMOSTASIS.....	13
1.1.1 Primary haemostasis.....	13
1.1.2 Secondary haemostasis	14
1.1.3 Fibrinolysis.....	14
1.2 VWF MOLECULE/STRUCTURE	16
1.2.1 VWF gene expression	16
1.2.2 VWF domain organisation and structure	16
1.2.2.1 VWF D regions	18
1.2.2.2 VWF A domains	19
VWF A1 domain	19
VWF A2 domain	21
VWF A3 domain	23
1.2.2.3 VWF C domains	24
1.2.2.4 VWF CTCK domain.....	25
1.2.3 VWF biosynthesis	25
1.2.3.1 VWF post-translational modifications and intracellular trafficking.....	25
1.2.3.2 VWF storage and secretion.....	26
1.3 VWF FUNCTION	29
1.3.1 Primary haemostasis.....	29
1.3.1.1 Platelet adhesion/capture.....	29
1.3.1.2 Platelet stable adhesion, activation and aggregation	31
1.3.1.3 VWF multimeric size and function.....	33
1.3.1.4 Factor VIII.....	35
1.4 VON WILLEBRAND DISEASE	35
1.5 ADAMTS13 MOLECULE/STRUCTURE.....	38
1.5.1 The discovery	38
1.5.2 ADAMTS13 gene expression.....	38
1.5.3 ADAMTS13 domain organisation and structure.....	39
1.5.3.1 Metalloprotease domain	41
1.5.3.2 Disintegrin-like domain	43
1.5.3.3 Thrombospondin-type 1 repeats	45
1.5.3.4 Cysteine-rich domain	46
1.5.3.5 Spacer domain	46
1.5.3.6 CUB domains	48
1.5.4 ADAMTS13 biosynthesis, post-translational modification and secretion	48
1.6 ADAMTS13 FUNCTION.....	49
1.7 ADAMTS13 IN DISEASE	53
1.7.1 Thrombotic thrombocytopenic purpura	53
1.7.1.1 Congenital TTP	53
1.7.1.2 Acquired TTP.....	54
1.7.1.3 Cardiovascular disease	55

1.8	VWF RECOGNITION AND PROTEOLYSIS BY ADAMTS13	56
1.8.1	The conformational activation of ADAMTS13	57
1.8.2	The conformational transition of the A2 domain activates cleavage	59
1.8.3	The role of exosite interactions in VWF recognition and proteolysis	61
1.8.3.1	Spacer domain binding to the unfolded A2 domain.....	61
1.8.3.2	Cysteine-rich domain binding to the unfolded A2 domain	61
1.8.3.3	Disintegrin-like domain binding to the unfolded A2 domain.....	62
1.8.3.4	Metalloprotease domain binding to the unfolded A2 domain	63
1.8.3.5	The modularity and portability of the exosite interactions	64
1.8.3.6	ADAMTS13 – a uniquely specific enzyme	64
1.9	Hypothesis and aims	66
2	MATERIALS AND METHODS	67
2.1	GENERATION OF VWF96 VARIANTS	67
2.1.1	Generation of pET SUMO-VWF96 bacterial expression vector	67
2.1.1.1	PCR amplification of VWF96 cDNA.....	67
2.1.1.2	Agarose gel electrophoresis	67
2.1.1.3	Agarose gel DNA extraction.....	67
2.1.1.4	Cloning of VWF96 cDNA into the pET SUMO vector	68
2.1.1.5	One Shot Mach1-T1 Chemically Competent <i>E. coli</i> transformation	70
2.1.1.6	Plasmid DNA isolation	70
2.1.2	Subcloning into the pET-25b(+) bacterial expression vector.....	71
2.1.2.1	PCR amplification of HisG-SUMO-VWF96 cDNA	71
2.1.2.2	Double restriction digest	71
2.1.2.3	DNA ligation.....	71
2.1.2.4	VWF96 mutagenesis by inverse PCR amplification of the pET-25b(+).....	74
2.1.2.5	Large-scale expression of the VWF96 variants.....	74
2.1.2.6	Purification of the VWF96 variants	75
	Immobilised Metal Ion Affinity Chromatography (IMAC)	75
	Anion Exchange Chromatography	75
2.1.2.7	SDS-PAGE.....	76
2.1.2.8	Coomassie staining	76
2.1.2.9	Western blotting	76
2.1.2.10	VWF96 variant quantification.....	77
2.1.2.11	VWF96 Enzyme-linked immunosorbent assay (ELISA)	77
	Optimised ELISA (anti-SUMO) conditions.....	77
	Optimised ELISA (anti-HisG) conditions	78
2.2	PRODUCTION OF ADAMTS13 and MDTCS	78
2.2.1	Mammalian protein expression.....	78
2.2.1.1	Expression of full-length ADAMTS13 in HEK 293 cells	78
2.2.1.2	Enzyme-linked immunosorbent assay (ELISA) for ADAMTS13 quantification.....	79
2.2.2	<i>Drosophila</i> Schneider (S2) insect cell expression of truncated ADAMTS13 variants (MDTCS and MDTCS(E225Q))	81
2.2.2.1	Thawing cells.....	81
2.2.2.2	Cell passage.....	81
2.2.2.3	Protein expression	81
2.3	VWF96 variant proteolysis by ADAMTS13	83
2.3.1	Qualitative analysis	83
2.3.2	Kinetic analysis	83
2.3.2.1	Catalytic efficiency by time course analysis of proteolytic reactions.....	83

2.3.2.2 Michaelis Menten kinetic analysis of VWF96 variant proteolysis	84
2.4 ELISA-based assays to measure equilibrium binding affinity between ADAMTS13 and VWF96 variants	85
2.4.1 Equilibrium binding affinity between immobilised VWF96 variants and soluble full-length ADAMTS13	85
2.4.2 Equilibrium binding affinity between immobilised VWF96 variants and MDTCS (E225Q)	86
2.5 Characterising the activating effect of anti-Spacer domain (3E4) and anti-CUB domain (17G2) monoclonal antibodies on the proteolysis of VWF96 by ADAMTS13	86
3 RESULTS: GENERATION OF VWF96 AND DEVELOPMENT OF VWF96 ELISA	87
3.1 INTRODUCTION	87
3.2 Cloning of VWF96 cDNA into the pET SUMO bacterial expression vector	90
3.3 Subcloning of HisG-SUMO-VWF96 cDNA into the pET-25b(+) by restriction digests ...	91
3.4 Expression and purification of VWF96	93
3.5 Qualitative assessment of VWF96 proteolysis by ADAMTS13	95
3.6 VWF96 ELISA development and optimisation	97
3.7 Analysis of VWF96 proteolysis by ELISA	103
3.8 An alternative VWF96 ELISA protocol for monitoring VWF96 proteolysis	105
3.9 DISCUSSION	109
4 RESULTS: GENERATION AND KINETIC CHARACTERISATION OF VWF96 VARIANTS	112
4.1 INTRODUCTION	112
4.2 Generation of VWF96 variants	114
4.3 Qualitative assessment of VWF96 variant proteolysis by ADAMTS13	116
4.4 'Massively parallel' kinetic analysis of VWF73 proteolysis by ADAMTS13	119
4.5 Generation of the VWF47, VWF57 and VWF96-Dis2 variants	122
4.6 Generation and proteolysis of the VWF87(Δ Spacer) variant	126
4.7 The revised panel of VWF96 variants	128
4.8 VWF96 ELISA for VWF96 variant quantification	132
4.9 Catalytic efficiency of VWF96 variants	133
4.10 Michaelis Menten kinetic analysis of VWF96 variant proteolysis	135
4.11 The equilibrium binding affinity (K_D) between ADAMTS13 and the VWF96 variants	141
4.12 The equilibrium binding affinity (K_D) between MDTCS(E225Q) and the VWF96 variants	143
4.13 DISCUSSION	145
5 RESULTS: KINETIC CHARACTERISATION OF THE ACTIVATING EFFECT OF ANTI-SPACER AND ANTI-CUB ANTIBODIES ON VWF96 VARIANT PROTEOLYSIS BY ADAMTS13	152
5.1 INTRODUCTION	152
5.2 Measuring the activating effect of the mAb 3E4 and mAb 17G2 antibodies on the catalytic efficiency of VWF96 proteolysis by ADAMTS13	154
5.3 The activating effect of mAb 3E4 and mAb 17G2 is dependent on the unfolding of ADAMTS13 C-terminal tail	155
5.4 The activating effect of the mAb 3E4 and mAb 17G2 is independent of the ADAMTS13 Spacer and Cys-rich domain exosite interactions	157
5.5 The mAb 3E4 and mAb 17G2 activate ADAMTS13 by increasing the k_{cat}	160
5.6 The conformational activation of ADAMTS13 is not induced by the recombinant D4-CK domains	163
5.7 DISCUSSION	165

6	DISCUSSION	170
6.1	Summary of hypothesis	170
6.2	Biochemical evidence of allosteric activation (summary of project results).....	170
6.3	Biophysical evidence of allosteric activation	173
6.4	Structural evidence of allosteric activation	175
6.5	How do ADAMTS13 domains communicate?	180
6.6	Conclusion – proposed model of allosteric activation	182
	REFERENCES	184
	APPENDIX I.....	207
	APPENDIX II	208

LIST OF FIGURES

Figure 1-1 Domain organisation of VWF.	17
Figure 1-2 The crystal structure of the VWF A1 domain (cartoon view).....	20
Figure 1-3 The crystal structure of the globular VWF A2 domain (cartoon view).....	22
Figure 1-4 The crystal structure of the VWF A3 domain (cartoon view).....	23
Figure 1-5 The D4-CK domains of VWF in complex with $\alpha_{IIb}\beta_3$, arranged in a dimeric bouquet structure.....	24
Figure 1-6 The polarity of the different endothelial VWF secretion pathways.	28
Figure 1-7 Haemostasis	32
Figure 1-8 The multimeric size composition of VWF in plasma.....	34
Figure 1-9 Schematic representation of the multimeric pattern of VWF in the plasma of different types of VWD.	37
Figure 1-10 ADAMTS13 domain organisation.....	40
Figure 1-11 ADAMTS13 MP domain model.....	42
Figure 1-12 ADAMTS13 MP-Dis domains.....	44
Figure 1-13 ADAMTS13 Cys-rich and Spacer domains	47
Figure 1-14 Post-translational modifications of ADAMTS13.....	49
Figure 1-15 Location-specific VWF unravelling promotes cleavage by ADAMTS13.....	52
Figure 1-16 The conformational activation of ADAMTS13.....	58
Figure 1-17 The conformational transition of A2 domain	60
Figure 2-1 pET-SUMO map.....	69
Figure 2-2 pET-25b(+) map	73
Figure 2-3 pcDNA TM 3.1 Mammalian expression vector map.....	80
Figure 2-4 pMT-BiP-PUR0 vector map.....	82
Figure 3-1 Agarose gel electrophoresis of VWF96 cDNA PCR amplification.....	90
Figure 3-2 Subcloning of HisG-SUMO-VWF96 cDNA from the pET-SUMO vector to the pET-25b(+)	92
Figure 3-3 VWF96 purification.....	94
Figure 3-4 Qualitative assessment of VWF96 and VWF115 proteolysis by ADAMTS13	96
Figure 3-5 VWF96 ELISA optimisation – ELISA #3	98
Figure 3-6 VWF96 ELISA optimisation – ELISA #6 and #7	99
Figure 3-7 VWF96 ELISA optimisation – ELISA#11 and ELISA#14.....	101
Figure 3-8 Analysis of VWF96 proteolysis using VWF96 ELISA.....	104
Figure 3-9 Optimisation of the VWF96 ELISA using anti-HisG antibody.....	106
Figure 3-10 Analysis of VWF96 proteolysis using the VWF96 ELISA (anti-HisG).....	108
Figure 4-1 Generation of VWF96 variants	115
Figure 4-2 Qualitative assessment of the proteolysis of VWF96 variants by ADAMTS13.....	118
Figure 4-3 Heat map illustrating the effect on the k_{cat}/K_m of substituting each residue in the VWF73 sequence.	121
Figure 4-4 Generation and proteolysis of VWF47 and VWF57	123
Figure 4-5 Quantitative analysis of VWF57 and VWF47 proteolysis by ADAMTS13 using the VWF96 ELISA.	124
Figure 4-6 Qualitative analysis of the proteolysis of the VWF96-Dis1 and VWF96-Dis2 variants by ADAMTS13.	126
Figure 4-7 Qualitative analysis of the proteolysis of the VWF96-Spacer and VWF87(Δ Spacer) variants by ADAMTS13.	127
Figure 4-8 The revised panel of VWF96 variants.....	130
Figure 4-9 Qualitative analysis of the proteolysis of the revised panel of VWF96 variants by ADAMTS13.....	131

Figure 4-10 Normalisation of VWF96 variant concentration by VWF96 ELISA.....	132
Figure 4-11 The catalytic efficiency of VWF96 variant proteolysis by ADAMTS13, as assessed by analysing the proteolytic time course of the reactions.....	134
Figure 4-12 Michaelis Menten kinetic analysis of the proteolysis of the VWF96 variants by ADAMTS13	138
Figure 4-13 Michaelis Menten kinetic analysis of the proteolysis of the VWF96 variants by ADAMTS13 plotted on the same graph.....	139
Figure 4-14 Equilibrium binding affinity between immobilised VWF96 variants and ADAMTS13 in solution	142
Figure 4-15 Equilibrium binding affinity between immobilised VWF96 variants and MDTCS(E225Q) in solution.....	144
Figure 5-1 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96 by ADAMTS13.	155
Figure 5-2 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96 by MDTCS	156
Figure 5-3 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF87(Δ Spacer) by ADAMTS13	158
Figure 5-4 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96-Cys by ADAMTS13	159
Figure 5-5 The effect of mAb 3E4 and mAb 17G2 on the Michaelis Menten kinetics of VWF96 proteolysis by ADAMTS13	161
Figure 5-6 The effect of recombinant VWF D4-CK domains on the catalytic efficiency of VWF96 proteolysis by ADAMTS13	163
Figure 5-7 The effect of recombinant VWF D4-CK domains (buffer exchanged) on the catalytic efficiency of VWF96 proteolysis by ADAMTS13	164
Figure 6-1 ITC experiments to investigate to binding affinity of tag-free VWF73 to MDTCS(E225Q)	174
Figure 6-2 Crystal structures of the ADAMTS4 active site, with and without active site inhibitor bound.....	176
Figure 6-3 Crystal structure of the stable complex between the Fab 3H9 and MDTCS(E225Q)	178

LIST OF TABLES

Table 3-1 VWF96 ELISA optimisation (varied conditions)	102
Table 4-1 Summary of the effect of ablating the ADAMTS13-VWF exosite interactions on the kinetics of proteolysis	140
Table 4-2 Summary of the equilibrium binding affinity (K_D) between immobilised VWF96 variants and ADAMTS13 or MDTCS(E225Q) in solution	144
Table 5-1 Summary of the effects of monoclonal antibodies on the kinetics of VWF96 (variant) proteolysis by ADAMTS13 and MDTCS.....	162

1 INTRODUCTION

1.1 OVERVIEW OF HAEMOSTASIS

The term haemostasis is derived from the Greek words haema (αἷμα) that means blood, and stasis (στάσις) that means to stop. It is a homeostatic process, involving complex interactions between the vessel wall, plasma proteins, and platelets, and aims at maintaining thrombohaemorrhagic balance (ie. maintaining the fluidity of blood while preventing its loss from sites of vessel damage). Under normal conditions, the endothelial cells lining the blood vessels are naturally antithrombotic (they present multiple components that assist in inhibiting haemostasis on their surface). Upon damage to the vascular endothelium, exposure of the thrombogenic subendothelial matrix (potent activators of haemostasis) and tissue factor-exposing cells to the circulation initiates blood clotting to prevent blood loss from the damaged site. The consequent haemostatic response can be divided into three phases; primary haemostasis, secondary haemostasis and fibrinolysis.

1.1.1 Primary haemostasis

The key players in primary haemostasis are platelets, the multimeric glycoprotein von Willebrand Factor (VWF) and collagen. Following vessel damage, primary haemostasis starts with local contraction of the vasculature to reduce the blood flow at the site of damage. At the same time, an unstable platelet plug forms to temporarily prevent bleeding. Circulating platelets in their resting state display multiple glycoproteins (Gp) on their surfaces that are important in recruiting platelets to the site of vessel damage to form the platelet plug. At low shear rates platelets can interact directly (via GpVI and $\alpha 2\beta 1$) with collagen present in the subendothelial matrix. However, at higher shear rates, platelets can form transient interactions (via their GpIb α) with collagen-bound VWF, which reduces their velocity and allows more stable interactions with collagen to take place. Collagen binding activates platelets, resulting in the release of their granule content, including VWF. Endothelial activation at the site of vessel damage also releases VWF. Release of endothelial- and platelet-derived VWF allows repeated VWF-platelet binding that is necessary to form the platelet plug. Activation of platelets also activates their membrane integrin $\alpha_{IIb}\beta_3$, which can interact with fibrinogen to facilitate platelet aggregation and the platelet plug formation. During platelet degranulation potent platelet agonists are released that amplify platelet activation within the growing platelet plug, where collagen is not accessible. Primary haemostasis must be a regulated process so that the

platelet plug does not grow indefinitely. The enzyme ADAMTS13 has the ability to regulate the growth of the platelet plug by proteolysing VWF and restricting platelet accumulation to the site of vessel damage.

1.1.2 Secondary haemostasis

Secondary haemostasis, or coagulation, is a proteolytic cascade involving the sequential activation of numerous enzymes (coagulation factors) from their circulating zymogen or procofactor form. The endpoint of this haemostatic phase is the generation of thrombin, which converts fibrinogen into fibrin, stabilising the haemostatic clot. At the site of vessel wall damage, tissue factor (TF) is exposed to the circulation, which can interact with coagulation factor VIIa (FVIIa) and initiate the coagulation cascade through activation of FIX and FX.

Coagulation is highly dependent on assembly of coagulation complexes on negatively charged phospholipid surfaces. Part of platelet activation within the growing plug is the conversion of their membrane into negatively charged surfaces (by presenting phosphatidylinositol and phosphatidylserine). These negatively charged phospholipid surfaces of activated platelets act as cofactors for the activation of coagulation factors and the propagation of coagulation.

Coagulation is very tightly regulated/coordinated. There are three anticoagulant pathways ensuring regulated coagulation, namely tissue factor pathway inhibitor (TFPI), antithrombin and protein C pathway. These inhibit different stages during coagulation.

1.1.3 Fibrinolysis

Formation of the blood clot enables the prevention of blood loss from the site of vessel damage while allowing wound healing. The clot is a transient structure and is eventually dissolved by a process called fibrinolysis. This is catalysed by the enzyme plasmin. Plasmin proteolytically degrades the fibrin within the clot into fibrin degradation products.

Thorough understanding of the molecular mechanisms that underpin the haemostatic system is important, as pathological situations (such as inflammation, trauma, surgical intervention, infection, haematological disorders, hypoxia, etc.) can challenge the haemostatic balance and cause thrombotic or bleeding complications. For example, it is well established that dysregulation of the VWF-ADAMTS13 axis can distort the haemostatic balance and cause both thrombotic and/or bleeding complications. As a result, the interaction between ADAMTS13

and VWF has been extensively studied over the last three decades. This thesis focuses on the molecular mechanisms that dictate the recognition and proteolysis of VWF by ADAMTS13.

1.2 VWF MOLECULE/STRUCTURE

1.2.1 VWF gene expression

Human *VWF* was sequenced in 1984. The gene is ~180kb and located on chromosome 12 (12p13.2). Out of a total of 52 exons, exon 1 is the 5' untranslated sequence, exons 2-18 encode the signal peptide and propeptide, and exons 19-52 encode the mature VWF protein. Expression of VWF only occurs in endothelial cells⁽¹⁾ and megakaryocytes.^(2, 3) This cell type specificity is the result of transcriptional regulation, controlled by a 734bp promoter region (-487 to +247 relative to the transcription start site).⁽⁴⁻¹²⁾ VWF is differentially expressed in endothelial cells of different vascular beds and tissues.⁽¹³⁻¹⁶⁾

1.2.2 VWF domain organisation and structure

The primary structure of the translated VWF mRNA consists of 2813 amino acids (aa); a 22aa signal peptide, a 741aa propeptide and a 2050aa mature subunit.⁽¹⁷⁾ Figure 1-1 illustrates how VWF is organised into different domains. The propeptide is made up of the D1 and D2 assemblies, and is followed by the mature subunit, which is organised into the D'D3 assemblies, three A domains (numbered A1 to A3), the D4 assembly, six C domains and the C-terminal cystine knot (CTCK).⁽¹⁸⁾ VWF multimerises intracellularly during synthesis, and its multimeric size dictates its ability to exist in either a globular fold or an unravelled string-like structure.^(19, 20) The globular conformation is adopted through weak intra-molecular interactions (hydrogen bonding). The transformation into an extended molecule is reversible and depends on rheological forces; this 'mechanically switchable' conformational transition has crucial physiological/pathophysiological implications.^(21, 22) The multidomain, modular structure of VWF subunits facilitates a range of physiological functions, including biosynthesis, post-translational modifications, dimer-/multimerisation, intracellular trafficking, storage, secretion, haemostasis and more.

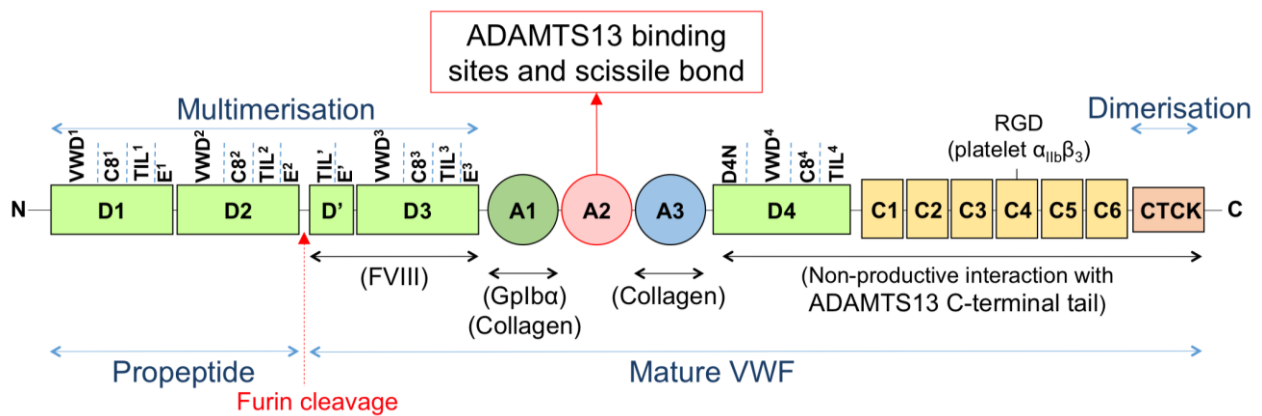


Figure 1-1 Domain organisation of VWF.

The D assemblies are made up of different modules, namely the VWD, C8, TIL and E and D4N. The D1D2 domains make up the propeptide, which is subject to furin cleavage. Dimerisation of VWF monomers depends on inter-molecular disulphide bond formation between the CTCK domains. Multimerisation depends on disulphide bond formation mediated by the N-terminal D assemblies. The plasma metalloprotease ADAMTS13 interacts with binding sites and cleaves the scissile bond within the VWF A2 domain, subject to A2 domain unravelling. VWF domains make functional interactions with multiple ligands, which are included in brackets.

1.2.2.1 VWF D regions

D regions are four distinct assemblies, namely the D1, D2, D'D3 and D4 assemblies. They have been termed assemblies, rather than domains, since they are made up of different modules, namely the Von Willebrand D (VWDⁿ), 8-cysteine (C8ⁿ), trypsin inhibitor-like (TILⁿ) and fibronectin type 1-like (Eⁿ) modules, which are present in each assembly in this order (see Figure 1-1). The modules are numbered (n) to refer to the assembly they belong to (eg. VWD¹, C8¹, TIL¹ and E¹ belong to the D1 assembly). The D' lacks a VWD' and a C8', while the D4 assembly lacks the E⁴ module but possesses a unique segment, D4N, which is disulphide bonded N-terminally to the VWD⁴. Electron microscopy analysis showed that D assemblies have a lobular structure. The correct folding of the modules within each D assembly seems to be interdependent, as their expression is only successful when all modules are present.⁽¹⁸⁾

The very large propeptide (D1 and D2 assemblies, 741aa) is not part of the mature protein as it is, eventually, proteolytically removed by furin in the *trans*-Golgi network.⁽²³⁻²⁵⁾ It is important for intracellular processing; it is directly involved in the multimerisation and storage of VWF, even when expressed *in trans*. For effective multimerisation, Weibel-Palade body (WPB) (endothelial-specific organelles that store VWF multimers prior to secretion, see section 1.2.3.2) formation and storage to take place, both D1 and D2 domains need to be present.^(17, 26-31)

The D'D3 assemblies are also critical for the multimerisation of VWF dimers (D1-D3 assemblies are the only VWF regions involved in multimerisation).⁽³²⁾ Cysteines in D1D2, and two cysteines in D3 (C1099 in C8³ and C1142 in TIL³) participate in an oxidoreductase mechanism that facilitates 'head-to-head' N-terminal multimerisation of VWF dimers.⁽³³⁻³⁵⁾ D1-D3 assemblies are also the minimum requirement for WPB formation and storage.^(36, 37) The D'D3 assemblies in mature VWF also bind and stabilise coagulation Factor VIII (FVIII) in circulation.⁽³⁸⁻⁴⁰⁾

The D4 assembly might form non-productive interactions (interactions that do not lead to proteolysis) with ADAMTS13, which are thought to prime ADAMTS13-mediated proteolysis^(41, 42) and to induce a conformational change in ADAMTS13 that enhances its activity (see section 1.8.1).⁽⁴³⁻⁴⁶⁾

1.2.2.2 VWF A domains

Von Willebrand A (VWA) domains are found in multiple proteins and are implicated in ligand binding in various processes, including haemostasis (A domains in VWF), the complement system (A domain of complement protein component C2a), cell adhesion (I domain in integrins) and cell surface receptors for anthrax toxins. VWA domains commonly have a central β -sheet, which is flanked by amphipathic α -helices. A common structural feature of these domains is a metal ion-dependent adhesion site (MIDAS) motif, which coordinates a Mg^{2+} at the C-terminus of the β -sheet, and facilitates interactions with ligands.⁽⁴⁷⁾ The structures of all A domains in VWF have been solved by X-ray crystallography,⁽⁴⁸⁻⁵⁴⁾ and show a similar arrangement of a central β -sheet (made up of six β -strands) and surrounding α -helices. Unlike other VWA domains, the A domains in VWF do not possess a classical MIDAS motif to coordinate a metal ion (the VWF A2 domain coordinates a Ca^{2+} in a different fashion), and they form interactions with their ligands at a different site in the domain. Both VWF A1 and A3 domains have an intra-domain long-range disulphide bond that stabilises the domain conformation; the absence of this disulphide bond in the VWF A2 domain accounts for increased domain flexibility, lower stability and the propensity to unfold, which are crucial to the regulation of VWF function by ADAMTS13.^(47, 55, 56)

VWF A1 domain

In the initial stages of haemostasis, the A1 domain forms a well-characterised interaction with platelet GpIb α receptors, which results in platelet tethering to the site of vessel damage.⁽⁵⁷⁻⁶¹⁾ The GpIb α -binding sites in A1 are naturally cryptic when VWF is globular, due to intra-molecular shielding by the D'D3 domains and the A2 domain, and become exposed when VWF unravels.⁽⁶²⁻⁶⁶⁾ The crystal structure of the A1 domain (see Figure 1-2) shows two points of contact between VWF A1 and platelet GpIb α , which are electrostatic interactions between oppositely charged sites in the two molecules. For the first interaction, the A1 domain α 3 helix, α 3 β 4 loop and β 3 strand interact with the β -switch around the leucine-rich repeats (LRR)5-8 of GpIb α . The second interaction involves the A1 domain loops α 1 β 2, β 3 α 2 and α 3 β 4 and the β -finger around the GpIb α LRR1.^(52, 59, 60, 67, 68) A third point of contact between the A1 domain and central LRR's in the GpIb α was predicted, as a result of a proposed shear-induced conformational change in the A1 domain.^(69, 70) Other binding partners of the A1 domain include collagen⁽⁷¹⁻⁸³⁾ and heparin.⁽⁷³⁻⁷⁹⁾

VWF A1 domain

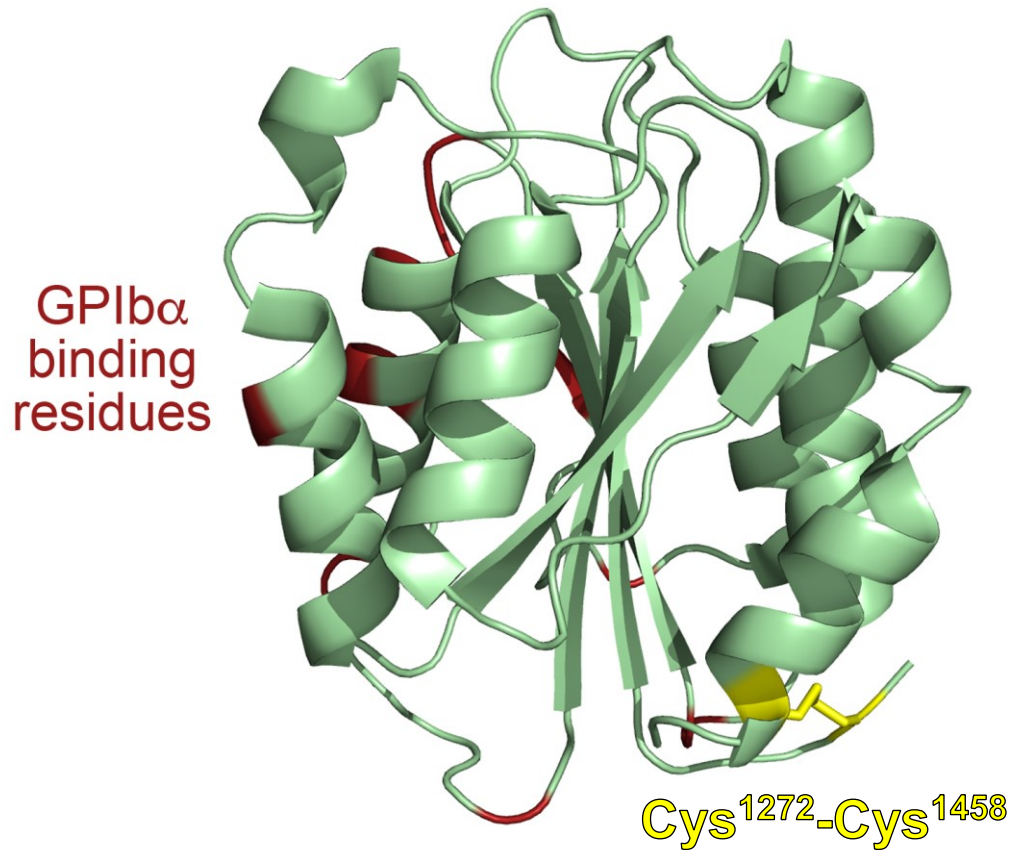


Figure 1-2 The crystal structure of the VWF A1 domain (cartoon view)

The binding sites that interact with GPIb α are coloured red. The intra-domain, long-range disulphide bond (C1272-C1458) is coloured yellow. The structure was obtained from ⁽⁶⁷⁾ and adapted.

VWF A2 domain

The VWF A2 domain is a structurally and functionally unique VWA domain. Unlike VWF A1, A3 and other VWA domains, it does not have an α 4-helix but an ' α 4-less' loop instead. It also lacks the intra-domain long-range disulphide bond seen in VWF A1 and A3 domains.^(54, 84) These features explain the ability of this domain to unfold.^(21, 55, 56) On the other hand, the VWF A2 domain uniquely possesses a vicinal disulphide linkage (C1669-C1670), a Ca²⁺-binding site (D1498, D1596, R1597, A1600 and N1602) and the N-linked glycan at N1574^(54, 85-87) (all annotated on Figure 1-3), which increase its thermostability and stabilise its globular structure, resisting the unfolding until a tensile force threshold is reached. A functional role in the domain's conformational stability was inferred for the N-linked glycan N1515, as loss of the glycan enhanced the clearance of VWF, but not when the domain was structurally constrained by introducing a long-range disulphide bond C1493-C1669.⁽⁸⁸⁾ These properties, therefore, make it a force-sensing domain, and the shear forces acting on it in the circulation determine whether it unfolds or not.^(53, 85, 89, 90) The conformational transition of the VWF A2 domain contributes to the extension and flexibility of the VWF multimers in a string-like arrangement that is a prerequisite for primary haemostasis to occur. Most importantly, the VWF A2 domain contains the cleavage site, as well as multiple interaction sites for ADAMTS13, which are only exposed for proteolysis upon domain unfolding.^(54, 56) How the conformation of the A2 domain dictates the haemostatic function of VWF and the balance in the VWF-ADAMTS13 axis will be discussed in section 1.8.2.

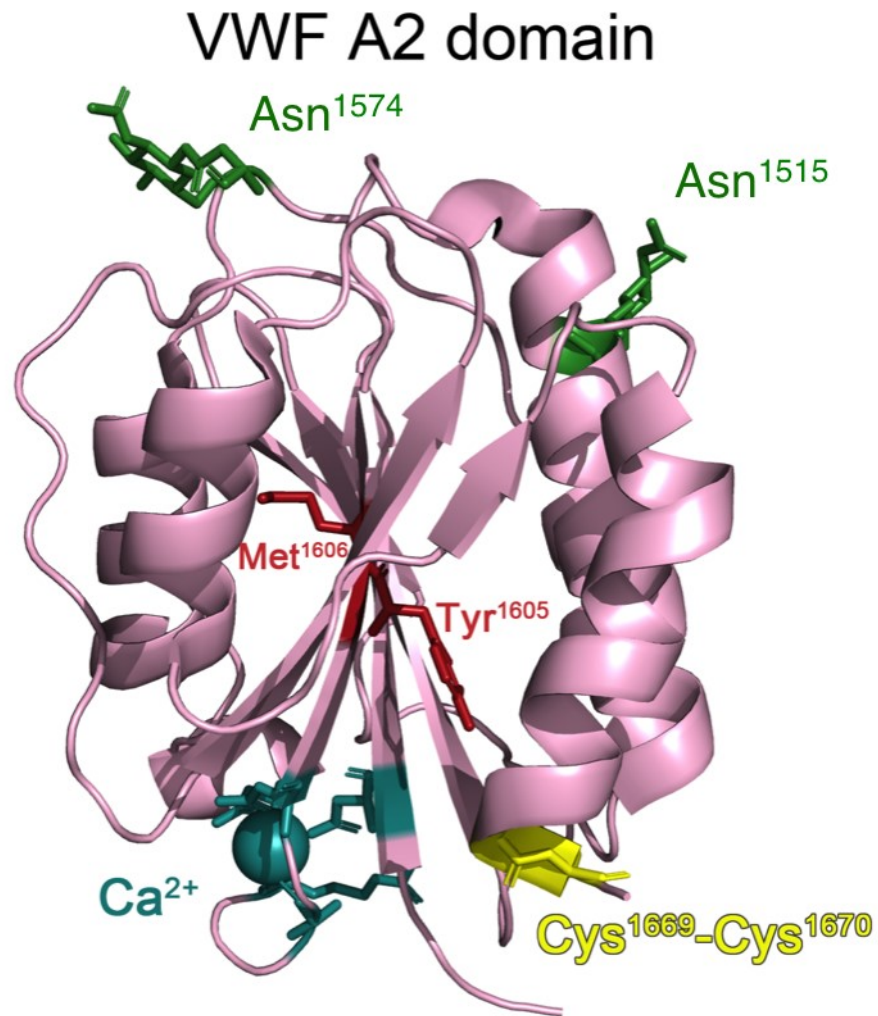


Figure 1-3 The crystal structure of the globular VWF A2 domain (cartoon view)

The scissile bond Y1605-M1606 (red), is buried in the centre of the globular A2 domain. The N-linked glycosylations (green), the Ca²⁺-binding site (teal) and the vicinal disulphide bond C1669-C1670 (yellow) stabilise the globular fold of the domain, until a tensile force threshold is reached. The structure was obtained from ⁽⁵³⁾ and adapted.

VWF A3 domain

The structure of the VWF A3 domain resembles that of the VWF A1 domain. It has a similar fold, it lacks the MIDAS motif seen in the VWA domains of integrins, it does not lack the $\alpha 4$ helix and it has an intra-domain long-range disulphide bond (see Figure 1-4).^(47, 84) Its importance in forming physiological cation-independent interactions with collagen during primary haemostasis has been established. For these, the residues Q1734, I1738, T1740, I1741, D1742, P1744, V1760, E1764, F1776, Y1780, S1783 and H1786 were shown to be important. All interacting residues are located at the front face of the domain.^(83, 91-97)

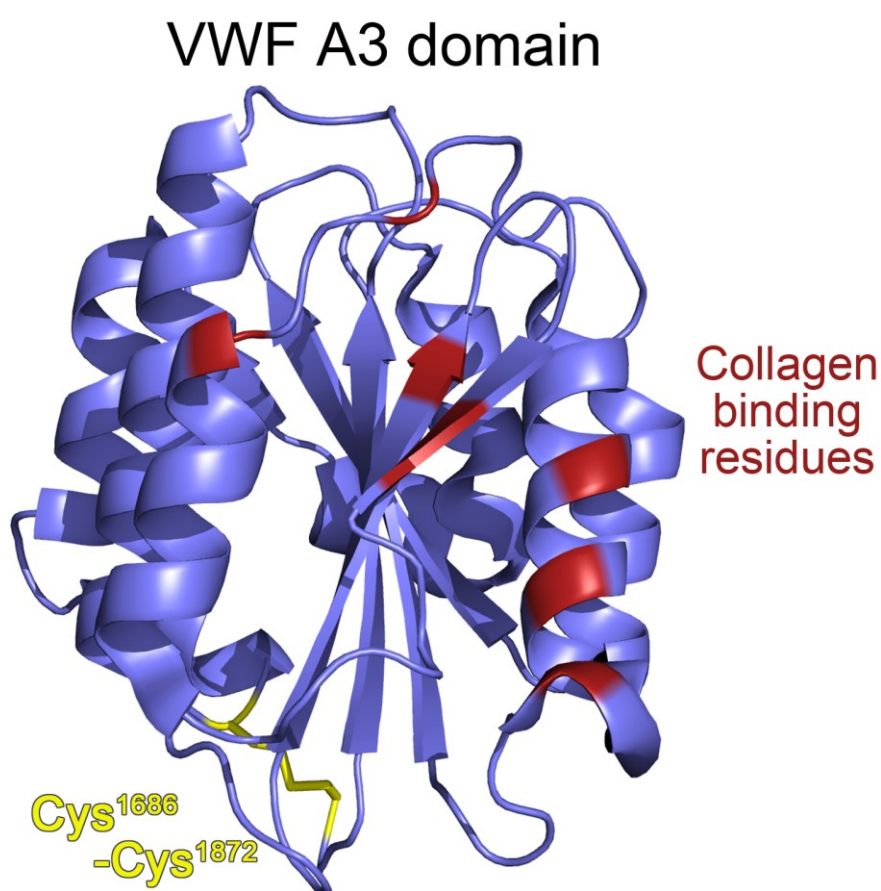


Figure 1-4 The crystal structure of the VWF A3 domain (cartoon view).

The binding sites in the VWF A3 domain that interact with collagen are coloured red. The intra-domain, long-range disulphide bond C1686-C1872 is coloured yellow. The structure was obtained from ⁽⁹⁷⁾ and adapted.

1.2.2.3 VWF C domains

The VWF C domains are six tandem homologous domains (C1 to C6), which were only recently annotated as distinct domains in the VWF structure.⁽¹⁸⁾ These domains are rich in cysteines and form multiple intra-domain disulphide linkages. In C1-C6, there are nine unpaired cysteines in total, which have been shown to be important for the secretion of VWF.⁽⁹⁸⁾ Electron microscopy identified that VWF dimers form a bouquet-like structure in the Golgi (see section 1.2.3.2)(Figure 1-5). In this bouquet, the C domains form the stem (~32nm) as a result of non-covalent inter-subunit interactions between the same C domains (C1-C1', C2-C2' etc.).⁽¹⁸⁾ The physiological function of the C domains is not clear. It is likely that they are structural components that make VWF long and flexible, and in combination with the lack of a hydrophobic core, they allow for the rope-like structure of VWF.^(18, 99, 100) The C4 domain contains an RGD sequence that can bind the activated $\alpha_{IIb}\beta_3$ integrin on platelets, and is implicated in the platelet plug formation during primary haemostasis.^(18, 101, 102)

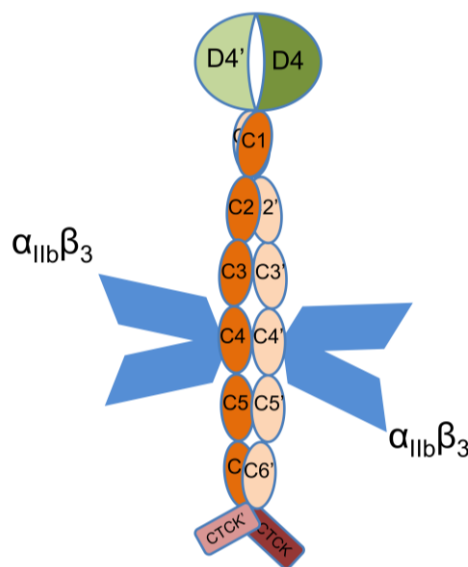


Figure 1-5 The D4-CK domains of VWF in complex with $\alpha_{IIb}\beta_3$, arranged in a dimeric bouquet structure.

The C1-C6 domains of one monomer form non-covalent, inter-subunit interactions with the respective C1'-C6' domains of the second monomer in the same dimer. One $\alpha_{IIb}\beta_3$ interacts with the RGD sequence of each of the C4 domains in the dimer.

1.2.2.4 VWF CTCK domain

CTCK domains are found at the C-terminus of various extracellular proteins, including the transforming growth factor- β , mucins and norrie disease protein. CTCK-containing proteins are typically known to form homo- or heterodimers. The CTCK also causes the proteins to adopt a folding that resists a globular conformation, exposing hydrophobic residues that mediate protein-protein interactions.^(103, 104) The CTCK domain in VWF possesses 11 cysteines, eight of which involved in intra-chain disulphide linkages. The remaining three cysteines are oxidized into three inter-chain disulphide bridges in the endoplasmic reticulum (ER) resulting in 'tail-to-tail' dimers with their long axes anti-parallel.^(18, 105, 106) Along with the C1-C6 domains, the CTCK domain forms the base of the stem in the bouquet-like homodimeric structure of VWF in the acidic compartments of the cell (Figure 1-5).⁽¹⁰⁰⁾

1.2.3 VWF biosynthesis

VWF biosynthesis and secretion are summarized in Figure 1-6.

1.2.3.1 VWF post-translational modifications and intracellular trafficking

The product of translation is preproVWF, which contains the signal peptide and the propeptide. After the signal peptide guides preproVWF to the ER, it gets cleaved, generating a 260kDa protein termed proVWF, which still has its propeptide covalently linked. ProVWF is N-linked glycosylated with high mannose carbohydrates (16 N-linked glycosylation sides; three in the propeptide and 13 in the mature monomer).⁽¹⁰⁷⁾ Dimerisation then occurs, involving 'tail-to-tail' covalent disulphide linkage of two proVWF molecules at the CTCK domains.^(105, 108, 109) In the Golgi, proVWF is O-linked glycosylated at 10 sides,⁽¹⁰⁷⁾ and its N-glycans further processed to convert high mannose carbohydrates into complex carbohydrates. The processing of the N-linked glycans increases the molecular weight of proVWF to 275kDa.⁽¹⁰⁹⁾

Multimerisation takes place in the low pH environment of the *trans*-Golgi network (TGN), and is independent of dimerization.⁽³²⁾ This occurs by inter-dimer disulphide bonds between the D3 domains of the proVWF dimers, in parallel orientation, and the two cysteines C1099 (in C8³) and C1142 (in TIL³) are important in the oxidoreductase mechanism.^(33, 34) The propeptide, D1-D2, is indispensable in the multimerisation. The mechanism of its involvement is similar to protein disulphide-isomerases; in the ER, propeptide cysteines facilitate transient intra-molecular disulphide bonds with the D3 domain that are later rearranged to permanent

multimeric bonds between the inter-dimer D3 cysteines. Furthermore, evolutionarily conserved His residues in the propeptide participate in the pH-sensing mechanism that drives multimerisation. Initially, the propeptide is still covalently linked to the mature subunit. Concurrently to multimerisation, furin proteolytically removes the propeptides, generating multimers constituted by mature subunits, which are now 220kDa. The propeptide remains non-covalently associated to the mature VWF and can still promote multimerisation *in trans*.^(23-25, 28-30, 33, 35, 108-113)

1.2.3.2 VWF storage and secretion

After post-translational processing, the VWF multimers are stored in the WPB, which are endothelial-specific, cigar-shaped organelles.^(114, 115) This maintains an intracellular pool of VWF that is releasable upon stimulation.⁽¹⁰⁸⁾ The D1-D3 domains are the minimum requirement for WPB formation.^(36, 37) Both D1-D2 propeptide domains need to be present for successful WPB formation and storage.^(30, 116, 117) The propeptide signals for multimerisation and storage are independent.⁽²⁹⁾

A model of the WPB formation has been proposed.^(84, 100) The lower pH of the Golgi favours the adoption of bouquet-like structures, as a result of non-covalent association between the CTCK-A2 domains of two monomers within a dimer; the A2, A3 and D4 resemble the flower, and the C1-6 and CTCK domains form the stem (Figure 1-5).⁽¹⁰⁰⁾ The VWF multimers assemble in a helical tubular fashion, with a high degree of organization and folding, and bud off to form WPB.^(84, 118-120)

VWF circulates in plasma at 5-10 μ g/ml and has a half-life of ~12 hours.^(121, 122) It is secreted from endothelial cells via three pathways: the constitutive pathway (non-stimulated basolateral secretion of mainly low molecular weight VWF multimers not packed in WPB), the basal pathway (apical secretion of WPB-derived VWF in the absence of stimulus) and the regulated pathway (secretion of WPB-derived VWF following a stimulus).⁽¹²³⁾ Upon a haemostatic stimulus (eg. thrombin, histamine), different modes of regulated WPB exocytosis are believed to occur; single WPB exocytosis (fusion of one WPB with plasma membrane), or multigranular exocytosis (intracellular homotypic fusion of several WPB to form a secretory pod containing pooled VWF).⁽¹²⁴⁾ During WPB content release, VWF has the potential to form platelet-coated strings on the endothelial cell membrane, which are made up of multiple multimers arranged in networks. The formation of these strings on the endothelial cell is

dependent on the tubular structure of VWF within the WPB.⁽³⁷⁾ ADAMTS13 prevents the persistence of such strings.⁽¹²⁵⁾

VWF is also synthesized in megakaryocytes.^(2, 3) Like endothelial cells, megakaryocytes can secrete VWF constitutively, or store it for regulated secretion. Megakaryocyte-derived VWF is stored in platelet α -granules, where it adopts a tubular arrangement that resembles the WPB. This pool of VWF is only released upon platelet activation, contributing to clot formation.^(126, 127)

WPB-^(123, 128) and α -granule-derived⁽¹²⁹⁾ VWF is composed of a range of multimeric species, but contains a higher proportion of unusually large multimers, which is estimated to reach up to ~100-200 subunits.^(17, 19-21, 56) Such enormous species, termed ultra-large VWF (ULVWF), are potent haemostatic activators. Since the multimeric size of VWF is not well regulated at the level of synthesis, this necessitates regulation in plasma; proteolytic regulation by ADAMTS13 in plasma ensures that ULVWF multimers do not accumulate in the circulation. How the enhanced haemostatic potential of ULVWF, as well as the proteolytic regulation by ADAMTS13, are manifest will be discussed in later sections.

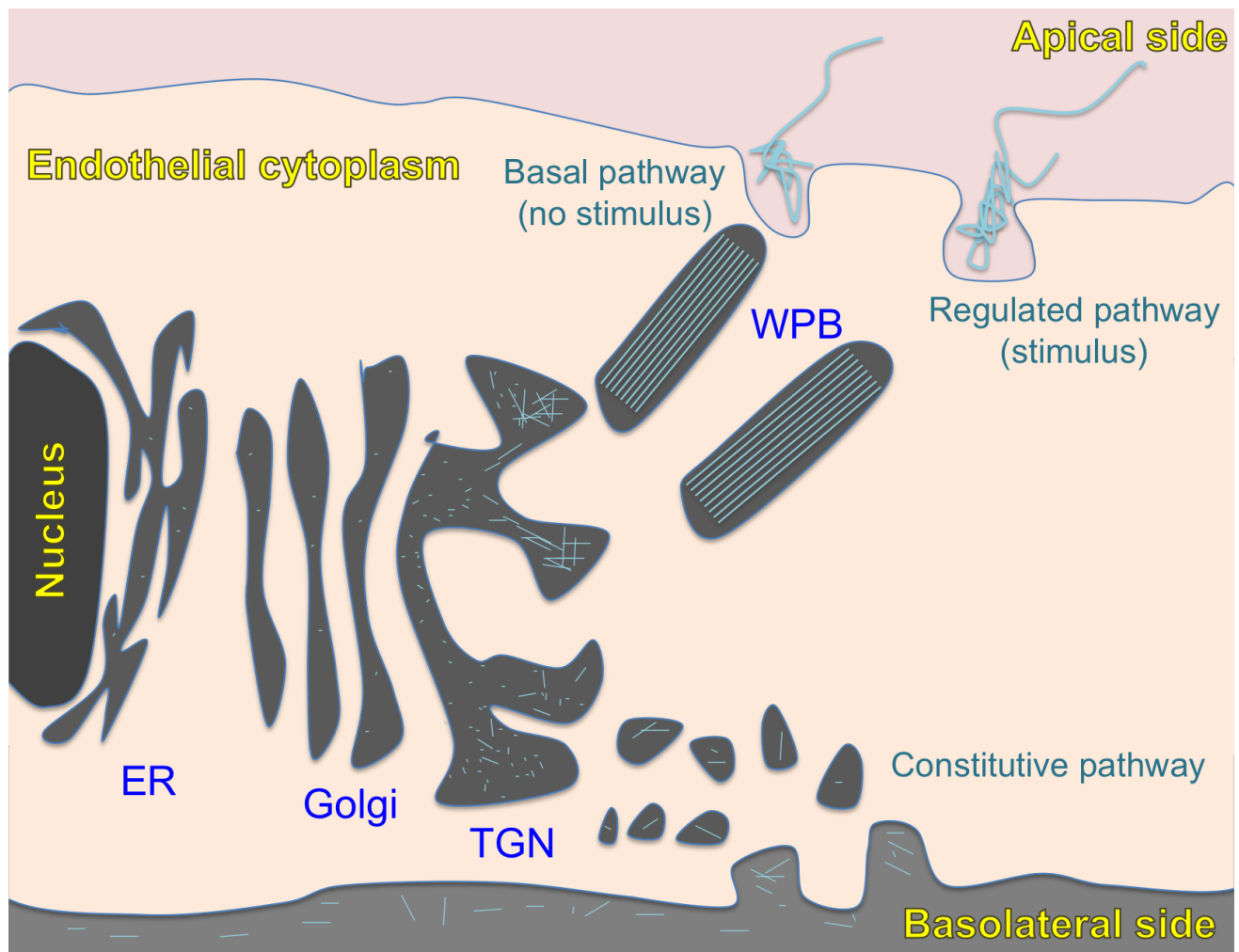


Figure 1-6 The polarity of the different endothelial VWF secretion pathways.

WPB-derived VWF is secreted apically, either following a stimulus (regulated pathway), like thrombin or histamine, or in the absence of stimulus (basal secretion). Apically secreted VWF is composed of higher molecular weight multimers. Lower molecular weight VWF that is not derived from WPB is secreted constitutively at the basolateral side of the endothelial cell, and is a natural constituent of the subendothelial matrix.

1.3 VWF FUNCTION

VWF has two main functions in haemostasis. Firstly, it is a key player in primary haemostasis, facilitating the formation of the platelet plug. Secondly, it is the carrier molecule for FVIII in circulation, which has implications in the coagulation cascade.

1.3.1 Primary haemostasis

The role of VWF in primary haemostasis is summarised in Figure 1-7.

1.3.1.1 Platelet adhesion/capture

Damage to the vessel wall results in extravascular structures (ie. the subendothelial matrix or deeper tissues) becoming exposed to the circulation. Thrombogenic extravascular molecules, such as collagens that are normally not in contact with blood, initiate primary haemostasis. The very first event after vessel damage is platelet attachment to these thrombogenic surfaces, and this can be either VWF-independent or VWF-dependent. Platelets can form interactions with collagen directly, independent of VWF. These interactions have been shown to rely on the platelet collagen-binding receptors GpVI⁽¹³⁰⁻¹³³⁾ and $\alpha_2\beta_1$.⁽¹³¹⁻¹³⁵⁾ With the help of synthetic receptor-specific collagen fragments, a primarily signaling role in platelet activation was assigned to GpVI at all shear conditions, whereas an adhesive role at low shear conditions was demonstrated for $\alpha_2\beta_1$.⁽¹³⁶⁾ Interactions of platelets with other extravascular substrates, such as laminin (via GpVI and $\alpha_6\beta_1$) and fibronectin (via $\alpha_5\beta_1$ and activated $\alpha_{IIb}\beta_3$),⁽¹³⁷⁾ as well as fibrinogen found within the growing plug (via non-activated $\alpha_{IIb}\beta_3$),^(138, 139) have been described.^(68, 137, 140) As these interactions cannot withstand higher shear forces, they can only mediate tethering at lower venous shears.

At elevated shear rates, it is now established that the primary mode of initial platelet recruitment to the site of vascular injury is VWF-dependent, with an absolute requirement of the interaction between platelet GpIb α and the A1 domain of VWF.^(57-61, 101, 141, 142) Soluble VWF (circulating in plasma) localises at the site of vessel damage in various ways. Firstly, VWF can sense the site of vessel damage and becomes immobilised by interacting with exposed collagen. For this to happen, the VWF A1 and A3 domains^(143, 144) interact with multiple collagen types, such as I, III^(76, 91-95, 97, 145, 146), IV⁽⁷¹⁾ and VI^(83, 147, 148). To date, no study has concluded on the relative importance of the A1- and A3-mediated interactions with the different collagens, but immobilisation of VWF is likely to be a complex process, where all the

types of interaction characterised act synergistically.⁽⁸¹⁾ Although other extravascular molecules could potentially interact with soluble VWF, none was proven to be physiologically important, leaving collagen as the most likely candidate for immobilising VWF. In response to endothelial cell and platelet activation as a result of a haemostatic trigger, VWF can also be released from WPBs and α -granules, respectively, which contributes to platelet adhesion to the site (see regulated pathway in section 1.2.3.2).⁽³⁷⁾ Basolaterally secreted VWF (through the endothelial constitutive secretion pathway) is a natural constituent of the endothelial extracellular matrix and becomes exposed when the vessel wall is impaired. Direct interactions have been reported between platelets and VWF within the extracellular matrix, but the physiological haemostatic relevance of this type of platelet adhesion is not clear.

High shear rates are important for VWF-dependent platelet adhesion. Following immobilisation, VWF undergoes a shear-dependent conformational change, in which its globular structure unfolds to a more extended, string-like arrangement.^(21, 22, 36, 37, 84) This exposes the platelet GpIb α binding sites within the VWF A1 domain (previously shielded by the D'D3 and A2 domains), which enables VWF to interact with circulating platelets.⁽⁶²⁻⁶⁶⁾ Apart from overcoming the intra-molecular shielding, shear rates were shown to have another, more direct role in this interaction. Increasing shear stress activates GpIb α , by inducing a conformational transition that changes its β -switch from a loop to a hairpin; this increases its affinity for the VWF A1 domain.^(149, 150) More recently, it was shown that the tensile force can also change the VWF A1 domain conformation, modulating its affinity for GpIb α in a 'two state flex-bond' fashion. The first state of this interaction is between the GpIb α β -switch and β -finger regions with different sites on the VWF A1 domain, and takes place at lower shear rates. As shear rates increase, the VWF A1 domain undergoes a conformational change, forming a second state interaction with a higher affinity site within GpIb α LRR2-4.^(69, 70)

The VWF A1-platelet GpIb α dissociation rate is relatively fast, which means that binding is transient and cannot sustain firm and stable platelet adhesion.⁽¹⁵¹⁾ The fast on and off rates of the GpIb α bonds give platelets a characteristic tethering or rolling movement over immobilised VWF in the same direction as the flow.^(136, 138, 151, 152) The GpIb α -A1 interaction is the only interaction that can occur at high shear rates to enable subsequent platelet adhesion. This is vital to stop bleeding at locations of naturally elevated shear rates (eg. arterioles). Furthermore, the narrowing of a vessel lumen during haemostasis (due to obstruction from the growing thrombus and vasoconstriction) necessitates an adhesion mechanism that can resist

the consequent higher shear rates.⁽¹³⁷⁾ While the first layer of VWF is immobilised by interacting with collagen, subsequent layers of VWF in the growing plug are immobilised by interacting with VWF and platelets.

1.3.1.2 Platelet stable adhesion, activation and aggregation

As already stressed, the platelet GpIb α -VWF A1 interaction is crucial for transient platelet adhesion under high shear rates and does not require platelet activation to occur. As it cannot sustain stable platelet adhesion, platelets roll over the immobilized VWF surface.^(138, 151) The velocity of rolling platelets is reduced, allowing interactions that require lower shear rates to take place. Such interactions include platelet $\alpha_2\beta_1$ with collagen,⁽¹⁵³⁾ and platelet GPVI with collagen,⁽¹⁵⁴⁾ and are necessary for stable platelet adhesion and optimal VWF-dependent response.^(133, 136) Platelet GpIb α is also a mechanosensitive domain that undergoes a shear-induced conformational change upon VWF A1 binding that induces platelet activation signals.⁽¹⁵⁵⁾ Together with the $\alpha_2\beta_1$ - and GPVI-mediated signaling, they contribute to the activation of platelet $\alpha_{IIb}\beta_3$; this integrin can now form essential interactions to facilitate stable platelet adhesion, platelet aggregation, and platelet plug and thrombus formation.^(136, 140, 141, 156, 157) The ligands for $\alpha_{IIb}\beta_3$ that support inter-platelet linkage and aggregation include fibrinogen,^(138, 158-161) fibrin,⁽¹⁶²⁾ and VWF (via the C4 domain RGDS).^(158, 163-170) Activated platelets secrete more VWF that can contribute to stabilising the clot.^(129, 171)

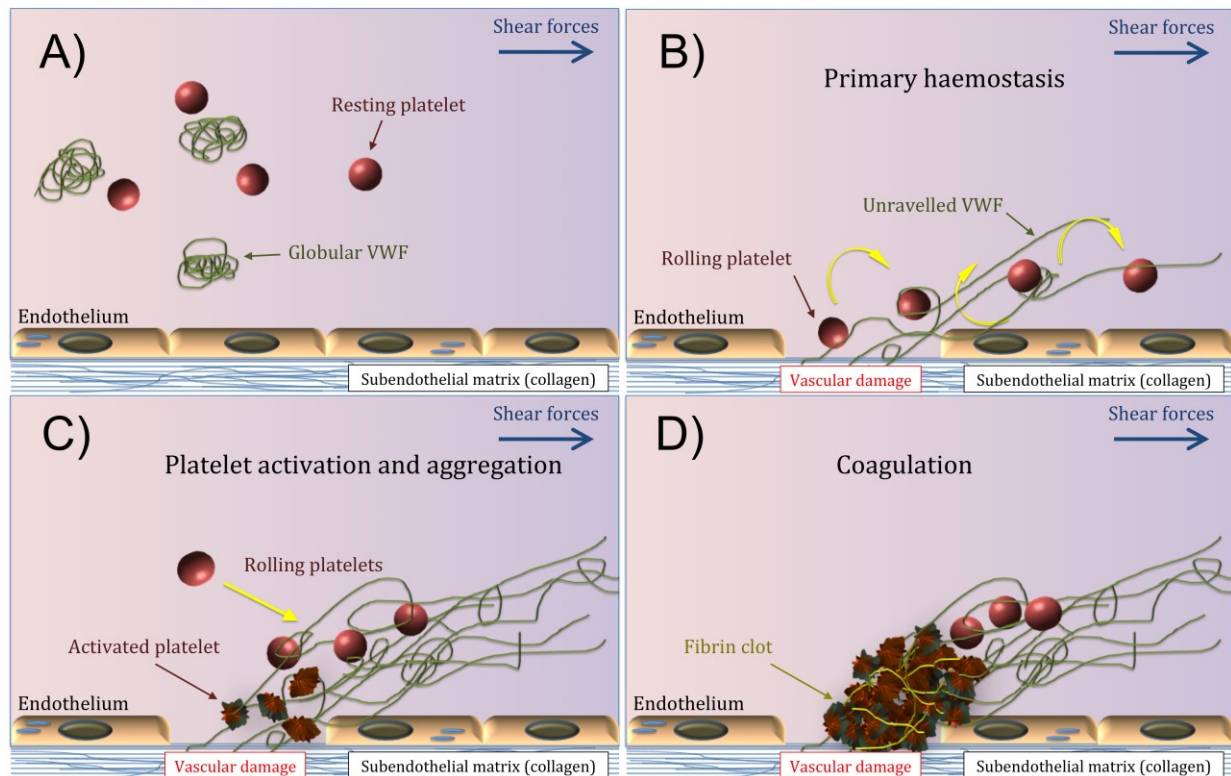


Figure 1-7 Haemostasis

(A) Healthy vessel, with intact endothelium and circulating resting platelets and globular VWF. (B) Upon vascular damage, thrombogenic extravascular molecules (eg. collagens) become exposed. Globular VWF interacts (via the VWF A1 and A3 domains) with exposed collagen, becomes immobilised, and undergoes shear-induced unravelling. This exposes the VWF A1 domain binding sites, which can interact with platelet GpIb α receptors under high shear rates and cause platelet adhesion. The VWF A1-GpIb α interactions are transient, giving platelets a rolling movement along the unravelling VWF. (C) At the inner layers of the growing thrombus, direct interactions of platelets (via GPVI and $\alpha_2\beta_1$) with collagen transduce activation signals. As a result of this, as well as activation signals following the conformational change of GpIb α , platelets become activated, change shape and release their granule content, which includes VWF (contributing to stabilising the clot) and more platelet activators (that induce platelet activation at the outer layers of the thrombus, where collagen is not available). Platelets aggregate as a result of inter-platelet interactions (crosslinking of $\alpha_{IIb}\beta_3$ by fibrinogen and VWF). More VWF immobilisation and platelet adhesion takes place at the outer layer of the thrombus. (D) Activated platelets present negatively charged phospholipid surfaces, which act as cofactors for the coagulation cascade. The end-product of coagulation, fibrin, stabilises the thrombus.

1.3.1.3 VWF multimeric size and function

The multimeric composition of VWF in circulation is heterogeneous, meaning that VWF circulates in a range of multimeric sizes (Figure 1-8). As already explained, the different secretory pathways secrete VWF of different molecular weight multimers. Endothelial-derived VWF that is constitutively secreted is of relatively lower multimeric size. In contrast, endothelial WPB-derived VWF, secreted apically, either basally or upon stimulation,^(123, 128) as well as VWF found in platelet granular content,⁽¹²⁹⁾ are of higher multimeric size (including ULVWF). From this, it is inferred that multimerisation of VWF is a heterogeneous process, yielding a range of VWF multimers. Furthermore, cleavage by ADAMTS13 upon VWF secretion and/or in circulation also creates VWF multimers of varying molecular weights (see section 1.6).

The multimeric size of VWF positively correlates with its haemostatic function; the larger the VWF multimers are, the greater the prothrombotic activity they possess. Larger multimers have higher binding affinity to collagen and extracellular matrix,^(172, 173) as well as to platelet GpIb α and $\alpha_{IIb}\beta_3$ receptors.^(139, 174, 175) Two mechanisms exist to explain this phenomenon. Firstly, larger VWF multimers have more platelet and collagen binding sites. This increases the local concentration/density of the domains available for binding, giving a multivalent nature to these interactions. Secondly (and probably most importantly), the tensile force acting on the molecule increases exponentially with increasing multimeric size;⁽⁵⁶⁾ as a result, ULVWF is more prone to unravelling, exposing its platelet-binding sites in the A1 domains. In fact, ULVWF can be adhesive even in the absence of shear stress.^(139, 176)

In pathological settings, a shift of equilibrium towards smaller or larger VWF multimers can cause bleeding disease (Von Willebrand disease, see section 1.4) or a thrombotic microangiopathy (Thrombotic Thrombocytopenic Purpura, see section 1.7.1), respectively. It is, therefore, vital that the multimeric composition of VWF is constantly regulated.

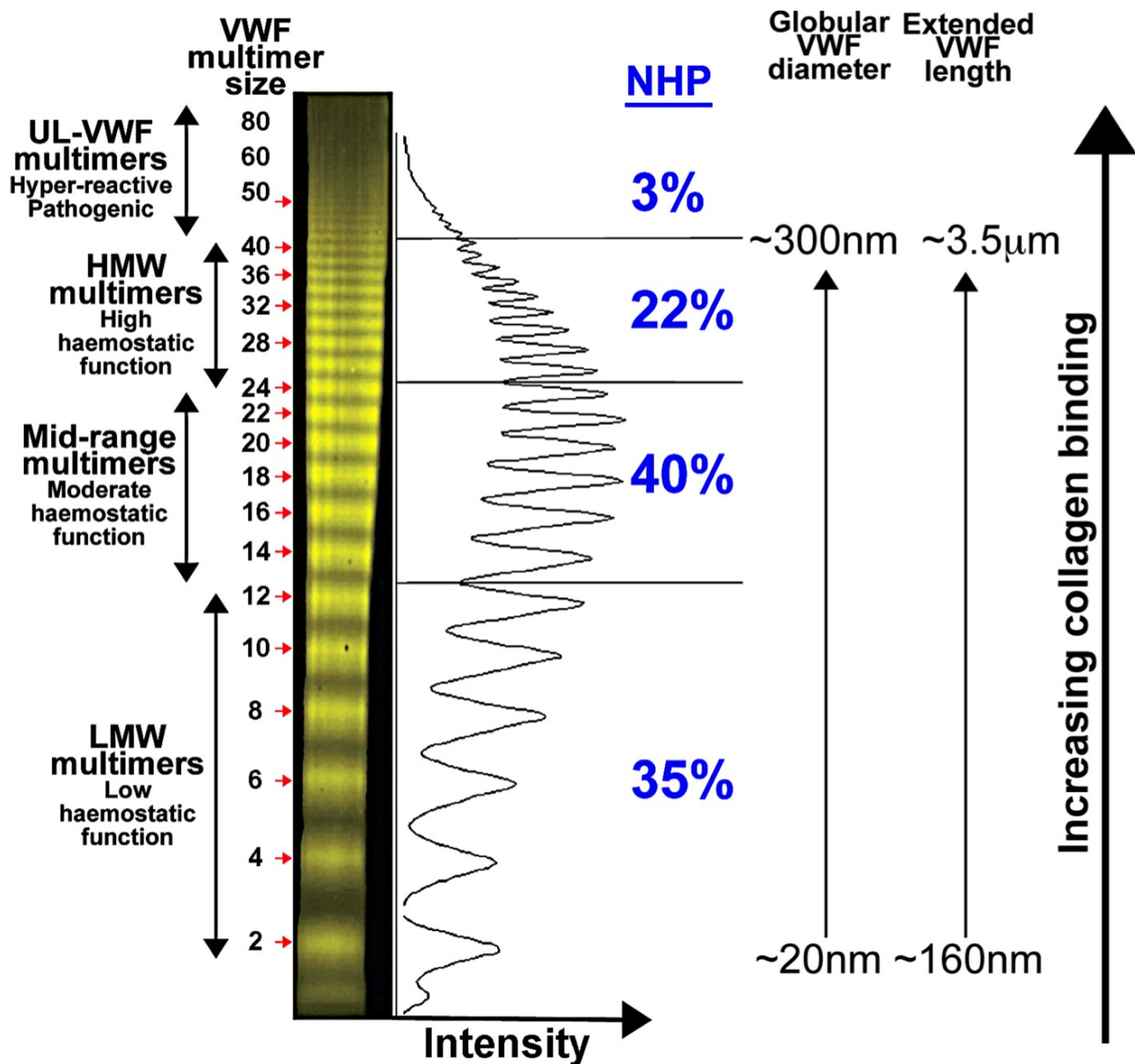


Figure 1-8 The multimeric size composition of VWF in plasma

Analysis of the multimeric composition of VWF by electrophoresis on 1% agarose gel. Each band corresponds to a linear VWF multimer molecule of different multimeric size. Densitometry was used for estimation of the relative percentage of low molecular weight VWF multimers (LMW, ~35%), mid-range molecular weight VWF multimers (~40%), higher molecular weight VWF multimers (HMW, ~22%) and unusually large VWF multimers (~3%) in circulation. VWF exists as a range of differently sized multimers, mainly as a result of the heterogeneity of the multimerisation process. Higher molecular weight multimers have increased haemostatic potential, due to possessing a greater number of collagen and platelet binding sites, as well as due to higher propensity to unravel. The figure was taken from ⁽¹⁷⁷⁾ and adapted (For permission, see Table B Appendix II).

1.3.1.4 Factor VIII

VWF is a carrier for FVIII in circulation through non-covalent high affinity bonds ($K_D=0.2-0.5\text{nM}$) involving the VWF D'D3 domains, with a stoichiometry of one FVIII molecule per 50-100 VWF subunits.^(39, 40, 178-180) This association is negatively regulated by the VWF D1D2 propeptide, as its proximity to D' may cause steric hindrance.⁽¹⁸¹⁻¹⁸³⁾ VWF binding enhances the stability and half-life of FVIII in the circulation, mainly via an immunoprotective effect, where VWF prevents the uptake of FVIII by dendritic cells.⁽¹⁸⁴⁾ Another way VWF offers stability is by preventing FVIII cleavage by proteases. VWF association prevents FVIII binding to phospholipids (phospholipid membranes act as cofactors for the proteolysis of coagulation factors),^(185, 186) and/or hinders protease binding sites, thus protecting FVIII from APC/Protein S-,^(179, 187-191) FXa- and FIXa-mediated proteolysis.^(192, 193) The physiological importance of VWF for FVIII function is apparent in the moderate haemophilia-like phenotype as a result of the functional deficiency of VWF in Von Willebrand disease (VWD)(see section 1.4). In type 3 VWD patients (quantitative deficiency of VWF), the FVIII levels are low, and are elevated following administration of VWF.^(194, 195) In type 2N VWD, mutations ablate FVIII binding to VWF, causing moderate haemophilia-like symptoms through FVIII clearance, while levels of VWF are normal.⁽¹⁹⁶⁾ Interestingly, FVIII binding to VWF seems to enhance VWF cleavage by ADAMTS13.^(197, 198)

1.4 VON WILLEBRAND DISEASE

Von Willebrand disease (VWD) refers to the inherited or acquired deficiency of VWF associated with a bleeding phenotype. The bleeding pattern is mainly mucosa-associated, including epistaxis, bruising, menorrhagia, haematoma, bleeding from minor wounds, and surgery- or trauma-associated bleeding, and varies according to sex and age, but also the severity of VWF deficiency. Unlike haemophilia, internal and joint bleeding is uncommon, but can be a type-specific symptom. VWD is divided into several types according to the causal mutations in the *VWF* gene, the pathophysiology and bleeding phenotype. Mutations are characterised by autosomal inheritance, and can have a dominant effect due to multimerisation. Rarely, VWD can be acquired; this can be due to excessive antibody-mediated VWF clearance, antibody-mediated interference with VWF functions (platelet or collagen binding), increased high molecular weight VWF multimer adsorption to abnormal cells associated with haematologic disorders (eg. myeloma cells), or excessive VWF proteolysis caused by high shear rates

associated with aortic valve stenosis (Heyde's syndrome).^(199, 200) Diagnosis of VWD is based on bleeding symptoms and family history of bleeding, as well as laboratory analysis of VWF and FVIII levels and function. Treatment strategies include administration of desmopressin to stimulate WPB release of VWF and FVIII, and administration of factor concentrate, which contains plasma-derived VWF and FVIII.⁽²⁰¹⁻²⁰³⁾

Type 1 VWD is used to describe the quantitative deficiency of VWF. It is the most common (70-80%), and at the same time most phenotypically heterogeneous, type of VWD. The majority of cases are the result of heterozygous missense *VWF* mutations that impair the transcriptional regulation, splicing, intracellular processing, trafficking, storage, secretion and/or clearance of VWF. Evidence implicates mutations in other loci that control intracellular trafficking of vesicles and exocytosis, as well as the ABO blood group, in the pathophysiology of VWD.⁽²⁰²⁾

In type 2 VWD, the VWF is functionally impaired, without necessarily a reduction in circulating levels. It is the second commonest type of VWD (20%), however, due to the existence of multiple mechanisms leading to dysfunctional VWF and pathogenicity, type 2 VWD has been further categorised into subtypes. Type 2A VWD is characterised by loss of high molecular weight VWF multimers (see Figure 1-9) in the circulation,⁽²⁰⁴⁾ as a result of mutations in the CTCK, propeptide (D1D2), D'D3 and A2 domains that impair multimer assembly, or mutations in the A2 domain that accelerate proteolysis by ADAMTS13.⁽³⁸⁾ For example, mutation of the two cysteines (C1099 and C1149) in the D3 domain, which are important in the oxidoreductase mechanism for multimerisation, lead to smaller circulating VWF multimers, due to impaired multimer assembly.^(33, 205) Heyde's syndrome is a form of acquired type 2A VWD; stenotic aortic valves increase the shear rate, enhancing the tendency of VWF A2 domain to unravel and get cleaved by ADAMTS13.⁽¹⁹⁹⁾

Mutations in the GpIb α -binding region of the VWF A1 domain enhancing VWF-platelet binding give rise to type 2B VWD.⁽³⁸⁾ Consequently, the greater tendency to form VWF-platelet complexes leads to increased VWF clearance, as well as proteolysis by ADAMTS13, thus, a reduction in the higher molecular weight multimer levels (see Figure 1-9).⁽²⁰⁴⁾ Platelet signaling defects have also been reported. A similar phenotype can arise from gain-of-function mutations in platelet GpIb α that enhance adhesion to VWF, causing the so-called platelet-type VWD. Loss-of-function mutations in the VWF A1 and A3 domains can impair the GpIb α -binding and collagen-binding capacity of VWF.⁽³⁸⁾ In this subtype, termed 2M VWD, supranormal

multimers can be found.^(204,206) Lastly, type 2N VWD is caused by mutations in, or proximal to, the FVIII-binding domains of VWF (ie. the propeptide and D'D3).⁽³⁸⁾ As already mentioned, defective binding to FVIII has implications in FVIII stability and survival, and presents as a mild haemophilia-like phenotype, while an abnormal multimeric pattern is only seen with specific mutations.⁽¹⁹⁶⁾

Type 3 VWD is the most severe, but at the same time most rare (<5%), type of VWD. Deletions or missense mutations affecting VWF synthesis account for essentially undetectable VWF (<5%)(see Figure 1-9), and suboptimally low FVIII (<10%) levels. Bleeding symptoms in this type of VWD lay at the most severe end of the spectrum, and include internal and joint bleeding.⁽²⁰¹⁾

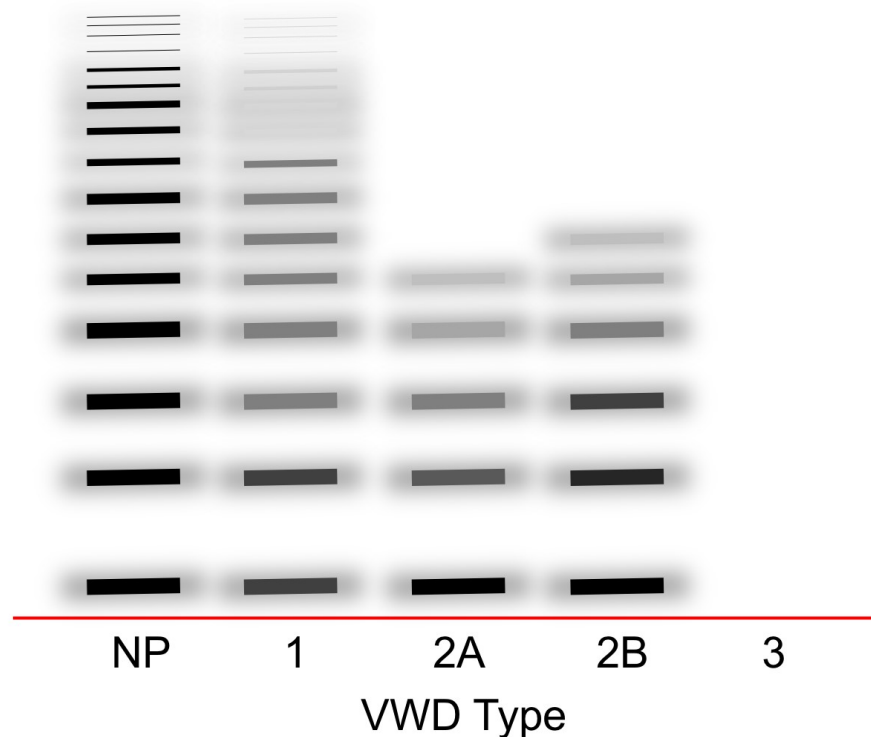


Figure 1-9 Schematic representation of the multimeric pattern of VWF in the plasma of different types of VWD.

The multimeric profile of VWF in plasma can be analysed by agarose/SDS gel electrophoresis and Western blotting. On the left is the multimeric pattern of VWF in normal plasma. Type 1 VWD is caused by quantitative deficiency of VWF, which can form normal multimers. In type 2A VWD, impaired multimer assembly or accelerated ADAMTS13-mediated proteolysis causes loss of high molecular weight multimers. Type 2B VWD is associated with reduced higher molecular weight VWF multimers due to mutations in the VWF A1 domain that enhance GpIb α -VWF binding, and, hence, VWF clearance and ADAMTS13 cleavage. Type 3 VWD arises from severe quantitative deficiency of VWF, which leads to undetectable levels of VWF in plasma.

1.5 ADAMTS13 MOLECULE/STRUCTURE

1.5.1 The discovery

It took around two decades to establish that VWF multimeric size is regulated by proteolysis in the circulation, and that certain bleeding (VWD) and thrombotic (thrombotic thrombocytopenic purpura, TTP) diseases are associated with an imbalance in the regulation of VWF multimeric size, and for ADAMTS13 to be recognised as the VWF-cleaving protease.

Initially, it was noted that VWF inside endothelial cells is of higher molecular weight/multimeric size than VWF circulating in plasma. This soon led to the understanding that a significant proportion of VWF was released as ULVWF, which was subsequently cleaved into smaller multimers in the circulation. Abnormalities in the proteolysis of circulating VWF were associated with VWD.⁽²⁰⁷⁻²¹²⁾ At the time, it had been known that another pathology of thrombotic phenotype, TTP (see section 1.7.1), was associated with the presence of circulating ULVWF.^(213,214) In 1990, the cleavage site in VWF was identified, and 140kDa and 176kDa fragments of VWF were shown to exist as a result of cleavage.⁽²¹⁵⁾ The importance of shear stress and calcium in VWF cleavage was also shown.⁽²¹⁶⁾

The first partial purification of the VWF-cleaving protease was published by two groups, Furlan *et al* and Tsai *et al*, in 1996. The isolated protease was dependent on the presence of divalent metal ions and shear stress, and cleaved VWF at the Y1605-M1606 bond, corroborating earlier results.^(217,218) The same groups later reported the functional deficiency of the VWF-cleaving protease,⁽²¹⁹⁾ as well as the presence of inhibitory autoantibodies,⁽²²⁰⁻²²²⁾ in TTP patient plasma.

Eventually, the VWF-cleaving protease was purified, its primary structure sequenced, and its gene found, identifying it as ADAMTS13.⁽²²³⁻²²⁵⁾ Since its discovery, ADAMTS13 has generated a wave of extensive research on its structure, its mode of action, its involvement in pathology and its potential therapeutic role in cardiovascular disease.

1.5.2 ADAMTS13 gene expression

ADAMTS13 belongs to the ADAMTS family of metalloproteases, which consists of 19 genes in humans.⁽²²⁶⁾ *ADAMTS13* is located on chromosome 9q34 and has a size of 37kb. It consists of 29 exons that are transcribed into a 4.7kb mRNA. Several alternatively spliced variants have been described, however, whether these are translated has not been determined.^(225, 227-229)

ADAMTS13 is mainly expressed in the hepatic stellate cells of the liver,^(228, 229) but expression in other cells, like cultured endothelial cells,^(230, 231) platelets,⁽²³²⁾ and glomerular podocytes,⁽²³³⁾ has also been reported.

1.5.3 ADAMTS13 domain organisation and structure

ADAMTS13 is translated into a precursor 1427aa protein and has a modular, multidomain structure that resembles that of other ADAMTS family members.⁽²³⁴⁾ From N- to C-terminus, ADAMTS13 possesses a signal peptide, a propeptide, the catalytic Metalloprotease (MP) domain, a Disintegrin-like (Dis) domain, a Thrombospondin type 1 repeat (TSP1), a Cysteine-rich (Cys-rich) domain, a Spacer domain, seven more TSP domains (TSP2-8) and two CUB (Complement components C1r/C1s, sea urchin *Uegf* protein, Bone morphogenic protein-1) domains (see Figure 1-10A).⁽²²⁷⁾ The crystal structure of the entire enzyme is yet to be solved. In 2009, the crystal structure of the proximal non-catalytic domains of ADAMTS13 (Dis to Spacer domains, DTCS) was published.⁽²³⁵⁾ Even though the MP and Dis domains of the ADAMTS1, -4 and -5 had been crystallised at the time,⁽²³⁶⁻²³⁸⁾ this was the first time that the crystal structure of the TSP1, Cys-rich and Spacer domains in any ADAMTS family member was solved.⁽²³⁵⁾ Based on that, and on homology modelling with other ADAMTS family proteases, a model structure for the MP domain fused to the crystal structure of the DTCS domains was created (Figure 1-10B).⁽²³⁹⁾

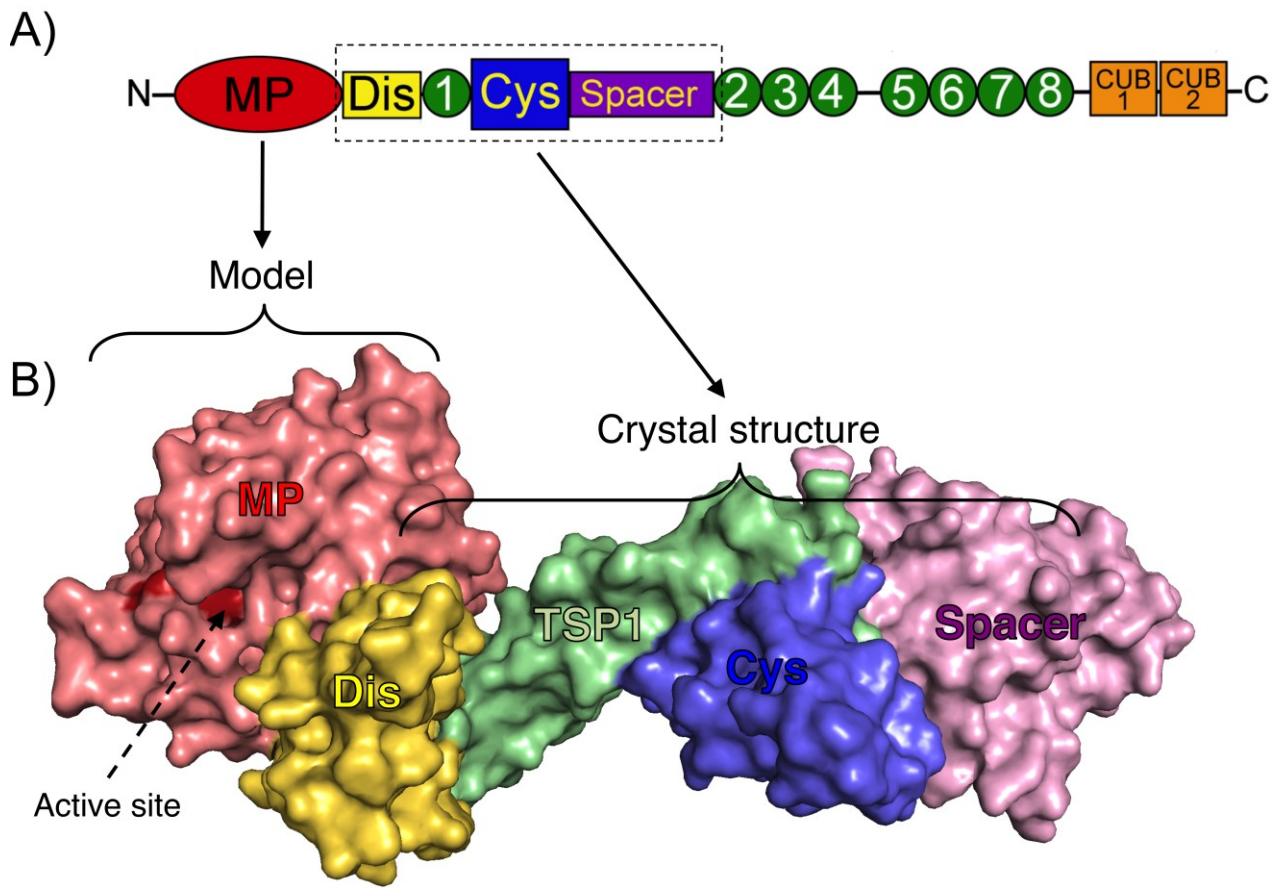


Figure 1-10 ADAMTS13 domain organisation.

(A) Domain organisation of ADAMTS13. From the N-terminus: the Metalloprotease (MP), the Disintegrin-like (Dis), one Thrombospondin type 1 repeat (TSP1), the Cysteine-rich (Cys-rich), the Spacer, seven more TSP domains (TSP2-8) and two CUB domains. All members of the ADAMTS family of metalloproteases possess the domains MP to Spacer. (B) The model structure of the MP domain, fused to the crystal structure of the Dis to Spacer domains, solved by Akiyama *et al.*⁽²³⁵⁾

1.5.3.1 Metalloprotease domain

Figure 1-11 shows the model of the ADAMTS13 MP domain, as predicted based on homology to ADAMTS1, -4 and -5, which have been crystallised.⁽²³⁶⁻²³⁸⁾

The ADAMTS13 MP domain contains structural elements that are conserved in the Reprolysin family of proteases.⁽²⁴⁰⁾ It has the Zn²⁺-binding motif, HEXHXXG/N/SXXHD, in which X is any residue and the three His residues (H224, H228, H234 in ADAMTS13) coordinate a Zn²⁺ ion.⁽²²⁶⁾ Zn²⁺ is essential for activity, and forms part of the catalytic centre.⁽²⁴¹⁾ Also perfectly conserved in this motif, a catalytic Glu residue (E225 in ADAMTS13) is indispensable in forming the active site and coordinating a water molecule for the proteolytic hydrolysis reaction to occur.^(242, 243) Adjacent to the Zn²⁺-binding motif, a perfectly conserved Met residue (M249 in ADAMTS13) forms a characteristic turn ('Met-turn'); this is a structural element that preserves the structural integrity of this family of enzymes.^(226, 227, 244)

For optimal activity, ADAMTS13 requires divalent cations. Beyond the Zn²⁺ in the active site, Ca²⁺ is also functionally important.^(218, 245) A low affinity Ca²⁺ cluster site exists, where two Ca²⁺ are predicted to be coordinated by the residues E83, D166, D173, C281 and D284, all of which are perfectly conserved in other ADAMTS proteases, like ADAMTS1, -4 and -5. A second, high-affinity Ca²⁺-binding site exists, where one Ca²⁺ is predicted to be coordinated by E212 and D182. The residues E184 and R190 are predicted to form a salt bridge, stabilising a loop that shapes this Ca²⁺ binding site. In ADAMTS1, -4 and -5 there are Cys residues at these positions, which form an analogous loop by a disulphide bond, and not a salt bridge. This Ca²⁺-binding site is adjacent to the active site and may offer conformational stability.^(241, 246, 247)

The MP domain alone does not bind nor cleave VWF specifically⁽²⁴⁸⁻²⁵¹⁾ as proteolysis is dependent on multiple interactions between exosites in the ADAMTS13 non-catalytic domains (Dis to Spacer domains) and the A2 domain of VWF.⁽²³⁹⁾ Nevertheless, the MP domain contains subsites that form low affinity interactions with residues around the cleavage site of VWF, which are crucial in directing proteolysis. Substitutions of the residues D187, R190 and R193 reduce proteolysis suggesting important contribution to the structure or function of ADAMTS13.⁽²⁴²⁾ The S1' subsite, which accommodates the P1' residue M1606 (where n' is the nth residue C-terminal to the scissile bond), is probably shaped by the residues D252-P256.⁽²⁴²⁾ Two more subsites, the S1 and the S3, were proposed to exist in the MP domain; the S1 is believed to be formed by L151/V195 and to accommodate the P1 residue Y1605 (where n is

the n^{th} residue N-terminal to the scissile bond), and the S3 to be formed by L198/L232/L274 and to accommodate the P3 residue L1603.⁽²⁵²⁾

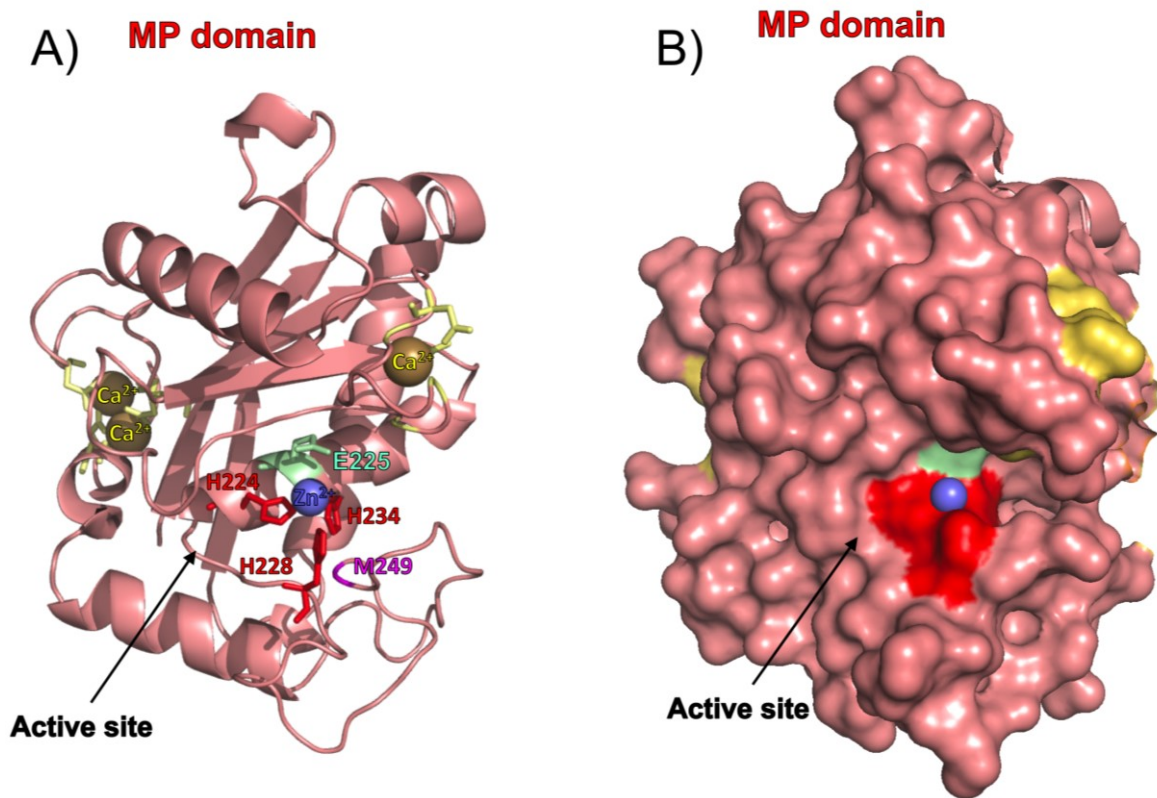


Figure 1-11 ADAMTS13 MP domain model

The low and high affinity Ca²⁺-binding sites are shown in yellow. The H224/H228/H234 (red) that coordinate the Zn²⁺ (blue) together with the catalytic E225 (teal), form the active site. The M249 that forms the characteristic 'Met-turn' is shown in magenta. (A) Cartoon view. (B) Surface view.

1.5.3.2 Disintegrin-like domain

The crystal structures of the ADAMTS1, -4 and -5 Dis domains were solved first,⁽²³⁶⁻²³⁸⁾ then the Dis domain of ADAMTS13 (residues 306-383) followed (Figure 1-12).^(235, 253) What is intriguing about the Dis domains of these ADAMTS proteases is that, despite the domain name, there is no homology to the Dis domain of ADAM proteases. Instead, they show a similar fold to the Cys-rich domains of ADAM proteases. Furthermore, while there is low sequence homology between ADAMTS13 Dis and Cys-rich domains, they have similar tertiary structures. ADAMTS13 Dis domain is made up of an α -helix, two pairs of antiparallel β -sheets and four disulphide bridges. There are also two poorly conserved regions, the variable (V)-loop and the hypervariable region (HVR).^(235, 254)

The Dis domain is functionally important for ADAMTS13 activity. While the MP domain by itself is not functional, addition of just the Dis domain is enough to restore specific, though inefficient, VWF cleavage.^(249, 251, 255) Mutagenesis of the variable regions identified the interaction between the R349 residue in the ADAMTS13 Dis domain HVR, and the D1614 located in the VWF A2 domain, which is part of a functional exosite interaction involving the Dis domain, that is important for VWF proteolysis by ADAMTS13.^(250, 256) Mutations in the vicinity of the Dis domain exosite, namely R349C and P353L, have been shown to be associated with the functional deficiency of ADAMTS13 in congenital TTP (see section 1.7.1).⁽²⁵⁷⁻²⁵⁹⁾

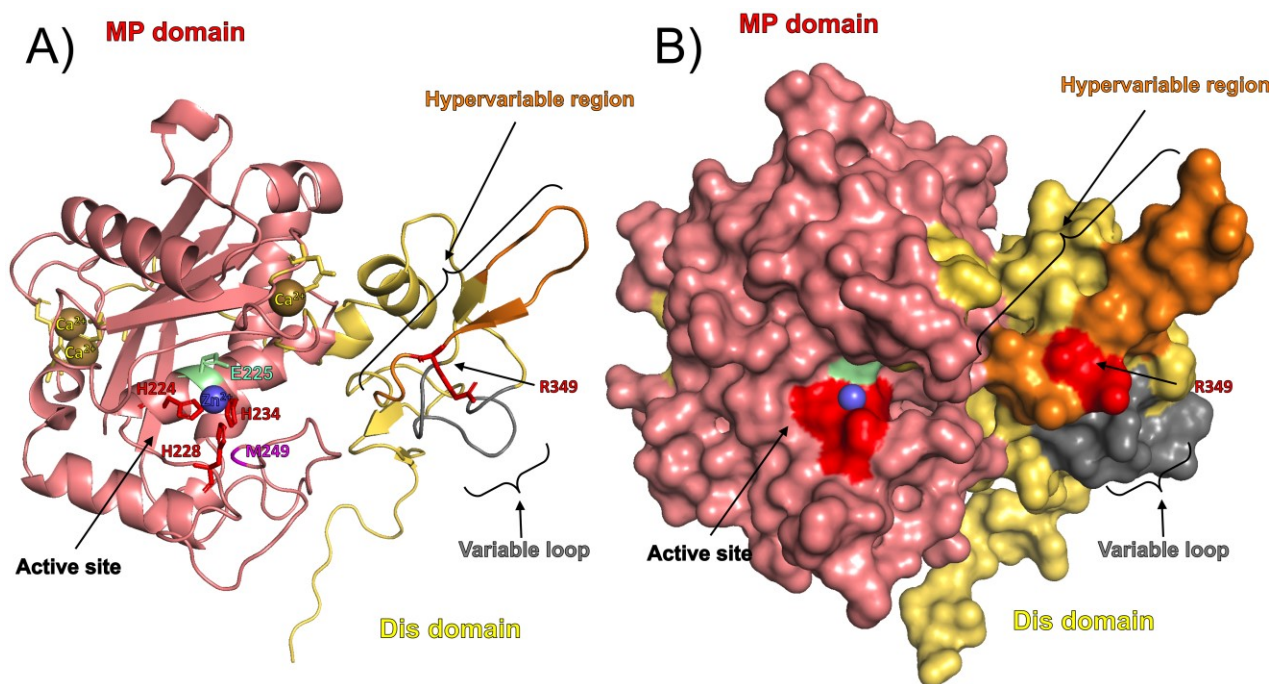


Figure 1-12 ADAMTS13 MP-Dis domains

The ADAMTS13 MP domain model (see Figure 1-11) was fused to the crystal structure of the Dis domain (yellow).⁽²³⁵⁾ The Dis domain is made up of an α -helix and two pairs of antiparallel β -sheets. The R349 residue (red) that interacts with the D1614 of the VWF A2 domain is located in the hypervariable region (orange). The variable loop is shown in grey. (A) Cartoon view. (B) Surface view.

1.5.3.3 Thrombospondin-type 1 repeats

All ADAMTS family proteases have their first TSP domain between the Dis and Cys-rich domains, but have a variable number of TSP domains C-terminal to the Spacer domain, with the exception of ADAMTS4, which has none.⁽²²⁶⁾ ADAMTS13 has eight TSP domains (TSP1-8), however only the crystal structure of TSP1 is available. It is an elongated domain, which resembles the structure of the second TSP in thrombospondin type 1 (which gave the name to the TSP domains). It is made up of three long and twisted strands, which are positioned anti-parallel to each other. There are two pairs of disulphide bonds in the structure. Hydrogen bonding involving three Ser residues stabilises the first strand. The remaining two strands fold into a β -sheet, which interacts with a β -sheet in the Cys-rich domain. The core of the TSP1 domain is made up of Trp, Arg and hydrophobic residues and is stabilised by two pairs of disulphide bonds; it was, thus, termed CWR-layered core. The sequence of the CWR-layered core is almost perfectly conserved between the TSPs of thrombospondin type 1 and the TSPs of the ADAMTS family of proteases.^(235, 260)

In other ADAMTS family members, like ADAMTS1, the TSPs mediate interactions with the extracellular matrix.⁽²⁶¹⁾ In ADAMTS4, the TSP is important for glycosaminoglycan binding in aggrecan, as well as for the enzymatic activity.⁽²⁶²⁾ For ADAMTS13, the data on the role of TSPs has not been very consistent. In the earlier studies using ADAMTS13 truncation variants, no obvious contribution of any of the TSPs could be demonstrated to the secretion of the enzyme, nor to the specificity and rate of cleavage.^(263, 264) A role in substrate binding and cleavage was later proposed for the TSP1, as it was shown to enhance interaction with VWF.^(248, 249) Using truncation variants of both ADAMTS13 and VWF, it was proposed that TSP1 interacts with the region Q1624-V1630 in the VWF A2 domain.⁽²⁵⁵⁾ Whether there is a functional exosite in the TSP1, which residues are implicated in the interaction with VWF, and how much this interaction contributes to VWF proteolysis, remains to be definitively concluded. Current thinking supports that TSP1 provides stability and spacing to the Dis/Cys-rich domains.

The role of the TSP2-8 has mostly been investigated together with the CUB domains, using ADAMTS13 C-terminal truncation variants that lack the domains C-terminal to the Spacer domain. The TSP2-8 region contains three linkers (L1 after the TSP2, L2 after the TSP4 and L3 after the TSP8), which give flexibility to these domains.⁽²⁶⁵⁾ This flexibility is important in allowing ADAMTS13 to exist in both folded and extended conformations that have functional

implications.^(43, 46) TSP8 was recently shown to be a major binding site for D4-CK domains in globular VWF; this interaction is believed to cause ADAMTS13 to transition from a folded to an extended fold⁽⁴⁴⁾ and will be covered in more detail in section 1.8.1.

1.5.3.4 Cysteine-rich domain

As already mentioned, the structure of the Cys-rich domain of ADAMTS13 is similar to its Dis domain (Figure 1-13); they both contain an α -helix, a V-loop, HVR and two pairs of antiparallel β -sheets. The Cys-rich domain differs in that it possesses a Cys-rich domain-specific protruding (P)-loop (12 residues), a Cys-rich unique (U)-loop, its V-loop is well defined in the crystal structure (in contrast to the disordered V-loop in the Dis domain), and it possesses three disulphide bonds (instead of four seen in the Dis domain).⁽²³⁵⁾

Several studies have shown the importance of the Cys-rich domain in ADAMTS13 for the specificity of VWF cleavage, which depends on the I1642-R1659 region in the VWF A2 domain, C-terminal to the cleavage site.^(248, 249, 251, 255, 263) De Groot *et al* identified the exosite in the Cys-rich domain non-conserved region (G471-V474), and its complementary binding site in the VWF A2 domain, which is formed by the hydrophobic residues I1642, W1644, I1649, L1650, I1651.⁽²⁶⁶⁾ Interestingly, the polymorphism P475S in ADAMTS13, immediately adjacent to the exosite identified, causes a 16% reduction in ADAMTS13 plasma levels, and a reduced affinity for VWF, which compromises the rate of proteolysis. The crystal structure of this polymorphic Cys-rich domain, solved by Akiyama *et al*, shows that the structure of the V-loop (where the identified exosite G471-V474 and the polymorphic residue P475 are located) is significantly altered, as compared to the wild-type domain; these structural alterations are in close proximity to the interacting exosite and may explain both the reduced affinity for VWF, as well as the reduced plasma levels due to misfolding.⁽²⁵³⁾

1.5.3.5 Spacer domain

The Spacer domain of ADAMTS13 contains no cysteines and is not homologous to any other known structural motif. It is made up of ten β -strands that form two anti-parallel β -sheets. Its globular, jelly-roll fold brings its two termini in close proximity (Figure 1-13).⁽²³⁵⁾

The Spacer domain contains an exosite that interacts with the VWF A2 domain to facilitate proteolysis. Creating truncation constructs of ADAMTS13 lacking the Spacer domain compromises cleavage significantly, by losing its affinity for VWF. In acquired TTP (see section

1.7.1) autoantibodies against the Spacer domain directly inhibit, or enhance the clearance of, ADAMTS13. Mapping the epitope of these antibodies in combination with mutagenesis, led to the contention that the residues R659, R660, Y661 and Y665 form an exosite in the ADAMTS13 Spacer domain that binds to the VWF A2 domain and promotes proteolysis.^(267, 268) The Spacer binding region, E1660-R1668, in the VWF A2 domain was the earliest exosite binding site to be discovered.^(248, 249, 251, 255, 263, 264, 269) Recent studies have also demonstrated an interaction between the Spacer domain and the C-terminal CUB domains, which gives ADAMTS13 a folded conformation. Disruption of this interaction was shown to extend the conformation of ADAMTS13 and enhance its activity by ~2-10 times (see section 1.8.1).^(43, 44, 46) That the Spacer domain contains the major epitopes targeted by autoantibodies in acquired TTP is, perhaps, explained by the presence of cryptic epitopes in this domain.^(263, 268, 270-274)

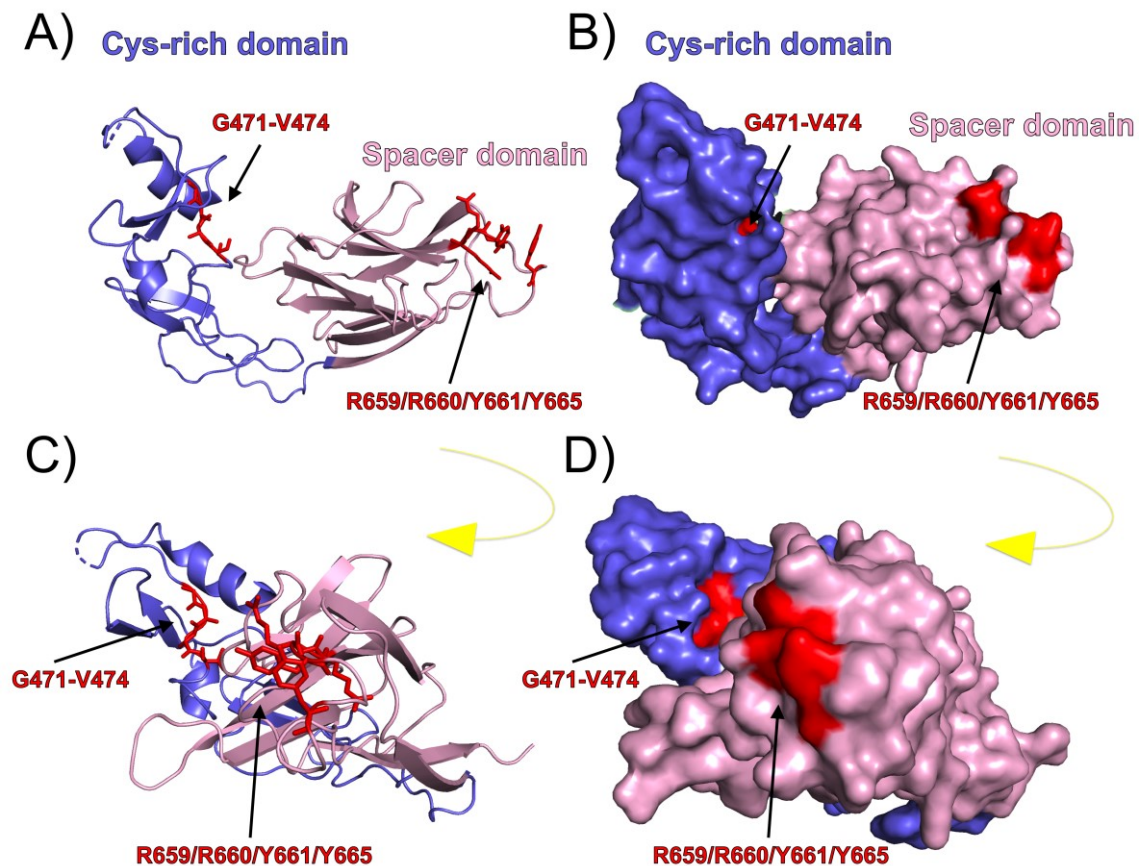


Figure 1-13 ADAMTS13 Cys-rich and Spacer domains

The crystal structures were solved by Akiyama *et al.*⁽²³⁵⁾ The Cys-rich domain (blue) is made up of an α -helix and two pairs of anti-parallel β -sheets. The hydrophobic region G471-V474 (red) interacts with the VWF A2 domain. The Spacer domain (pink) contains ten β -strands that form two anti-parallel β -sheets. The solvent exposed residues R659/R660/Y661/Y665 (red) were proposed to interact with the VWF A2 domain. (A) Cartoon view. (B) Surface view. (C) Cartoon view, rotated. (D) Surface view, rotated.

1.5.3.6 CUB domains

The CUB domains are ~110aa long and, based on crystallization studies, they contain two pairs of disulphide bonds, a Ca²⁺-binding site, and are arranged in two β -sheets.⁽²⁷⁵⁻²⁷⁸⁾ ADAMTS13 is unique amongst the ADAMTS family members to possess CUB domains.⁽²²⁶⁾ The two CUB domains of ADAMTS13 are located at the C-terminus of the enzyme.

As already mentioned, ADAMTS13 CUB domains were proposed to interact with the Spacer domain of the same molecule, maintaining the folded conformation of ADAMTS13, which, when disrupted, enhances the activity of ADAMTS13. Binding of the TSP8-CUB2 domains of ADAMTS13 to the VWF D4-CK domains was proposed to interfere with the Spacer-CUB interaction and unfold ADAMTS13 to its more active, extended conformation (see section 1.8.1).^(43, 44, 46)

The CUB domains of ADAMTS13 may be implicated in intracellular trafficking and secretion. In endothelial cells, apical secretion was demonstrated; a nucleotide insertion, 4143-4144insA, that deletes the second CUB domain results in reduced secretion with amended polarity.⁽²³¹⁾ The same insertion occurs in congenital TTP.⁽²⁷⁹⁾

1.5.4 ADAMTS13 biosynthesis, post-translational modification and secretion

ADAMTS13 undergoes extensive post-translational modifications, with at least one glycan present in each of its domains. Ten N-linked glycans, mostly of the complex type, were reported; two in the MP, one in the Cys-rich, three in the Spacer, one in the TSP2, one in the TSP4 and two in the CUB domains. Five out of a total of six O-linked glycans identified are attached to a Ser residue, and are present in the MP domain, TSP4/5 linker, TSP6 and CUB domains. One O-linked glycan that is attached to a Thr residue was found in the Dis domain. There are eight O-fucosylation sites, one in the Dis domain, and the rest in the TSP domains (except TSP4).^(280, 281) Altering the O-fucosylation sites in the TSP domains was shown to impair ADAMTS13 secretion, highlighting that correct post-translational modifications and folding of the TSP domains has functional implications.⁽²⁸²⁾ Lastly, three C-mannosylation sites are present in the TSP1, TSP4/5 linker and TSP7.^(280, 281) Modification of the C-mannosylation site in the TSP1 reduced the expression and proteolytic activity of ADAMTS13.⁽²⁸³⁾ Glycosylated ADAMTS13 is secreted into the blood and circulates at a concentration of 5nM (1 μ g/ml).^(264, 284-286)

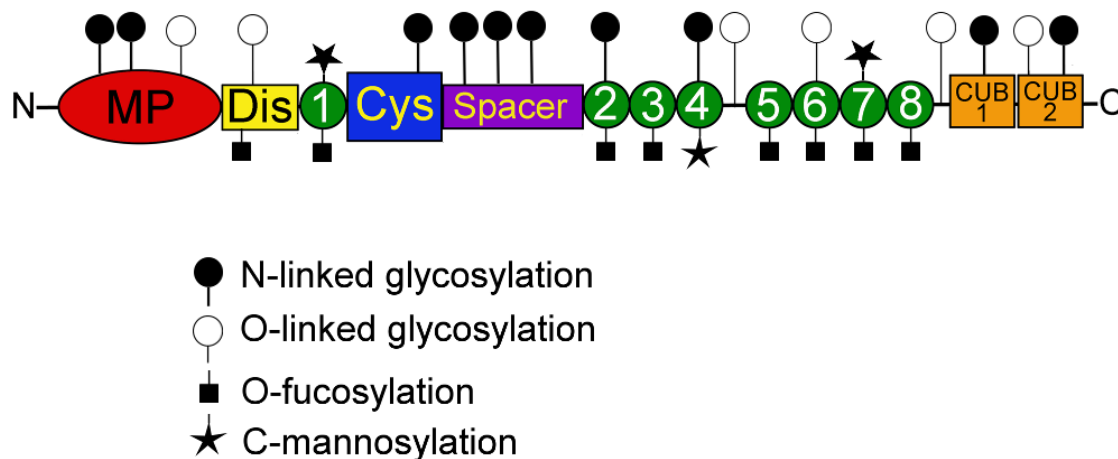


Figure 1-14 Post-translational modifications of ADAMTS13

ADAMTS13 possesses ten N-linked glycans, six O-linked glycans, eight O-fucosylation sites and three C-mannosylation sites. The figure was developed based on the results of ^(280, 281).

1.6 ADAMTS13 FUNCTION

The physiological function of plasma ADAMTS13 is to cleave VWF, and in so doing regulate its multimeric size. As already explained, the multimeric size of VWF correlates positively with its haemostatic potential. Therefore, dysregulation of VWF cleavage can shift the equilibrium towards VWF species of higher or lower multimeric sizes than physiological, causing TTP or VWD, respectively.

VWF circulates in a folded, globular conformation, and undergoes a shear-dependent extension, which is a prerequisite for platelet adhesion and primary haemostasis to occur. Platelet adhesion is dependent on VWF unfolding for abolishing the inter-domain steric hindrance, to expose the Gplb α binding sites in the VWF A1 domain. Intriguingly, the proteolytic activity of ADAMTS13 is also dependent on shear-induced VWF unfolding, however, a distinct molecular mechanism exists. This is the ability of the A2 domain to undergo a shear-dependent conformational transition, which extends the previously globular domain, to expose binding sites that interact with different exosites in ADAMTS13, directing proteolysis.

Additionally, the scissile bond is cryptic within the globular A2 domain of VWF and is only exposed upon domain unravelling (see section 1.8.2).⁽²³⁹⁾

Factors that promote VWF unravelling enhance its proteolysis by ADAMTS13. Such factors are fluid shear stress on VWF (the physiological mechanism that unravels VWF),⁽²¹⁶⁾ as well as chemical (non-physiological) denaturants. *In vitro* proteolysis of multimeric VWF is minimal unless flow or chemical denaturants are provided.^(217, 218) Platelets enhance the forces exerted on VWF, contributing to its shear-dependent unravelling. VWF proteolysis is also enhanced by the presence of FVIII. As expected, platelets, FVIII and fluid shear act synergistically to enhance VWF proteolysis by ADAMTS13.^(197, 287-289)

Since VWF unravelling is a prerequisite for proteolysis, it follows that ADAMTS13 activity is location-specific, and occurring at physiological scenarios, where VWF is expected to have a higher propensity to favour an extended, as opposed to a globular, conformation:

a) During ULVWF multimer secretion from the endothelial WPB, unravelling as a result of the shear stress causes VWF string formation. The string-like VWF structures can potentially adhere to, and become decorated with, platelets in a GpIb α -dependent fashion. ADAMTS13 interacts with the ULVWF strings, cleaving and releasing heterogeneously smaller VWF multimers; this cleavage of WPB-derived VWF is believed to be a physiological mechanism of multimeric size regulation at secretion.^(125, 290-295) It is important that ULVWF is processed as soon as it is secreted, as its high multimeric size makes it hyperactive and able to cause platelet adhesion/aggregation spontaneously, even under low shear.⁽¹⁷⁶⁾

b) ULVWF that is not cleaved upon secretion, as well as the higher molecular weight VWF multimers that arise as a result of ULVWF cleavage, circulate in a globular conformation but have higher propensity to unravel and cause platelet adhesion. Spontaneous, fluid-phase unravelling can be more common at locations of relatively elevated shear stresses, like the microvasculature, in the absence of collagen, surface interactions or other haemostatic agonists. Proteolysis by ADAMTS13 is thought to be protective against the generation of VWF-platelet aggregates resulting from the spontaneous unravelling of VWF at these sites.⁽²³⁹⁾ In TTP, ULVWF can persist (no cleavage at ULVWF secretion due to ADAMTS13 deficiency) and cause pathological thrombi in the microvasculature (also due to deficient regulation of spontaneous ULVWF unravelling by ADAMTS13 at these sites).⁽²⁹⁶⁾ Fluid-phase platelet-VWF complexes enhance VWF proteolysis, independent of surface interactions. While this is likely to

act as a feedback inhibition mechanism for regulating spontaneous platelet adhesion at these sites, it is also believed to contribute to maintaining the physiological VWF multimeric composition in circulation.⁽²⁸⁸⁾

c) ADAMTS13 regulates primary haemostasis at the site of vascular damage, where collagen initiates the VWF-dependent platelet adhesion. At the initial stages of primary haemostasis after deendothelisation and exposure of the subendothelial matrix, ADAMTS13 regulates the adhesion of resting platelets to immobilised VWF. This could be due to cleavage of subendothelial (basolaterally secreted) VWF, or regulation of soluble VWF immobilisation to the site.⁽²⁹²⁾ The platelet plug grows outwards by repeated VWF immobilisation and further platelet adhesion. Concurrently to this, coagulation, activation of platelets and thrombin generation in the core of the thrombus encourage VWF-independent platelet adhesion/aggregation, making ADAMTS13 regulation of the growing thrombus irrelevant at these inner layers. With further growth of the thrombus size, away from the site of vessel injury, the local concentration of thrombin and platelet activators is reduced, and the effect of the endothelial cell-associated anticoagulant pathways is more potent. At these sites ADAMTS13 can regulate the size of the growing thrombus, to avoid vessel occlusion and thrombosis, by cleaving VWF and inhibiting further VWF-dependent platelet adhesion. This regulatory role of ADAMTS13 was illustrated *in vitro*, by flowing blood over immobilised collagen, which showed ADAMTS13-specific regulation of the platelet plug growth size during thrombogenesis.^(239, 297, 298) The ability of ADAMTS13 to limit thrombus growth was also shown *in vivo*; ADAMTS13 deficiency in mice significantly reduces the time for thrombus formation and vessel occlusion following arteriolar injury.⁽²⁹²⁾

Figure 1-15 illustrates ADAMTS13 function in circulation.

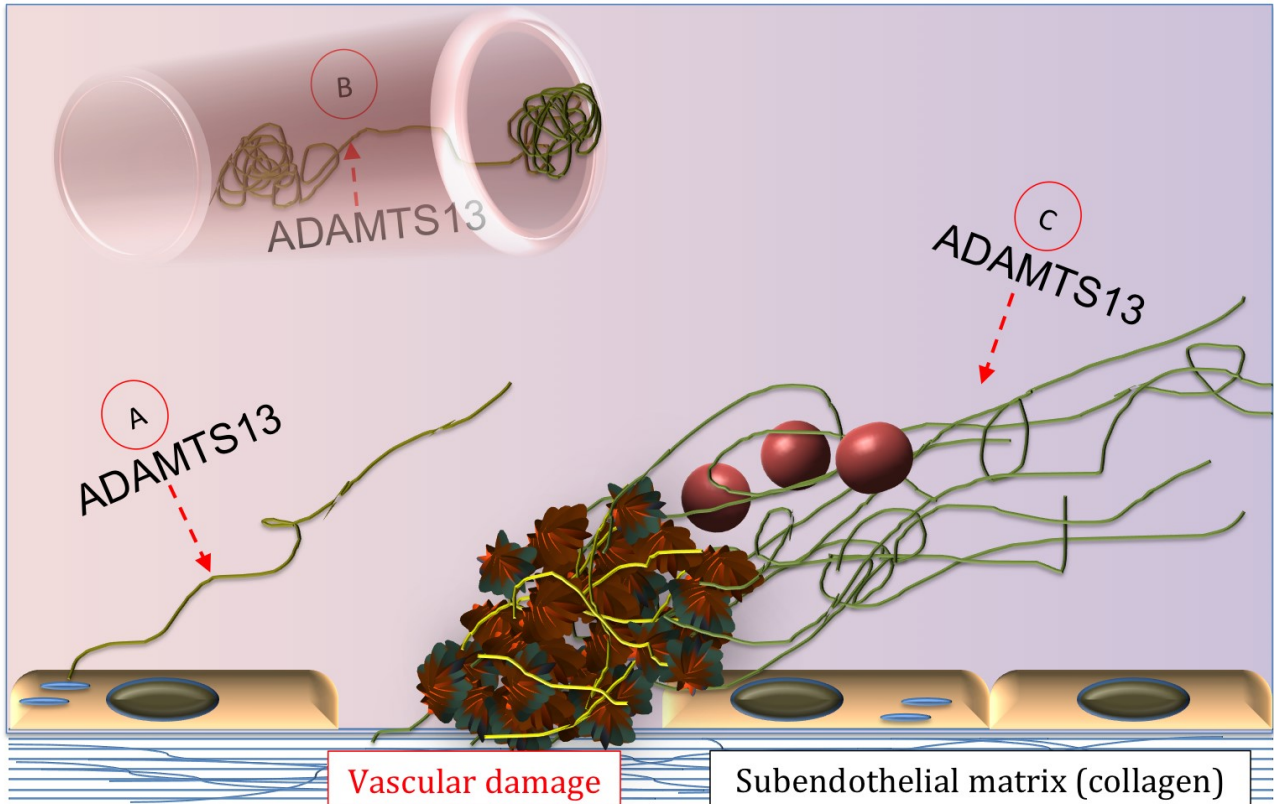


Figure 1-15 Location-specific VWF unravelling promotes cleavage by ADAMTS13

(A) WPB-derived ULVWF strings adhere to the endothelium upon secretion and are subject to cleavage by ADAMTS13. (B) In the microvasculature, elevated shear stress causes spontaneous fluid-phase unravelling of higher molecular weight VWF species, allowing cleavage by ADAMTS13. Deficiency of the protective effect of ADAMTS13 in TTP results in the accumulation of pathological thrombi. (C) ADAMTS13 regulates primary haemostasis by preventing the growth of the thrombus away from the site of vessel damage.

1.7 ADAMTS13 IN DISEASE

1.7.1 Thrombotic thrombocytopenic purpura

TTP was described almost a century ago, and the original definition included a pentad of clinical and laboratory characteristics: fever, microangiopathic haemolytic anaemia with schistocytosis, thrombocytopenia, neurological complications, renal failure.⁽²⁹⁹⁻³⁰¹⁾ It is caused by the functional deficiency of ADAMTS13, resulting in the persistence of hyperactive ULVWF species that cause excessive platelet adhesion and aggregation, and, consequently, microvascular thrombosis.

The appearance of ULVWF had been associated with TTP from as early as 1982, many years before the discovery of ADAMTS13.⁽²¹³⁾ It was not until around 15 years later that the functional deficiency of ADAMTS13 (then referred to as the VWF-cleaving protease) was linked to the impaired processing of ULVWF released from the endothelium.^(217, 219, 221) The pentad of clinical findings has been the basis of diagnosis, until recently, when greater understanding of the pathophysiology led to the inclusion of severe ADAMTS13 deficiency of <10% in the definition. In fact, now severe ADAMTS13 deficiency of <10% specifically diagnoses TTP.⁽³⁰²⁻³⁰⁴⁾

The incidence of TTP is rare, ~6 cases per million in the UK.⁽³⁰⁵⁾ Untreated, 90% of the cases lead to death, which can be as early as within 24 hours from presentation. This was the mortality rate for many years until it was understood that TTP could be treated by plasma therapy, which impressively reduced the mortality rate to 10-20%.⁽³⁰⁵⁻³⁰⁷⁾ TTP can be either congenital (cTTP) or acquired (aTTP).

1.7.1.1 Congenital TTP

cTTP, also known as Upshaw-Schulman syndrome,^(308, 309) is the most rare type of TTP, accounting for only ~5% of all cases.^(305, 310) It is caused by mutations in the *ADAMTS13* gene that impair the secretion and activity of ADAMTS13.⁽³¹¹⁻³¹⁵⁾ The first report of mutations in the *ADAMTS13* gene, and their association to TTP, was published in 2001.⁽²²⁵⁾ Since then, >100 mutations have been reported in the coding regions for all domains of ADAMTS13 apart from the signal peptide. The majority (>60%) of the reported mutations are missense, and the rest include frameshift, nonsense and splice site mutations,⁽³¹⁶⁾ which cause misfolding and intracellular retention of ADAMTS13. Some mutations allow secretion, but have an additional

functional deficit in ADAMTS13 activity. Notably, the mutations R349C and P353L affect the Dis domain exosite (see section 1.8.3.3) that is involved in the productive interaction between ADAMTS13 and the unravelled VWF A2 domain.⁽²⁵⁷⁻²⁵⁹⁾

cTTP has an autosomal recessive inheritance with most cases being compound heterozygous, and a minority being homozygous.^(296, 313) cTTP mainly presents at neonatal and childhood age, and only 20% of the cases present later than 18 years of age.⁽³¹³⁾ Adult-onset cTTP is most frequently associated with pregnancy.⁽³¹⁰⁾ Besides the characteristic clinical presentation, the severe ADAMTS13 deficiency <5% and the absence of antibodies, there are currently molecular tests that detect causal mutations in *ADAMTS13* to aid the diagnosis of cTTP.⁽³⁰⁷⁾

In cTTP patients, the levels of ADAMTS13 activity are always low (<5%); most frequently, ADAMTS13 antigen levels are also very low. Acute episodes generally occur as a result of concurrent precipitating factors, like infections. Further episodes can be prevented by prophylactic plasma infusions.⁽³⁰⁷⁾ Recombinant ADAMTS13 (commercially known as BAX930) is being trialled as a potential therapy in cTTP patients. A prospective phase 1 clinical trial (single-dose study) demonstrated that BAX930 was a safe treatment for cTTP, and was well tolerated by all patients. BAX930 treatment increased the ADAMTS13 antigen and activity levels in patient plasma within 1 hour, reduced the large VWF multimers detected in plasma, and no immune response against the administered recombinant ADAMTS13 was reported in any patient. The pharmacokinetics and plasma half-life of BAX930 were similar to those associated with administration of plasma-derived ADAMTS13.⁽³¹⁷⁾

1.7.1.2 Acquired TTP

Most commonly, TTP presents as an acquired disorder, which accounts for >95% of adult-onset TTP episodes.⁽³¹⁰⁾ It is primarily caused by the appearance of anti-ADAMTS13 autoantibodies. IgG autoantibodies in TTP were first described in 1998.⁽²²⁰⁻²²²⁾ Autoantibodies, mainly directed against the Spacer domain, can be inhibitory, reducing the activity of ADAMTS13. Interestingly, non-inhibitory antibodies have been shown to be associated with severely reduced ADAMTS13 antigen levels in aTTP patient plasma. Non-inhibitory autoantibodies probably enhance the clearance of ADAMTS13.⁽²⁷³⁾ In epitope mapping studies it was shown that multiple domains are targeted, however, the Spacer domain is now considered to be the major target of these autoantibodies.^(270, 273, 274) The Spacer residues R660, Y661 and Y665, which purportedly bind to the unravelled A2 domain of VWF and are

important for proteolysis (see section 1.8.3.1), are part of the epitope targeted by the antibodies. This offers an explanation as to how the inhibitory effect of the autoantibodies is manifest. Residues R568 and F592 were also shown to be targets of these autoantibodies.^(268, 318) Besides the IgG positive cases, a minority of aTTP patients have no detectable IgG, and have been referred to as 'aTTP of unknown cause'.⁽³¹⁰⁾

While >50% of the cases are idiopathic, aTTP can also appear secondary to another precipitating factor (eg. autoimmune disease, HIV, drugs, infection, cancer, transplantation). In contrast to the congenital form, aTTP is very uncommon before the age of 18. Other predisposing factors for the acquired disease are ethnicity (black), gender (women), underlying autoimmune disease and genetic background.^(305, 310, 319, 320)

aTTP is primarily treated with plasma exchange. The aim of this treatment is to increase the ADAMTS13 levels, and also remove pathogenic factors, like autoantibodies and ULVWF. Other treatments are used in combination with plasma exchange, which aim at targeting the autoimmune component of the disease; such treatments are corticosteroids and rituximab.⁽³⁰⁷⁾

1.7.1.3 Cardiovascular disease

VWF levels were first associated with myocardial infarction (MI) and coronary heart disease (CHD) in the 1990s.⁽³²¹⁻³²³⁾ The discovery of ADAMTS13 as the VWF-cleaving protease generated interest into whether the VWF-ADAMTS13 axis has a role in cardiovascular disease (CVD). In 2003, the first report of the association of low ADAMTS13 activity with CVD was published,⁽³²⁴⁾ and, since then, many case-control, prospective and meta-analysis studies have linked high VWF,⁽³²⁵⁻³⁴⁰⁾ low ADAMTS13,^(325-330, 333, 334, 339-342) and/or high VWF:ADAMTS13 ratio^(339, 343) to the risk of MI, ischaemic stroke (IS) and/or CHD. In studies where both VWF and ADAMTS13 levels were associated (positively and negatively, respectively) with CVD, their risks were independent and synergistic.^(333, 340-342) Denorme *et al* analysed the composition of thrombi obtained from stroke patients; VWF was distinctly one of the main components of these pathologic thrombi, further corroborating the involvement of VWF-ADAMTS13 axis in thrombus formation and the pathophysiology of CVD.⁽³⁴⁴⁾

Studies in mice appear to be in agreement with the results of the abovementioned clinical studies in associating VWF and ADAMTS13 with CVD. VWF deficiency (VWF^{-/-}) and neutralization of VWF by inhibitory polyclonal anti-VWF antibodies reduced the volume of infarcts in mouse models that had been subjected to cerebral or myocardial

ischaemia/reperfusion injuries (by middle cerebral artery occlusion or left anterior descending artery ligation, respectively).^(345, 346) In contrast, ADAMTS13 deficiency (ADAMTS13-/-) increased the volume of the infarcts,⁽³⁴⁵⁻³⁴⁷⁾ and this effect was VWF-dependent.^(345, 346) In addition, impaired spontaneous endogenous thrombolysis was observed in ADAMTS13-/- mice, which suggested that ADAMTS13 has a role in thrombolysis and recanalization of the obstructed vessels.⁽³⁴⁴⁾ To test its thrombolytic potential, recombinant human (rh) ADAMTS13 was administered to mice with occluded middle cerebral artery or left anterior descending artery; this reduced the infarct size,^(344, 345, 347, 348) dose-dependently,⁽³⁴⁴⁾ and even at delayed administration.^(344, 348) The thrombolytic activity of rhADAMTS13 was compared to tissue plasminogen activator (tPA), currently the only approved treatment for acute ischaemic stroke. While tPA can induce haemorrhagic complications if administered outside the therapeutic time window, rhADAMTS13 was not associated with any haemorrhagic complications,^(344, 345, 348) which suggested a wider therapeutic time window and highlighted its potential suitability in therapy. rhADAMTS13 was also shown to reverse platelet crosslinking in *in vitro* VWF-platelet aggregates.⁽³⁴⁴⁾

The role for VWF-ADAMTS13 in inflammation and atherosclerosis is also being studied. A significant association between the degree of atherosclerosis and VWF levels was shown in patients with transient ischaemic attack and ischaemic stroke.⁽³⁴⁹⁾ In mice treated with rhADAMTS13 a significantly lower degree of neutrophil infiltration was demonstrated.⁽³⁴⁷⁾ On the other hand, greater degree of inflammation and atherosclerosis was observed in ADAMTS13-deficient mice,^(346, 350-352) which occurred via a VWF-dependent mechanism.⁽³⁵⁰⁾

1.8 VWF RECOGNITION AND PROTEOLYSIS BY ADAMTS13

ADAMTS13 has attracted an enormous amount of scientific attention, owing to its extraordinary specificity for VWF; no other known physiological substrate for ADAMTS13 exists. It is unusual for a plasma protease to exhibit no known off-target proteolysis. For this reason, plasma proteases are usually specifically activated and inactivated, and further regulated by co-factors. The biochemistry of VWF recognition and proteolysis by ADAMTS13 has been extensively studied. It is now understood that, for a productive proteolytic event to take place, the VWF A2 domain must undergo a shear-dependent unfolding that exposes the previously cryptic scissile bond and binding sites for ADAMTS13. ADAMTS13 contains exosites in its proximal non-catalytic domains (Dis, Cys-rich and Spacer domains), which interact with

the exposed binding sites within the VWF A2 domain; these exosite interactions play a crucial role in proteolysis. The research has not been limited to solely identifying the contributing exosite residues; multiple studies aimed at also characterising the contribution of the exosite interactions to the rate and kinetics of proteolysis. A more recent discovery is that the activity of ADAMTS13 is regulated by a conformational activation mechanism, which involves unfolding of the C-terminal tail of the enzyme (TSP2-CUB2 domains).

1.8.1 The conformational activation of ADAMTS13

ADAMTS13 has recently been shown to be a conformationally flexible molecule, with the ability to adopt both folded and extended conformations (Figure 1-16). The works of South *et al* and Muia *et al* suggested that the folded conformation depends on interactions between the Spacer and CUB domains.^(43, 44, 46) Three linker regions, present between the TSP2-CUB2 domains, give ADAMTS13 its conformational flexibility.⁽²⁶⁵⁾ Various conditions abolish the Spacer-CUB interaction, extending the ADAMTS13 molecule; these are lower pH, certain anti-ADAMTS13 monoclonal antibodies, mutations in ADAMTS13 Spacer domain and/or the interaction with the VWF D4(-CK) domain(s). Interestingly, this extension enhances ADAMTS13 activity ~2-to-10-fold, which was suggested to be due to abolishing the negative effect of the CUB domain interaction proximal to the Spacer exosite that is implicated in VWF binding and proteolysis (see section 1.8.3.1).^(43, 44, 46)

While both CUB1 and CUB2 interact with the Spacer domain, the interaction between CUB1 with, most probably, Y661/Y665, is of most relevance to the folded conformation of ADAMTS13.⁽⁴⁴⁾ The D4-CK domain in globular VWF was already known to be involved in non-productive interactions with the C-terminal tail of ADAMTS13 and to enhance the proteolysis of multimeric VWF under flow.^(41, 42) South *et al* demonstrated that the ADAMTS13 TSP8 domain is the major binding site for the D4-CK domains of VWF, and that this interaction disrupts the Spacer-CUB interaction to unfold ADAMTS13 and enhance its activity.⁽⁴⁴⁾

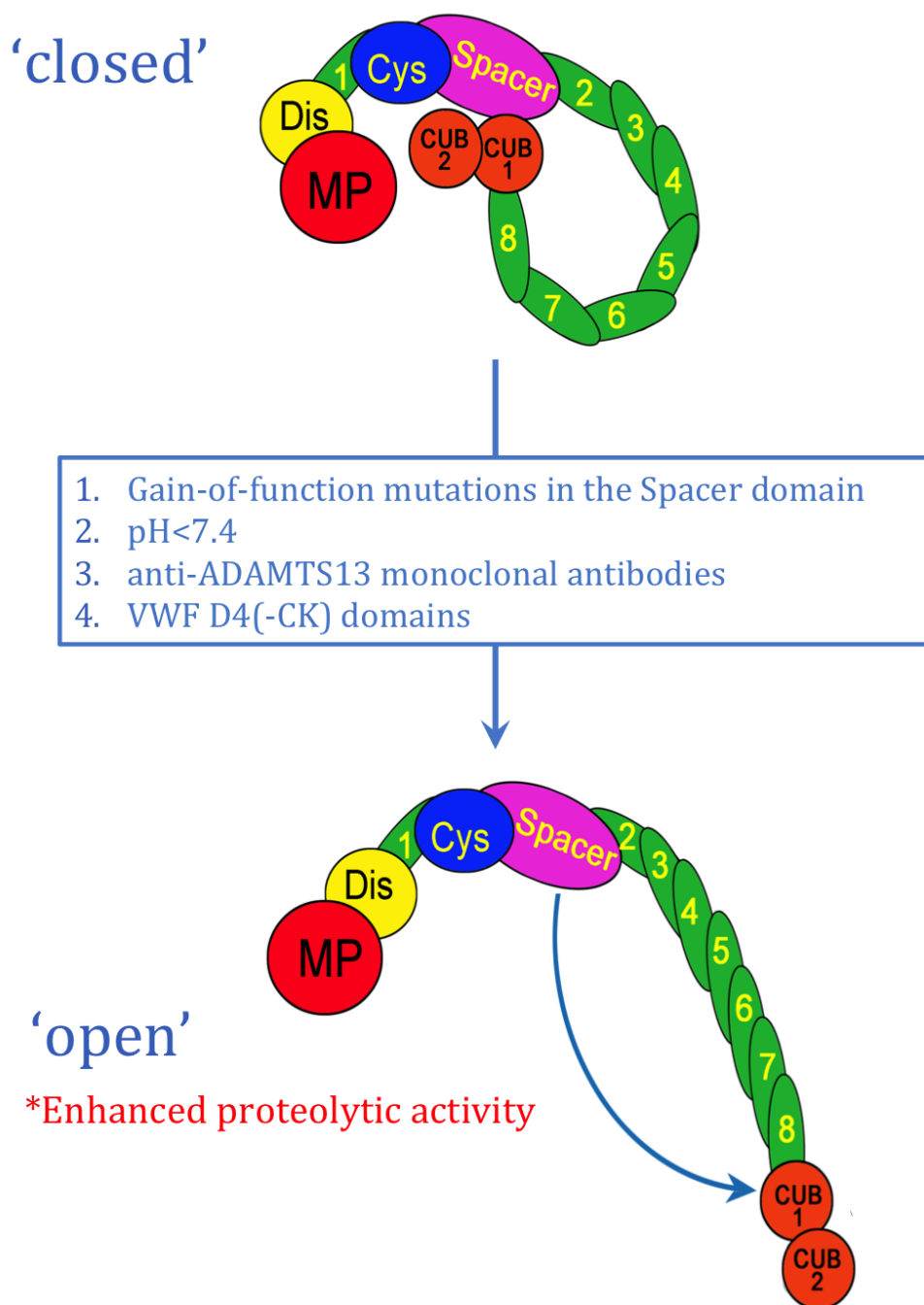


Figure 1-16 The conformational activation of ADAMTS13.

ADAMTS13 adopts a folded conformation, mediated by inter-domain interactions between the C-terminal tail and the N-terminal domains (proposed Spacer-CUB interaction). Mutations in the Spacer domain, lower pH, anti-ADAMTS13 monoclonal antibodies and binding of the VWF D4(-CK) domain(s), can disrupt the interactions and cause extension of the molecule. This was termed conformational activation, as it enhances the activity of ADAMTS13 ~2-to-10-fold.

1.8.2 The conformational transition of the A2 domain activates cleavage

It has already been mentioned that VWF unravelling dictates when and where proteolysis will take place. The ability of the A2 domain, under flow conditions, to undergo an intra-domain conformational adaptation that exposes its previously cryptic ADAMTS13 binding sites and scissile bond is now established to be the first molecular event and an absolute requirement for proteolysis to occur. Various observations have led to this conclusion. Firstly, VWF cleavage occurs under shear flow but not static conditions, which is in accordance with a shear-dependent VWF unravelling mechanism that allows cleavage by ADAMTS13.^(216, 218) Secondly, platelets in complex with VWF enhance cleavage; this was explained by an increase in the hydrodynamic forces acting on the VWF molecule, which increase the propensity of VWF to unravel.^(287, 288) Thirdly, in static conditions, chemical denaturants allow the cleavage of the otherwise resistant multimeric VWF by ADAMTS13 by chemically exposing cryptic cleavage and binding sites for ADAMTS13 and mimicking the physiological shear-dependent conformational transition mechanism.^(55, 218) Lastly, mutations in the A2 domain are known to increase its susceptibility to cleavage by ADAMTS13, and, as a result, cause type 2A VWD.⁽³⁵³⁾ Molecular modelling of the effect of type 2A mutations hypothesized structural alterations that destabilise the A2 domain structure and make the scissile bond and binding sites for ADAMTS13 more accessible for proteolysis.⁽³⁵⁴⁻³⁵⁶⁾ Xu *et al* ⁽³⁵⁷⁾ and Lynch *et al* ⁽³⁵⁸⁾ showed experimentally that type 2A VWD mutations compromised the VWF A2 domain stability, which would explain the enhanced susceptibility to proteolysis by ADAMTS13.

All of the above, taken together, support that the A2 domain is conformationally elastic; it exists as a folded domain that is resistant to proteolysis, but this resistance is abolished by a shear-dependent conformational change that unfolds the domain. The nature of this conformational transition was first described definitively in 2009. The crystal structure of the A2 domain revealed that the cleavage site is buried in a hydrophobic core, and that unfolding is required for proteolysis to occur. The structure also suggested that unfolding would begin from the C-terminus.⁽⁵⁴⁾ Single molecule experiments to study the effect of elongational forces on isolated A2 domains confirm that the A2 domain is a mechanosensitive domain, which, above a tensile force threshold, transitions from a folded to an unfolded structure. What is more, this study confirmed that cleavage by ADAMTS13 depends on VWF A2 domain unfolding. The tensile force threshold was predicted to be reachable during the transit of free circulating VWF through healthy vessels.⁽⁵⁶⁾ As explained in section 1.2.2.2, the unique force-sensing properties

of the A2 domain are attributed to various unique structural features (' α 4-less' loop, lack of intra-domain long-range disulphide bond, vicinal disulphide linkage (C1669-C1670), a Ca^{2+} -binding site (D1498, D1596, R1597, A1600 and N1602) and an N-linked glycan N1574-GlcNAc), which determine its tensile force threshold and influence its stability. Figure 1-17 shows the structural transition that extends the VWF A2 domain, exposing the binding sites for the ADAMTS13 domain exosites, as well as the scissile bond.

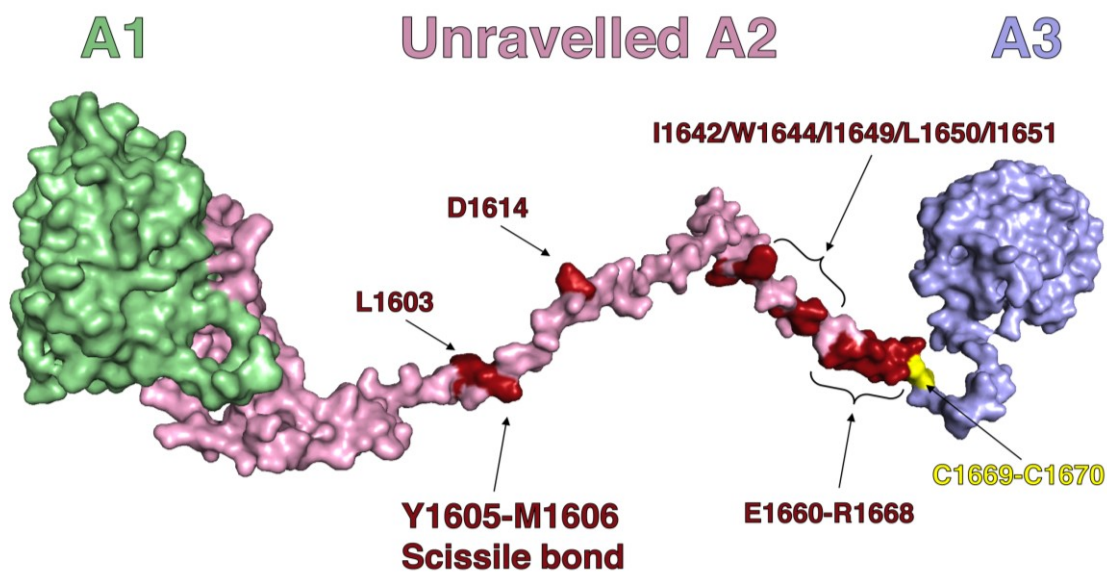


Figure 1-17 The conformational transition of A2 domain

A model of the VWF A1 (globular, green), A2 (unravelling, pink) and A3 (globular, blue) domains. The VWF A2 domain is a mechanosensitive domain that unravels as a result of tensile forces acting upon the molecule. Domain unravelling exposes previously cryptic binding sites that form functional interactions with exosites in the Spacer (E1660-R1668), Cys-rich (I1642/W1644/I1649/L1650/I1651), Dis (D1614) and MP (L1603) domains of ADAMTS13. These interactions lead to proteolysis of the scissile bond, Y1605-M1606. The binding sites and scissile bond within the unravelling VWF A2 domain are shown in red. The vicinal disulphide linkage (C1669-C1670) is shown in yellow.

1.8.3 The role of exosite interactions in VWF recognition and proteolysis

1.8.3.1 Spacer domain binding to the unfolded A2 domain

The Spacer domain was the first domain whose functional importance in ADAMTS13 activity was established. Removal of the Spacer domain significantly reduced the rate of VWF proteolysis, still maintaining the specificity of the reaction (cleavage at the scissile bond).^(249, 263, 264, 269) This was later corroborated by equilibrium binding experiments, where losing the Spacer domain caused a ~10-fold reduction in the equilibrium binding affinity.⁽²⁴⁸⁾ The Spacer exosite residues involved in the interaction with the VWF A2 domain were identified by looking into the Spacer domain epitope targeted by anti-ADAMTS13 antibodies in aTTP patients (Y658-Y665 region).⁽³⁵⁹⁾ Mutagenesis identified the solvent exposed R659, R660, Y661 and Y665 residues as the putative exosite residues that interact with the VWF A2 domain.^(235, 267, 268)

The Spacer domain exosite binding site in the VWF A2 domain spans the region E1660-R1668.^(251, 255, 360) Removal of this region causes a reduction in the rate of proteolysis, similar to that seen when removing the Spacer domain in ADAMTS13; this functional deficit is lost when using an ADAMTS13 variant truncated after the Cys-rich domain (MDTC), confirming the functional binding between this region and the Spacer domain exosite. Furthermore, short peptides spanning the region P1645-R1668 (includes the Spacer exosite binding site, E1660-R1668) are inhibitory to the proteolysis.⁽³⁶¹⁾ Kinetic analysis showed that the Spacer exosite interaction contributes ~15-to-20 fold to the rate of proteolysis, mainly by influencing the functional substrate binding affinity (K_m of the reaction).^(251, 255) Despite these biochemical data the precise interactions are not known.

1.8.3.2 Cysteine-rich domain binding to the unfolded A2 domain

In early ADAMTS13 truncation studies, the contribution of the Cys-rich domain to the activity of ADAMTS13 was not obvious.^(248, 263, 264) In pull-down assays, purified isolated Cys-rich domains could bind to VWF A2 domain-derived peptides; whether this binding contributed to the productive interaction between ADAMTS13 and VWF was still unclear.⁽²⁴⁹⁾ It was later shown that the Cys-rich domain contains an exosite that contributes to ADAMTS13 activity; the contribution of the entire domain was measured to be ~10-fold.⁽²⁵⁵⁾ The Cys-rich domain exosite spans a hydrophobic region, G471-V474, which is poorly conserved in the ADAMTS family of proteases. Using engineered N-linked glycans in this region, as well as swapping this

region with the corresponding sequence in ADAMTS1, a ~6-to-11 fold reduction in the rate of proteolysis was measured.⁽²⁶⁶⁾ Furthermore, the polymorphism P475S increases the K_m of the proteolysis reaction, showing that the region identified contributes to the functional binding between VWF and ADAMTS13.⁽²⁵³⁾

The functionality of the Cys-rich exosite was initially shown to be dependent on the VWF A2 domain region I1642-R1659, which indicated the location of the complementary binding site.⁽²⁵⁵⁾ Based on the hypothesis that a functional hydrophobic interaction exists, the hydrophobic residues in this region were substituted to Ser residues (IWILI1642/1644/1649-51SSSSS). This reduced the binding affinity and the rate of proteolysis by ~12-fold. No additional proteolytic deficit was observed when the G471-V474 were mutated in ADAMTS13, confirming a functional exosite interaction, of hydrophobic nature, between the G471-V474 residues in the Cys-rich domain and I1642/W1644/I1649/L1650/I1651 in the VWF A2 domain.⁽²⁶⁶⁾

1.8.3.3 Disintegrin-like domain binding to the unfolded A2 domain

Different studies reported that the isolated MP domain lost its specificity for VWF, but addition of just the Dis domain could restore the specificity.⁽²⁴⁹⁻²⁵¹⁾ This highlights the highly important role of the Dis domain for specific proteolysis to occur. A panel of point mutations in the variable regions of the Dis domain showed that the R349, L350 and V352 residues (in the ADAMTS13 Dis domain HVR) are important for ADAMTS13 activity; mutating these residues individually reduced the rate of proteolysis by up to ~20-fold. Creating the D1614A substitution in the VWF A2 domain, the functional deficit associated with a mutation at R349 was lost, demonstrating the functional interaction between the ADAMTS13 Dis domain R349 and the VWF A2 domain D1614. This interaction contributes to the functional binding affinity between VWF and ADAMTS13 (ie. K_m of the reaction). The D1614A mutation in the VWF A2 domain does not abolish the functional deficit associated with mutating L350 and V352 in ADAMTS13 Dis domain.⁽²⁵⁰⁾ Furthermore, mutating other residues in the VWF A2 domain in proximity to the D1614 (E1615/K1617) also reduces the binding affinity and the rate of proteolysis.⁽²⁵⁶⁾ These indicate that the Dis domain exosite interaction has not been fully characterised, and more residues, in both ADAMTS13 and the VWF A2 domain, are likely to be identified to contribute to this interaction.

1.8.3.4 Metalloprotease domain binding to the unfolded A2 domain

The loss of the proximal, non-catalytic domains of ADAMTS13 (Dis to Spacer domains) by truncation, whose great contribution towards binding affinity and specific proteolysis is now established, leaves the isolated MP domain unable to bind/cleave VWF specifically (at least detectably).^(248-251, 263, 264) Nevertheless, the MP domain must form specific, lower affinity functional interactions with the VWF A2 domain for proteolysis to occur, since mutating VWF A2 domain residues that are predicted to interact with the ADAMTS13 MP domain is detrimental to the rate of proteolysis. Mutating the P1 and P1' residues that make the scissile bond in the VWF A2 domain (Y1605-M1606) reduced the rate of cleavage by up to 300-fold.⁽³⁶²⁾ Furthermore, deletion of the P9-P2 residues, R1597-V1604, in the VWF A2 domain abolishes proteolysis.⁽²⁵⁵⁾

Subsites were identified in the MP domain, which form lower affinity interactions with the VWF A2 domain, close to the cleavage site, contributing to the specificity of the reaction. Swapping non-conserved regions in the MP domain with the corresponding sequences in ADAMTS1 and ADAMTS2, in combination with point mutations, reduced/abolished proteolysis. Mutating the residues D187, R190 and R193 in the MP domain independently reduced the rate of proteolysis (10-fold, 2-fold and 4-fold, respectively). Swapping the M252-L256 region in ADAMTS13 with the corresponding sequence in ADAMTS1, enhances the ability of the enzyme to cleave VWF substrates with a substituted P1' residue, M1606A. Based on this, the V252-L256 region was proposed to be part of the S1' pocket that accommodates the P1' residue, M1606.⁽²⁴²⁾

Residues in the VWF A2 domain located N-terminal to the cleavage site (P9-P2 residues, R1597-V1604) are critical for proteolysis,⁽²⁵⁵⁾ which suggests that they act as a docking site for ADAMTS13. Substituting the P3 residue, Leu1603, with Ser, Asn, Ala and Lys almost abolished proteolysis (up to >400-fold reduction); substitutions with the hydrophobic residues Met and Phe did not have a significant influence on the rate of proteolysis. That the synthetic peptide spanning D1596-Y1605 inhibited proteolysis, but less when it contained the L1603A substitution, confirmed the functionality of the P3 residue as a functional exosite binding site. Multiple residues, estimated to be in proximity to the active site in the MP domain, were targeted by mutagenesis individually to identify the S1 and S3 subsites that accommodate the Y1605 and L1603 residues, respectively. Two clusters of residues, namely L198/L232/L274

and V195/L151, appeared to influence the activity of ADAMTS13. However, further evidence demonstrating direct interactions involving these residues is required.⁽²⁵²⁾

1.8.3.5 The modularity and portability of the exosite interactions

Gao *et al* were the first to describe the modular nature of the interactions between ADAMTS13 domain exosites and their complementary binding sites in the VWF A2 domain.⁽²⁵⁵⁾ Instead of an extended interaction site, distinct segments/modules of interactions exist. These exosite interactions are portable and act independently of each other. Proteolysis can occur even after internally deleting any of the Dis to Spacer domains.⁽²⁴⁹⁾ Furthermore, certain internal deletions that change the relative distances between the exosite binding sites in the VWF A2 domain do not prevent proteolysis from taking place. That the exosite interactions are independent events could be of functional relevance. The VWF unfolding (a requirement for cleavage) is thought to involve multiple conformational transitions, during which the exposure of the interaction sites may not always happen in the same order; each interaction being an independent event would allow any exosite binding site exposed first to interact, irrespective of whether another exosite interaction has taken place.⁽²⁵⁵⁾

In a recent study by Kretz *et al*, all possible substitutions were introduced to the D1596-R1668 region in the VWF A2 domain by oligonucleotide synthesis. Using substrate phage display and high-throughput sequencing, they measured the effect of all possible point mutations to the rate of proteolysis, which was used to generate a heat map. This clearly showed the modularity of the exosite binding sites within the VWF A2 domain sequence; there are four distinct regions, in which mutations compromise the rate of proteolysis, corresponding to the binding sites for the MP, Dis, Cys-rich and Spacer domains of ADAMTS13. While prior studies using conventional mutagenesis were limited by the number of mutations analysed, this study impressively provided a complete dataset of the effect of any mutation at any position in the VWF A2 domain region of interest, redefining the boundaries of the exosite binding sites important for the specificity of ADAMTS13 proteolysis.^(363, 364) This will be discussed further in Chapter 4.

1.8.3.6 ADAMTS13 – a uniquely specific enzyme

Perhaps the most intriguing fact about ADAMTS13 is its unprecedented specificity; it is only proteolytically active against a single physiological substrate, VWF. This specificity becomes more striking when considering some unique properties that characterise ADAMTS13.

ADAMTS13 is secreted and circulates as a constitutively active enzyme. This is different to other haemostatic proteases, which circulate as zymogens and are specifically activated on demand by proteolytic removal of their activation peptide. ADAMTS proteases do possess a propeptide, which regulates their function, until it is eventually cleaved for activation.⁽³⁶⁵⁾ In ADAMTS13, the propeptide is unusually short, non-conserved, and functionally redundant.⁽³⁶⁶⁾ Also quite uniquely, ADAMTS13 has a long active plasma half-life of several days.⁽³⁶⁷⁾ If ADAMTS13 persists active for such a long period (with its active site 'open' and accessible) one would expect some inhibition (at least non-specific) by the large range of inhibitors found in plasma. However, no natural inhibitors are known to regulate ADAMTS13 function. *In vitro*, known endogenous inhibitors of MMPS, ADAMs, ADAMTSs proteases (tissue inhibitors of metalloproteases, TIMPs) and broad-spectrum MMP inhibitors (batimastat and iliomastat) do not seem to inactivate ADAMTS13.⁽³⁶⁸⁾ It is unlikely that a physiological cofactor for ADAMTS13 exists (purified recombinant ADAMTS13 retains its specific activity *in vitro*, in the absence of plasma).

The well-characterised exosite interactions that precede proteolysis have a central role to play in the specificity; they give ADAMTS13 its affinity to interact with VWF, and also direct the cleavage site into the active site with precision. We know that the activity of the isolated MP domain is almost abolished, and on some occasions it was shown to lack specificity. Addition of the proximal non-catalytic domains (Dis to Spacer domains) restores the catalytic efficiency and specificity of ADAMTS13. Could the exosite interactions facilitate an allosteric activation mechanism that opens and reshapes the active site for efficient and specific cleavage? Conformational flexibility within the active site has already been demonstrated in the crystal structures of ADAMTS4. In the presence of an active site inhibitor, the active site adopts an open conformation. In contrast, in the absence of an inhibitor, the active site adopts a rather closed conformation.⁽²³⁷⁾

1.9 Hypothesis and aims

I hypothesize that the active site of ADAMTS13 is flexible, and can exist in an active/'open' or a latent/'closed' state. Whether proteolysis occurs or not depends on where the equilibrium between these two conformational states lies; is an active/'open' conformation or a latent/'closed' conformation favoured? Upon one or more exosite interactions, a conformational change is transduced to the active site that shifts the equilibrium from favouring a latent/'closed' state (that cannot cleave VWF), to favouring an active/'open' state (that can cleave VWF) – a model of **allosteric activation**. This would explain the resistance of ADAMTS13 to off-target proteolysis (the specific exosite binding sites are only present in the VWF A2 domain and are exposed upon domain unravelling) and inhibition (active site is otherwise latent/'closed', inaccessible by inhibitors). The combination of this hypothetical exosite-dependent allosteric activation mechanism of ADAMTS13, with the need for a shear-dependent unravelling of the VWF A2 domain, and the C-terminal tail-mediated conformational activation of ADAMTS13, would explain the enormous specificity that characterises the ADAMTS13-VWF axis.

In this project, the precise contribution of each exosite interaction to the recognition of VWF and its proteolysis by ADAMTS13 will be investigated. A new VWF A2 domain peptide substrate, VWF96, will be generated as a substrate for ADAMTS13. Variants of this substrate, which have the entire exosite binding sites mutated individually, will also be generated; these will ablate the entire exosite interactions without interfering with the structure of ADAMTS13. I hypothesize that, if an exosite interaction is important in allosterically activating ADAMTS13, ablating that interaction not only will it influence the binding affinity between VWF and ADAMTS13, but it will also significantly influence the functionality of the enzyme's active site. Certain kinetic parameters can be used as measures of active site functionality and the substrate binding affinity; the k_{cat} (substrate turnover number, measures the functionality of the active site), the K_m (Michaelis constant, measures functional substrate binding affinity) and the K_D (dissociation constant, measures equilibrium binding affinity). In-house ELISA-based assays will be developed that monitor the proteolysis of the VWF96 variants to derive these kinetic constants. If an exosite that allosterically activates ADAMTS13 exists, then its ablation would be expected to significantly reduce the k_{cat} of proteolysis.

2 MATERIALS AND METHODS

2.1 GENERATION OF VWF96 VARIANTS

2.1.1 Generation of pET SUMO-VWF96 bacterial expression vector

2.1.1.1 PCR amplification of VWF96 cDNA

The cDNA for VWF96 (G1573-R1668) was generated by PCR amplification, using the OneTaq HotStart Quick load 2x Master Mix with standard buffer (NEB), according to the manufacturer's instructions. 1ng of the pET-100 vector, which had the cDNA for a longer VWF A2 domain fragment (VWF115, E1554-R1668) inserted and was already available in the lab,⁽²⁵⁶⁾ was used as the DNA template. The primers were produced by Thermo Fisher Scientific and were used at 0.2μM, in a final reaction volume of 50μl ('VWF96 PCR amp' primers in Table A, Appendix I). The conditions of the PCR reaction were: initial denaturation at 94°C for 30 seconds, then 28 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 15 seconds and extension at 68°C for 15 seconds, and then a final extension step at 68°C for 5 minutes. The PCR products were separated from the rest of the reaction components by agarose gel electrophoresis (section 2.1.1.2). The bands were excised and DNA was extracted from the gels (section 2.1.1.3).

2.1.1.2 Agarose gel electrophoresis

1.2% (w/v) agarose gels (Sigma) were prepared using Tris-Borate-EDTA buffer (89mM Tris-borate and 2mM EDTA, pH 8.3, Sigma) mixed with 5μg/ml SYBR safe gel stain (Invitrogen). DNA samples were mixed with Gel Loading Dye 6X (NEB) and loaded on the gel. The Quick-Load 2-Log DNA ladder (NEB) was used as a reference for molecular size. Electrophoresis was performed at 100V for 35 minutes and DNA bands were visualised on a Safe Imager blue light transilluminator (Invitrogen).

2.1.1.3 Agarose gel DNA extraction

The QIAquick Gel Extraction Kit (QIAGEN) was used to extract the DNA from the agarose gels. DNA bands of the desired molecular size were excised from the agarose gel, weighed and placed into a 1.5ml Eppendorf tube. 3 volumes of Solubilisation Buffer QG were added to 1 volume gel (where 1mg gel ~ 1μl) followed by incubation at 50°C until the gel completely dissolved. 1 gel volume of isopropanol was added and mixed. The mixture was transferred to a QIAquick spin column and centrifuged at 13000rpm for 1 minute to allow the DNA to bind to

the column. This was followed by centrifugation with 750µl of the ethanol-containing PE buffer at 13000rpm for 1 minute to wash the column. An extra centrifugation step ensured removal of any remaining ethanol from the column. To elute the bound DNA, 50µl Elution Buffer EB was added to the column and allowed to stand at room temperature for 1 minute to equilibrate the membrane, followed by centrifugation at 13000rpm for 1 minute.

2.1.1.4 Cloning of VWF96 cDNA into the pET SUMO vector

The PCR-amplified VWF96 cDNA was inserted into the pET SUMO bacterial expression vector, which fused N-terminal HisG and SUMO (small ubiquitin-related modifier) peptides (map in Figure 2-1). The pET SUMO TA cloning kit (Invitrogen) was used according to the manufacturer's instructions. The pET SUMO vector was supplied linearized, and contained single 3' deoxythymidine residues (T). The VWF96 cDNA, which had 3' deoxyadenosine (A) overhangs added by the *Taq* polymerase during PCR amplification, was cloned into the TA cloning site of the pET SUMO vector in a single step. In 5µl of ligation reaction, 1µl of PCR product, 0.5µl of ligation buffer, 1µl of pET SUMO vector, 0.5µl of T4 DNA Ligase (Invitrogen) and 2µl of sterile water, were used. The reaction was incubated at room temperature for 30 minutes.

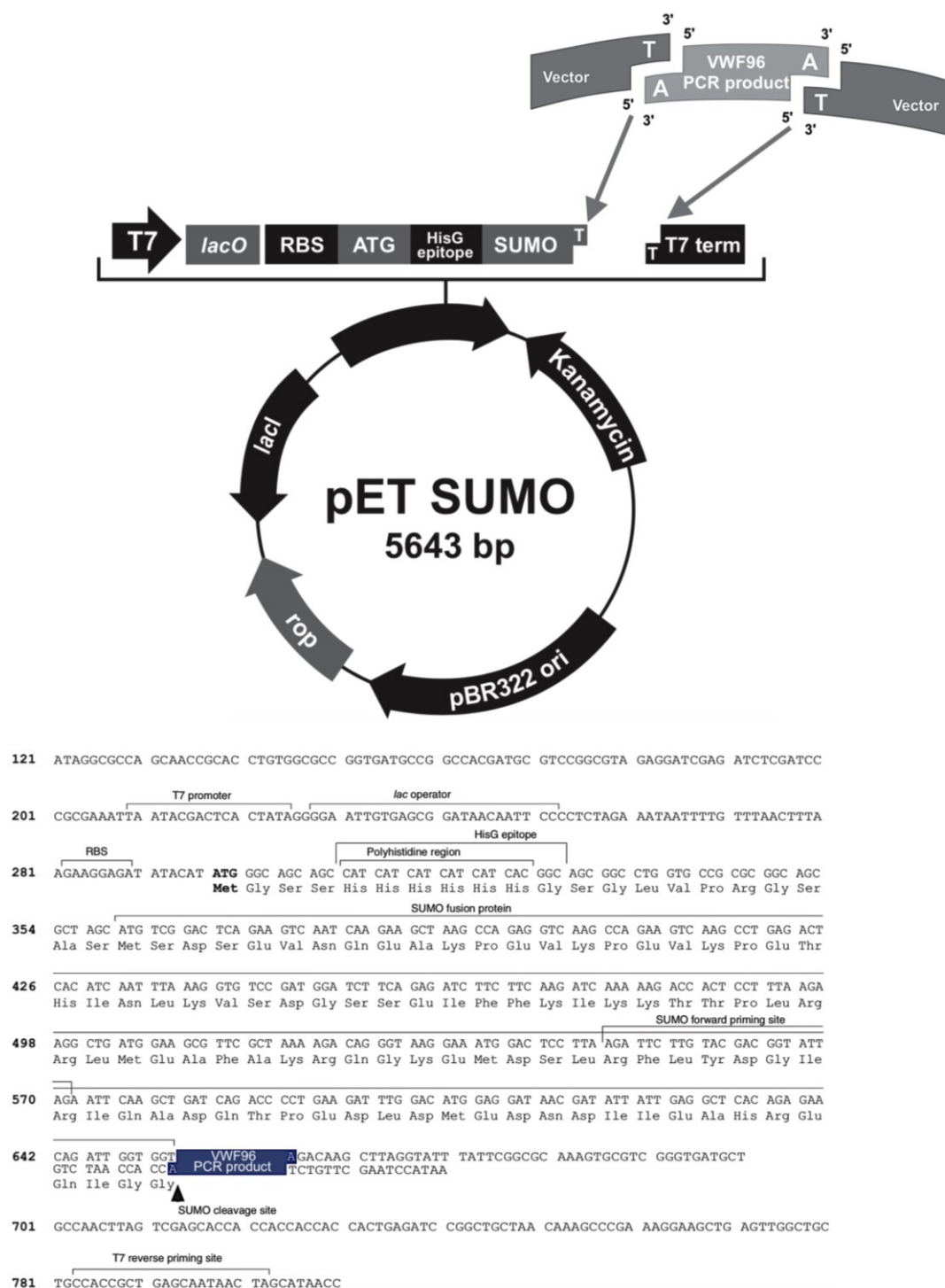


Figure 2-1 pET-SUMO map.

Features: T7 promoter (T7), lac operator (lacO), ribosome binding site (RBS), initiation codon (ATG), HisG epitope, SUMO ORF, TA cloning site, T7 terminator (T7 term), Kanamycin resistance gene, pBR322 origin, *ROP* ORF, *lacI* ORF. **TA cloning site:** pET SUMO is supplied linearised with a deoxythymidine (T) at its 3' ends. This allows quick and efficient one-step ligation of a *Taq*-amplified insert, which has one deoxyadenosine (A) at the 3' ends.

2.1.1.5 One Shot Mach1-T1 Chemically Competent *E. coli* transformation

The ligation reaction product (VWF96 pET SUMO vector) (section 2.1.1.4) was used to transform One Shot Mach1-T1 Chemically Competent *E. coli* (Invitrogen). After 50µl of bacteria were thawed on ice, 2µl of the ligation reaction was added, mixed gently and incubated on ice for 30 minutes. The mixture was then incubated at 42°C for 30 seconds to heat shock the bacteria. 250µl of S.O.C. outgrowth medium (NEB) was added and the bacteria were cultured at 37 °C, with shaking, for 1 hour. 150µl of the culture was spread on LB Agar (Sigma) medium. As the pET SUMO vector possesses a kanamycin resistance gene for selection in *E. coli*, 50µg/ml kanamycin was included in the LB Agar.

2.1.1.6 Plasmid DNA isolation

The QIAprep Spin Miniprep kit (QIAGEN) was used for DNA plasmid isolation. Single colonies were picked from the LB Agar plates to inoculate 5ml of LB Broth (Sigma) containing 50µg/ml kanamycin, and were grown overnight at 37°C with shaking. The overnight cultures were centrifuged at 13000rpm for 3 minutes at room temperature, keeping the pellets and discarding the supernatants. The pellets were resuspended in 250µl of RNase-containing resuspension buffer (P1) and transferred to a 1.5ml microcentrifuge tube. 250µl of lysis buffer (P2) was added to the resuspended sample, mixed and incubated for 5 minutes at room temperature to allow complete bacterial lysis. 350µl of neutralisation buffer (N3) was added followed by mixing and centrifugation at 13000rpm for 10 minutes. The pellet was discarded and the supernatant was applied to a QIAprep spin column and spun for 1 minute, discarding the flow-through. 750µl of buffer PE washed the column by centrifugation at 13000rpm for 1 minute. An extra centrifugation step was performed to remove any remaining ethanol from the column. The bound plasmids were eluted by adding 50µl of elution buffer (EB) to the column, standing for 1 minute at room temperature to equilibrate the membrane and eluting by centrifugation at 13000rpm for 1 minute. Successful cloning of the VWF96 cDNA into the pET SUMO vector was verified by sequencing ('Sequencing' primers in Table A, Appendix I).

2.1.2 Subcloning into the pET-25b(+) bacterial expression vector

2.1.2.1 PCR amplification of HisG-SUMO-VWF96 cDNA

The verified VWF96 pET SUMO vector was used as template for PCR amplification (section 2.1.1.1) of the sequence encoding the HisG-SUMO-VWF96 peptide, to subclone this into the bacterial expression vector pET-25b(+), which fuses C-terminal HSV and 6xHis tags (map in Figure 2-2). The primers for the PCR amplification introduced a 3' XhoI restriction site for subcloning ('VWF96-XhoI' primers in Table A, Appendix I). The PCR components were separated by agarose gel electrophoresis (section 2.1.1.2) and extracted from the gel (section 2.1.1.3).

2.1.2.2 Double restriction digest

Double restriction digests were set up, using the restriction enzymes XhoI and NdeI (NEB). In 50µl and 30µl restriction digest reactions, 43µl of the PCR product (HisG-SUMO-VWF96 cDNA with 3' XhoI restriction site)(section 2.1.2.1) and 6µl of the pET-25b(+) vector, respectively, were digested. Each reaction contained 1µl of each restriction enzyme and 1X CutSmart buffer (NEB), and was incubated at 37°C for 3 hours. The DNA fragments were separated by agarose gel electrophoresis (section 2.1.1.2) and extracted from the gel (section 2.1.1.3).

2.1.2.3 DNA ligation

DNA ligation was performed to ligate the NdeI- and XhoI-digested pET-25b(+) vector and HisG-SUMO-VWF96 cDNA insert (molar ratio 1 vector : 3 insert). In a final reaction volume of 20µl, 1µl T4 DNA Ligase (NEB) and 1X ligase buffer were used, according to the manufacturer's instructions. A 'no insert' control ligation reaction was performed to assess vector self-ligation. After incubation at room temperature for 1 hour, 4µl of each of the reactions were transformed into 50µl of NEB Turbo competent *E. coli* (NEB)(section 2.1.1.5). The pET-25b(+) vector possesses an ampicillin resistance gene for selection in *E. coli*, therefore, 100µg/ml ampicillin was added to the media. The emergence of colonies from the 'no insert' control ligation reaction indicated self-ligation of the parent vector and, hence, the presence of empty vectors. For this reason, multiple colonies were screened by PCR for successful ligation. Single colonies were picked and directly suspended in a PCR reaction containing OneTaq HotStart Quick load 2x Master Mix and 0.4µM T7 forward and reverse primers ('Sequencing' primers in Table A, Appendix I), at a final volume of 50µl. The following conditions were used: initial denaturation

at 94°C for 2 minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 68°C for 45 seconds, and then a final extension step at 68°C for 5 minutes. The PCR reactions were analysed by agarose gel electrophoresis (section 2.1.1.2). Plasmids from the positively screened colonies were isolated as described (section 2.1.1.3), using 100µg/ml ampicillin in the media, and verified by sequencing ('Sequencing' primers in the Table A, Appendix I).

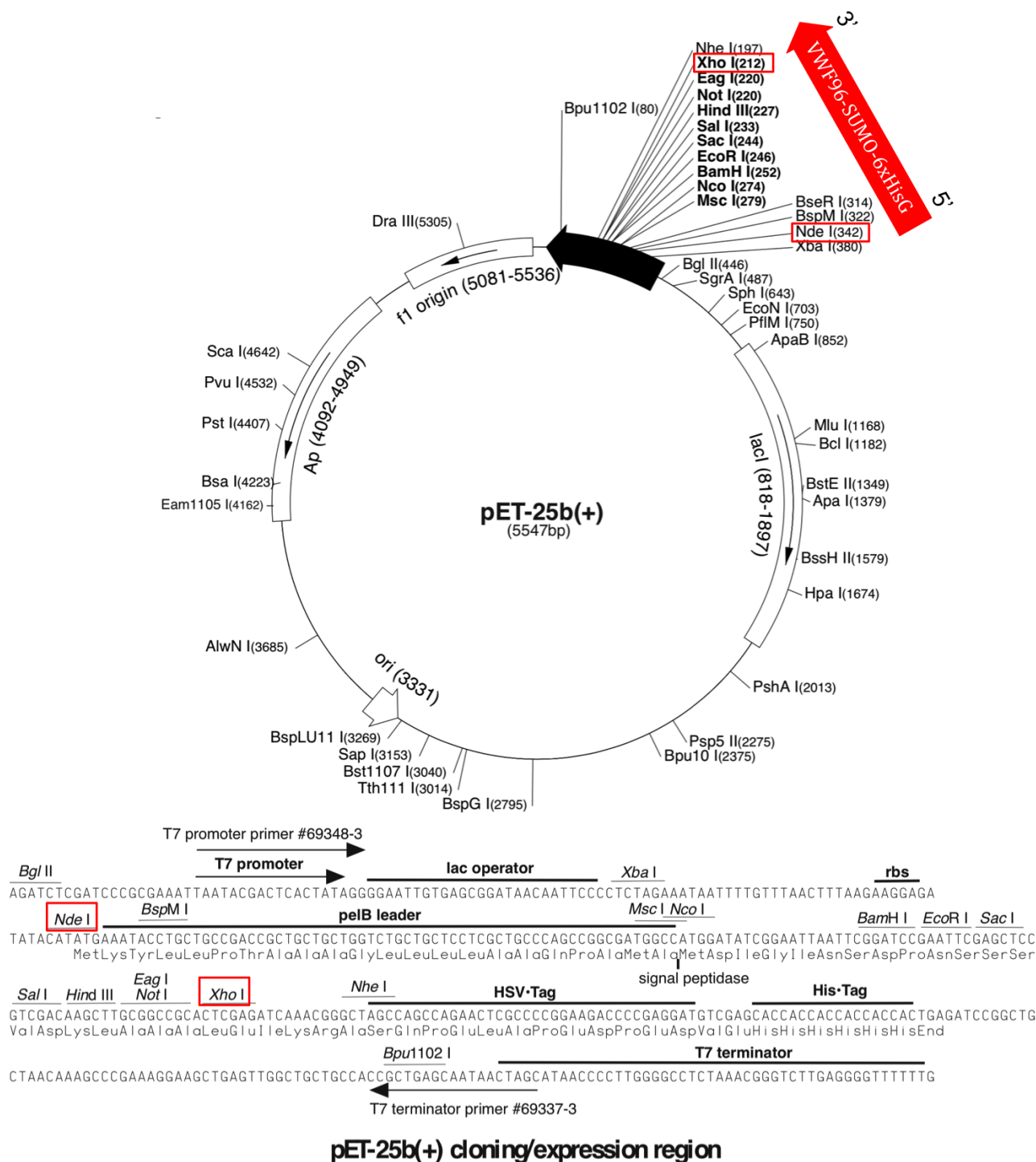


Figure 2-2 pET-25b(+) map

The HisG-SUMO-VWF96 cDNA was subcloned into the T7 cloning/expression region (sequence at the bottom) of the pET-25b(+) vector, using the NdeI and XhoI restriction sites (squared red). The cloning/expression region includes the following features: T7 promoter, lac operator, ribosome binding site (rbs), HSV tag ORF, 6xHis tag ORF, T7 terminator. Other features of the pET-25b(+) vector: pBR322 origin (ori), ampicillin resistance gene (Ap 4092-4949).

2.1.2.4 VWF96 mutagenesis by inverse PCR amplification of the pET-25b(+)

pET-25b(+) expression vectors expressing variants of VWF96 were generated by inverse PCR, using primers that introduce mutations/deletions into the VWF96 cDNA sequence. In a final volume of 50µl, 1ng of template DNA (VWF96-pET-25b(+)) (section 2.1.2.3), 2mM deoxynucleotides, 25mM magnesium sulphate, 0.3µM of forward and reverse primers ('Inverse PCR' primers in Table A, Appendix I), 1X buffer for KOD polymerase (Merck Millipore) and 1µl KOD DNA polymerase (Merck Millipore), were added. The following conditions were used: initial denaturation at 95°C for 2 minutes, then 25 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 10 seconds and extension at 70°C for 3 minutes, and then a final extension step at 70°C for 5 minutes. The PCR products (~6065bp) were separated by agarose gel electrophoresis (section 2.1.1.2) and extracted from the gel (section 2.1.1.3). The products of inverse PCR using the KOD polymerase were linear, with non-phosphorylated blunt ends. To circularise the gel extracted PCR products, 5' phosphorylation followed by ligation reactions were set up. 8µl of gel extracted PCR products were mixed with 10µl of Quick Ligase buffer (NEB) and 1µl of T4 PNK (NEB), and were incubated at 37°C for 30 minutes. At the end of the incubation, 1µl of Quick Ligase was added to the same tubes followed by a 5-minute incubation at room temperature. The reactions were used to transform One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) using 100µg/ml ampicillin in the media for selection (section 2.1.1.5). Multiple colonies were picked and used for plasmid isolation (section 2.1.1.6), and successful mutagenesis was verified by sequencing ('Sequencing' primers in Table A, Appendix I).

2.1.2.5 Large-scale expression of the VWF96 variants

50µl BL21 (DE3) *E. coli* were transformed with the pET-25b(+) vectors containing the inserts encoding VWF96 variants, and plated on LB Agar medium containing 100µg/ml ampicillin (section 2.1.1.5). Single colonies were picked to inoculate 10ml of fresh LB Broth containing 100µg/ml ampicillin, which were grown at 37°C for 5 hours with shaking. The 10ml cultures were used to inoculate 200ml of fresh LB Broth containing 100µg/ml ampicillin, followed by shaking overnight at 37°C. The overnight cultures were used to inoculate 2.5L of fresh LB Broth containing 100µg/ml ampicillin and incubated with shaking at 37°C. At log phase (~2 hours after inoculation), VWF96 variant expression was induced by the addition of 1mM IPTG, followed by further incubation for 5 hours. The cultures were centrifuged at 3500g for 30

minutes, discarding the supernatants and freezing the bacterial pellets at -80°C overnight. The pellets were thawed and lysed with BugBuster™ Protein Extraction Reagent (NOVAGEN), containing Benzonase (25 units per ml of BugBuster mix) (NOVAGEN), Chicken Egg White Lysozyme (NOVAGEN) and Protease Inhibitor Cocktail for use in purification of Histidine-tagged proteins (Sigma Aldrich), according to the manufacturer's instructions. BugBuster is a mixture of non-ionic detergents, which can perforate the cell wall of bacteria. Benzonase is a nuclease that degrades DNA and RNA, and is used to reduce the viscosity of the bacterial lysate for easier processing. Chicken Egg White Lysozyme catalyses the hydrolysis of polysaccharides in the peptidoglycan of the bacterial cell wall, facilitating lysis. The Protease Inhibitor Cocktail inhibits endogenous bacterial proteases and phosphatases, which could degrade the extracted VWF96. 2.5ml of the BugBuster mix was used per 50ml of culture. Insoluble cell debris was removed by centrifugation at 10000rpm at 4°C for 30 minutes, followed by filtration of the supernatant using the 0.2µm Minisart® Syringe Filters, keeping the filtrate.

2.1.2.6 Purification of the VWF96 variants

Immobilised Metal Ion Affinity Chromatography (IMAC)

VWF96 variants were initially purified from the bacterial lysates (soluble fraction) (section 2.1.2.5) using Nickel HiTrap Chelating HP columns (GE Healthcare Life Sciences) and the ÄKTA start FPLC system. The column was charged with 0.1M Nickel(II) sulphate heptahydrate (Sigma Aldrich) and equilibrated with 20mM Tris (pH 7.6)/500mM NaCl/20mM Imidazole (Sigma Aldrich). The bacterial lysates were passed over the column to allow 6xHis-tagged proteins (VWF96 variants have two 6xHis tags, one at each terminus) to bind the Ni²⁺ on the column, and the column was washed with 20mM Tris (pH 7.6)/500mM NaCl/20mM Imidazole. Elution of the VWF96 variants was performed with 20mM Tris (pH 7.6)/500mM NaCl/300mM Imidazole and the eluted fractions were dialysed in 20mM Tris (pH 7.6)/50mM NaCl twice at room temperature for 3 hours, and once at 4°C overnight, using the 10kDA MWCO SnakeSkin™ Dialysis Tubing (Thermo Fisher Scientific). The efficiency of the purification was assessed by SDS-PAGE (section 2.1.2.7) and Coomassie staining using Imperial protein stain (Thermo Fisher Scientific)(section 2.1.2.8).

Anion Exchange Chromatography

A second purification step was performed using HiTrap Capto Q ImpRes anion exchange column (GE Healthcare Life Sciences). The column was equilibrated with 20mM Tris (pH

7.6)/50mM NaCl, the samples were passed over and elution steps were performed with increasing NaCl concentrations in the buffer (20mM Tris (pH 7.6)/0.05-2M NaCl) to separate the VWF96 variants from impurities. VWF96 variants eluted at 540mM NaCl. The purified samples were analysed using SDS-PAGE (section 2.1.2.7) and Coomassie staining using Imperial stain (Thermo Fisher Scientific)(section 2.1.2.8), and dialysed in 20mM Tris (pH 7.6)/50mM NaCl/5mM CaCl₂ twice at room temperature for 3 hours, and once at 4°C overnight, using the 12-14kDa MWCO D-Tube™ Dialyzers (Merck Millipore).

2.1.2.7 SDS-PAGE

Proteins were separated according to molecular weight by SDS-PAGE. Samples were first diluted in NuPAGE 4X LDS Sample Buffer (Invitrogen) and incubated at 95°C for 5 minutes to cause protein denaturation. Denatured protein samples were loaded on NuPAGE Bolt Bis-Tris Plus 4-12% or 12% precast polyacrylamide gels (Invitrogen) and electrophoresed in MES Running Buffer (Invitrogen) at 200V for 25 minutes.

2.1.2.8 Coomassie staining

After SDS-PAGE (section 2.1.2.7), three 5-minute gel washes were performed with water on a shaking platform and at room temperature. The proteins on the gel were stained with Imperial protein stain (Thermo Fisher Scientific) by shaking at room temperature for 1 hour. The gels were destained with water by shaking at room temperature until all stained protein bands on the gel were clearly visible, and the background staining was removed.

2.1.2.9 Western blotting

The purified VWF96 variants were analysed by Western blotting. Following SDS-PAGE (section 2.1.2.7), proteins were transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) 'MIXED MW' pre-defined protocol (1.3A, up to 25V, for 7 minutes). The membranes were blocked in blocking buffer: 5% (w/v) non-fat milk (PanReac AppliChem) in phosphate buffered saline (PBS)(Sigma) for 1 hour on a shaking platform. To detect the C-terminal HSV tag on VWF96 variants, the membranes were incubated with goat polyclonal (pAb) anti-HSV whole IgG conjugated with horseradish peroxidase (HRP)(A190-136P, Bethyl). The N-terminal SUMO peptide was detected using chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) followed by 3 washes in PBS containing 0.1% Tween (PBS/0.1%Tween), and then incubating with HRP-conjugated goat pAb anti-chicken IgY

(abcam). Alternatively, the N-terminal HisG tag was detected using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) followed by 3 washes in PBS/0.1% Tween, and then incubating with HRP-conjugated rabbit pAb anti-mouse IgG (Dako). All antibodies were diluted 1:5000 in blocking buffer containing 0.1% Tween, and were incubated with the membrane for 1 hour with shaking. Following 5 washes, chemiluminescent detection was carried out using the Immobilon Western Chemiluminescent HRP substrate (Merck), and the ChemiDoc™ Imaging System (BIO-RAD).

2.1.2.10 VWF96 variant quantification

Purified VWF96 variants were quantified using predicted extinction coefficients and absorbance at 280nm (Nanodrop, Thermo Fisher Scientific), as well as using the in-house developed ELISA (section 2.1.2.11).

2.1.2.11 VWF96 Enzyme-linked immunosorbent assay (ELISA)

As part of this project, an ELISA assay was developed and optimised to detect VWF96 variants, exploiting the terminal SUMO and HSV peptides. Optimisation of the ELISA assay conditions involved identifying sensitive, commercially available antibodies, establishing the orientation of the ELISA antibodies, antibody concentrations, and a VWF96 concentration range that is linearly associated with absorbance. Details of all the conditions/parameters that were optimised are provided in Chapter 3 (section 3.6).

Optimised ELISA (anti-SUMO) conditions

In a Nunc Maxisorp™ 96 well ELISA plate (Thermo Fisher Scientific), 100µl of affinity-purified, chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors), diluted in 50mM sodium bicarbonate buffer (pH 9.6) to a concentration of 0.5µg/ml, were incubated overnight at 4°C. The coated wells were washed three times with 400µl PBS/0.1% Tween and blocked with 300µl 3% BSA/TBS buffer (20mM Tris pH 7.6/50mM NaCl containing 3% (w/v) Bovine Serum Albumin) for two hours, at room temperature, with shaking. The wells were washed, and 100µl of VWF96 diluted in 1% BSA/TBS buffer (20mM Tris pH 7.6/50mM NaCl) to the concentrations of 0nM, 0.01nM, 0.03nM, 0.05nM, 0.08nM, 0.1nM, 0.13nM and 0.15nM were added and incubated at room temperature for 1.5 hours, with shaking. Following washing, 100µl of pAb HRP-conjugated anti-HSV goat whole IgG (A190-136P, Bethyl), diluted in 1% BSA/TBS buffer (20mM Tris pH 7.6/50mM NaCl) to a concentration of 0.5µg/ml, were added and incubated for

one hour, at room temperature, with shaking. The antibodies were removed by washing and 170µl SIGMAFAST™ OPD (*o*-Phenylenediamine dihydrochloride) HRP substrate was used for the detection of VWF96. Colour development was stopped using 2.5M H₂SO₄ and measured spectrophotometrically at 492nm. A 'buffer-only' sample, to which no VWF96 was added, was used as blank for each plate, and was used to remove the background absorbance from all samples in the same plate.

Optimised ELISA (anti-HisG) conditions

An alternative ELISA protocol was developed, exploiting the N-terminal HisG tag of VWF96 (as opposed to the SUMO tag) for capturing VWF96 on the plate. This was because the affinity of the chicken pAb anti-SUMO/SUMOstar (AB7002, Life Sensors) for the SUMO tag on VWF96 varied across batches/LOTs, rendering the VWF96 ELISA too insensitive to monitor proteolysis.

The optimised ELISA protocol above was modified. Mouse mAb anti-HisG IgG2a (1.18µg/ml) (R940-25, Thermo Fisher Scientific) were immobilized on the 96-well plate for capturing VWF96. To generate the standard curve, 0nM, 0.1nM, 0.2nM, 0.4nM, 0.6nM, 0.8nM, 1nM and 1.2nM VWF96 were used.

2.2 PRODUCTION OF ADAMTS13 and MDTCS

2.2.1 Mammalian protein expression

2.2.1.1 Expression of full-length ADAMTS13 in HEK 293 cells

Human embryonic kidney (HEK) 293 cells stably transfected with the ADAMTS13 pcDNA 3.1/myc-his construct (map in Figure 2-3) were already available and stored in 10% Dimethyl sulfoxide (DMSO) in liquid nitrogen. Cells were thawed at room temperature. The DMSO and dead cells were removed by centrifugation at 100g for 5 minutes, discarding the supernatant and resuspending the pelleted cells in fresh growth medium (Eagle Minimal Essential Medium (Sigma) supplemented with 10% fetal bovine serum (FBS)(Labtech), 5mM L-Glutamine (Gibco Life Technologies), 1X MEM non-essential amino acids (Gibco Life Technologies) and 5mM penicillin/streptomycin (Sigma)). Cells were seeded in cell culture flasks and incubated overnight at 37°C, 5% CO₂. At >70% confluence, cells were washed with PBS, detached from the flask using TrypLE™ Express Enzyme (Gibco Life Technologies), resuspended into growth media containing FBS, and seeded for further expansion.

When enough cells were available at 70% confluence, cells were washed with PBS, growth medium was replaced by OptiMEM reduced serum medium (Gibco Life Technologies) with 5mM penicillin/streptomycin, and cells were incubated for three to five days at 37°C, 5% CO₂. All tissue culture was performed using aseptic technique.

The conditioned media containing ADAMTS13 were harvested, filtered using MF Millipore 0.45µm MCE filter units (Merck Millipore) and concentrated through a 10kDa MWCO Pellicon® XL Ultracel®-10 membrane (Merck Millipore) using the Labscale® TFF System (Merck Millipore). The samples were further concentrated using the Amicon Ultra Centrifugal Filter Units (Merck Millipore).

2.2.1.2 Enzyme-linked immunosorbent assay (ELISA) for ADAMTS13 quantification

To establish the concentration of full-length ADAMTS13 in conditioned media, an ELISA assay was used, which had been optimised prior to this project.⁽³³⁰⁾ In a Nunc Maxisorp™ 96 well ELISA plate (Thermo Fisher Scientific), 100µl of rabbit pAb anti-ADAMTS13 (depleted of anti-ADAMTS13 TSP2-4 domain antibodies), diluted in 50mM sodium bicarbonate buffer (pH 9.6) to a concentration of 5µg/ml, were incubated overnight at 4°C. The coated wells were washed three times with 400µl PBS/0.1% Tween and blocked with PBS/3% BSA for two hours, at room temperature, with shaking. The wells were washed, and 100µl of various dilutions (in PBS/1% BSA) of concentrated conditioned media containing ADAMTS13 were added and incubated at room temperature for 1.5 hours, with shaking. To generate a standard curve, dilutions of normal human plasma (Technoclone, Austria) were used instead (the concentration of ADAMTS13 in normal pooled human plasma was previously measured to be 900ng/ml).⁽³³⁰⁾ Following washing, 100µl of biotinylated rabbit pAb against ADAMTS13 TSP2-4 domains (diluted in PBS/1% BSA to a concentration of 0.2µg/ml) were added and incubated for one hour, at room temperature, with shaking. The antibodies were removed by washing, and 100µl of Pierce™ Streptavidin-HRP (Thermo Fisher Scientific) diluted 1:1000 in PBS/1% BSA was added to the wells and incubated for 1 hour, at room temperature, with shaking. A final wash was performed and 170µl SIGMAFAST™ OPD (*o*-Phenylenediamine dihydrochloride) HRP substrate was used for detection of ADAMTS13. Colour development was stopped using 2.5M H₂SO₄ and was measured spectrophotometrically at 492nm. A buffer-only well was used as blank in each plate, and the background absorbance was subtracted from all samples on the same plate.

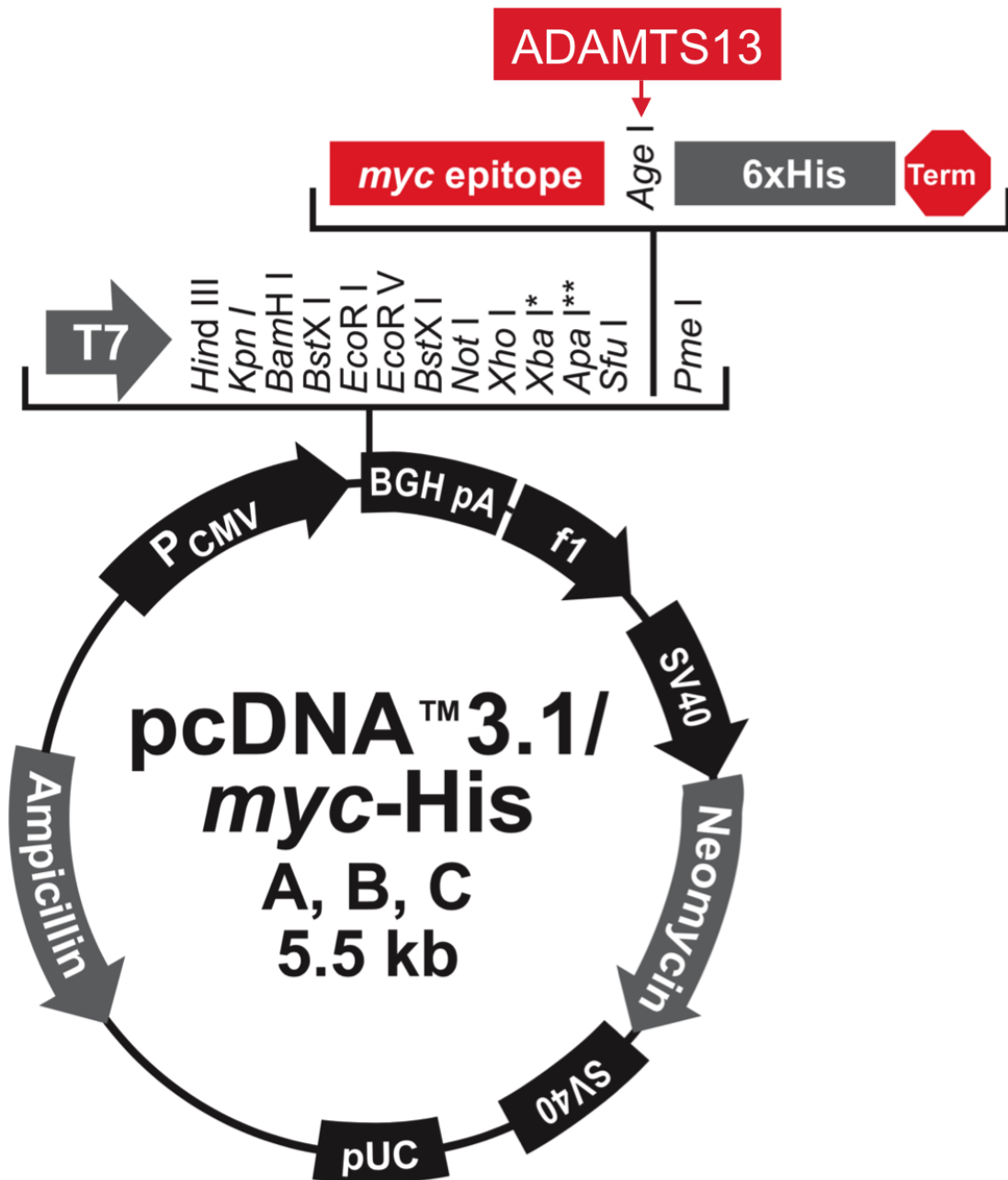


Figure 2-3 pcDNA™3.1 Mammalian expression vector map.

Features: cytomegalovirus enhancer-promoter (PCMV) for expression in mammalian cells, multiple cloning site, myc epitope, polyhistidine tag (6xHis), BGH polyadenylation signal and transcription termination sequence for mRNA stability, SV40 origin of replication/promoter/polyadenylation signal, neomycin resistance gene, ampicillin resistance gene, pUC origin for replication in bacteria.

2.2.2 Drosophila Schneider (S2) insect cell expression of truncated ADAMTS13 variants (MDTCS and MDTCS(E225Q))

2.2.2.1 Thawing cells

The cDNA for the MDTCS domains of ADAMTS13 lacking the signal peptide/propeptide (G78-P682, with or without the inactivating E225Q substitution) was cloned into the pMT-BiP-PUR0 (map in Figure 2-4),⁽³⁶⁹⁾ which was used to stably transfect Drosophila Schneider (S2) insect cells using calcium phosphate (by Dr Frank Xu). Stably transfected S2 insect cells were already available and stored in 10% DMSO in liquid nitrogen. Cells were thawed at room temperature, added to a T25 flask containing 5ml of room temperature Schneider's Drosophila Medium, Modified, with L-Glutamine (Lonza), supplemented with 10% FBS, 10µg/ml Gibco™ Sterile Puromycin Dihydrochloride (Life Technologies) and 5mM penicillin/streptomycin, and incubated at room temperature in the dark for 30 minutes. After detaching the cells from the flask by pipetting, the cultures were centrifuged at 100g for 2-3 minutes, discarding the supernatant to remove DMSO and dead cells. The cells were resuspended in 5ml of fresh complete Schneider's Drosophila medium, added to a T25 flask and incubated at room temperature in the dark, until reaching a density of 6-20x10⁶ cells/ml.

2.2.2.2 Cell passage

Any cells that attached to the flask were detached by pipetting. Cells were washed twice by centrifugation at 100g (keeping the supernatant from the conditioned media aside) and resuspending the pellet in fresh room temperature complete Schneider's Drosophila medium. The cells were passaged into new culture flasks using 20% conditioned media and 80% fresh room temperature complete Schneider's Drosophila medium, splitting them so as to reach a density of 2-4x10⁶ cells/ml, and incubated at room temperature in the dark. This procedure was repeated to expand the culture from T25 to T75 to T175 flasks.

2.2.2.3 Protein expression

Prior to protein expression induction, the cells from each T175 flask were washed and resuspended in 250ml EX-CELL® 420 Serum-Free Medium for Insect Cells with L-Glutamine (Sigma), supplemented with 10µg/ml Gibco™ Sterile Puromycin Dihydrochloride and 5mM penicillin/streptomycin, and transferred to 2L Corning® Erlenmeyer Shake Flasks with Vent Cap (Corning). The cultures were incubated in the dark, at 28°C with shaking until reaching a

density of $6\text{-}20 \times 10^6$ cells/ml, when more media were added to reduce cell density to a density of $2\text{-}4 \times 10^6$ cells/ml. Protein expression was induced with the addition of 0.5mM CuSO_4 . 7 days after protein expression induction, the conditioned media were harvested, centrifuged at 5000rpm for 20 minutes and filtered through a $0.2\mu\text{m}$ filter.

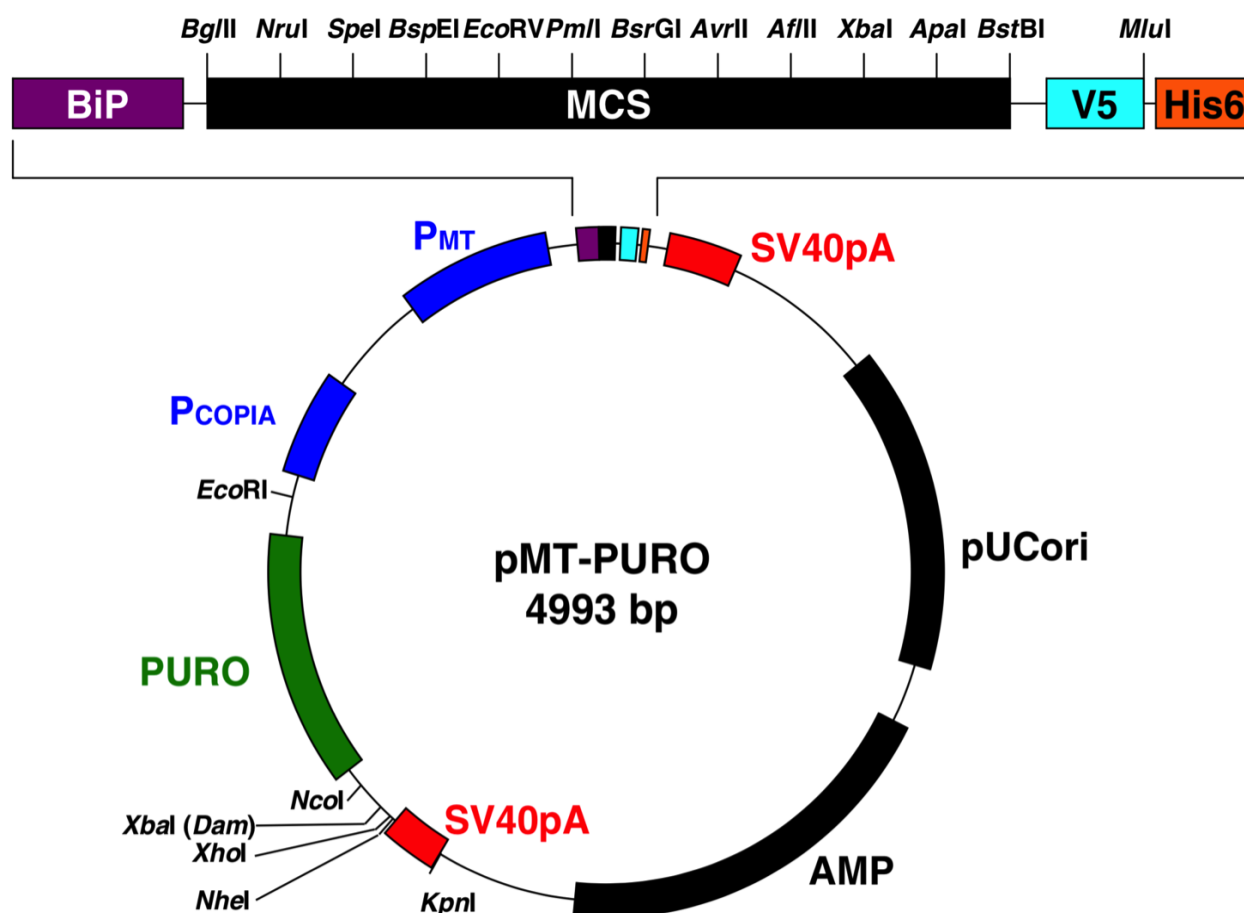


Figure 2-4 pMT-BiP-PURO vector map

Features: Metallothionein promoter (PMT), BiP signal sequence; multiple cloning site (MCS) V5 peptide tag, polyhistidine tag (His6), SV40 late polyadenylation signal (SV40 pA), copia promoter (PCOPIA), ampicillin resistance gene (AMP), pUC origin for replication in bacteria (pUCori), puromycin N-acetyltransferase gene for puromycin resistance (PURO).

2.3 VWF96 variant proteolysis by ADAMTS13

2.3.1 Qualitative analysis

Concentrated ADAMTS13 in conditioned medium (section 2.2.1.1) and purified VWF96 variants (section 2.1.2.6) were incubated separately in TBSC buffer (20mM Tris pH 7.6/50mM NaCl/5mM CaCl₂) at 37°C for 15 minutes. Reactions (final volume 135µl) were set up between 0-53nM ADAMTS13 and 3µM VWF96 variants in TBSC buffer (20mM Tris pH 7.6/50mM NaCl/5mM CaCl₂). 25µl reaction sub-samples were stopped every 30 minutes for 1.5 hours by mixing with 55mM EDTA. The sub-samples were analysed on 4-12% or 12% Bis-Tris gels by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8).

2.3.2 Kinetic analysis

2.3.2.1 Catalytic efficiency by time course analysis of proteolytic reactions

The catalytic efficiency of VWF96 proteolysis by ADAMTS13 was quantified using the novel VWF96 ELISA protocols developed as part of this project.

ADAMTS13 in conditioned medium and purified VWF96 variants were incubated separately in 1% BSA/TBSC buffer (20mM Tris pH 7.6/50mM NaCl/5mM CaCl₂) at 37°C for 15 minutes. Reactions (final volume 100µl) were set up between 0.75-187.5nM ADAMTS13 and VWF96 variants (for each VWF96 variant I chose concentrations that were lower than the K_m) in 1% BSA/TBSC buffer (20mM Tris pH 7.6/50mM NaCl/5mM CaCl₂). 10µl reaction sub-samples were stopped between 0-120 minutes by mixing with 25mM EDTA buffer/1% BSA/TBS buffer (20mM Tris pH 7.6/50mM NaCl). The stopped sub-samples were diluted to 0.09nM VWF96 in 1% BSA/TBS buffer (20mM Tris pH 7.6/50mM NaCl) and analysed by VWF96 ELISA using the chicken pAb anti-SUMO/SUMOstar (AB7002, Life Sensors) for capturing VWF96 by the N-terminal SUMO tag. Alternatively, the stopped sub-samples were diluted to 0.75nM VWF96 and analysed by VWF96 ELISA using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) for capturing VWF96 by the N-terminal HisG tag (section 2.1.2.11). The concentration of uncleaved substrate at each time point was measured and, from this, the fraction of substrate proteolysed was calculated and plotted as a function of time. The catalytic efficiency, k_{cat}/K_m , was determined using Graphpad Prism by fitting the data from the time course reactions into the equation $P = 1 - \exp(-1 \times [ADAMTS13] \times t \times k_{cat}/K_m)$.⁽²⁵⁶⁾

$[ADAMTS13]$ is the concentration of ADAMTS13 (μM), t is the time in seconds, and P is the proteolysed substrate fraction.

2.3.2.2 Michaelis Menten kinetic analysis of VWF96 variant proteolysis

To obtain the separate kinetic constants, k_{cat} and K_m , for VWF96 variant proteolysis, multiple time course reactions were performed as described above (section 2.3.2.1), between ADAMTS13 (0.2-505nM) and VWF96 variants (0.2-350 μM). From each time course analysis, the initial rate of proteolysis (at <15% proteolysis) was calculated (in nMs^{-1}), normalized per unit (nM) of enzyme in the reaction, and plotted as a function of substrate concentration. The Michaelis Menten equation was used to fit the data in Graphpad Prism:

$$V_i = \frac{V_{\text{max}} \times [\text{substrate}]}{K_m + [\text{substrate}]}$$

V_i is the initial rate of proteolysis (nMs^{-1}). V_{max} is the maximum rate of proteolysis (nMs^{-1}) that can be achieved (ie. the highest initial rate that occurs at enzyme saturation). $[\text{substrate}]$ is the concentration of VWF96 variant in the reaction (μM). K_m , or Michaelis constant, is the concentration of substrate that achieves half maximal rate of proteolysis ($\frac{V_{\text{max}}}{2}$)(μM).

The k_{cat} (s^{-1}) is the catalytic constant, or turnover number, which measures the functionality of the active site of each unit of enzyme (ie. the number of reactions each active site catalyses per unit of time). It can be calculated by dividing the V_{max} by enzyme concentration ($k_{\text{cat}} = \frac{V_{\text{max}}}{[\text{ADAMTS13}]}$). Since the initial rates of proteolysis had been normalized (per unit of enzyme) prior to plotting and fitting into the Michaelis Menten equation, $[\text{ADAMTS13}]$ is equal to 1, and k_{cat} is numerically equal to the V_{max} .

2.4 ELISA-based assays to measure equilibrium binding affinity between ADAMTS13 and VWF96 variants

2.4.1 Equilibrium binding affinity between immobilised VWF96 variants and soluble full-length ADAMTS13

In a Nunc Maxisorp™ 96 well ELISA plate (Thermo Fisher Scientific), 100µl of purified VWF96 variants, diluted in 50mM sodium bicarbonate buffer (pH 9.6) to a concentration of 100nM, were incubated overnight at 4°C. The coated wells were washed three times with 400µl PBS/0.1% Tween and blocked with 300µl PBS/3% BSA for two hours, at room temperature, with shaking. The wells were washed, and 100µl of concentrated conditioned media containing ADAMTS13, diluted to 0-600nM in PBS/1% BSA/80mM EDTA (EDTA was included in the buffer to prevent proteolysis of the immobilized VWF96 variants by ADAMTS13), were added and incubated at room temperature for 1.5 hours, with shaking. The same concentrations of ADAMTS13 were also added to uncoated, blocked wells, to later subtract the absorbance associated with the non-specific binding of ADAMTS13 to the wells. A buffer-only sample was added to a coated well to be used as blank in each plate, and was used to remove the background absorbance from all samples in the same plate. Detection of bound ADAMTS13 was performed as described in section 2.2.1.2. After subtracting the absorbance from the blank and non-specific binding control wells, the data were fitted in Graphpad Prism using the one site binding equation,

$$B = \frac{B_{max} \times [ligand]}{K_D + [ligand]}$$

B is the percentage specific binding (after subtracting the absorbance associated with non-specific binding and blank, the absorbance associated with specific binding is expressed as percentage of absorbance at B_{max}). B_{max} is the point where all binding sites are occupied (the maximum absorbance associated with specific binding that can be reached, plotted as 100% binding). $[ligand]$ is the concentration of the soluble ligand (full-length ADAMTS13). K_D is the equilibrium dissociation constant, which is the concentration of the soluble ligand that gives half maximal binding ($\frac{B_{max}}{2}$), in the same units as $[ligand]$.

2.4.2 Equilibrium binding affinity between immobilised VWF96 variants and MDTCS (E225Q)

The protocol in section 2.4.1 was adapted to measure the equilibrium binding affinity between immobilised VWF96 variants and soluble MDTCS (E225Q). 0-600nM MDTCS (E225Q) (expressed in *Drosophila* Schneider S2 insect cells and purified to homogeneity by Dr Frank Xu) were used instead of full-length ADAMTS13, and no EDTA was included in the buffers, as the E225Q mutation inactivates the active site of the enzyme. MDTCS (E225Q) binding to the immobilised VWF96 variants was detected using 1.29µg/ml rabbit pAb anti-ADAMTS13 (depleted of anti-ADAMTS13 TSP2-4 domain antibodies), followed by 0.125µg/ml HRP-conjugated goat pAb anti-rabbit IgG (P0448, Dako).

2.5 Characterising the activating effect of anti-Spacer domain (3E4) and anti-CUB domain (17G2) monoclonal antibodies on the proteolysis of VWF96 by ADAMTS13

The protocols in sections 2.3.2.1 and 2.3.2.2 were adapted to measure the activating effect of the anti-Spacer domain (mAb 3E4) and anti-CUB domain (mAb 17G2) monoclonal antibodies on the catalytic efficiency (k_{cat}/K_m) and Michaelis Menten kinetics (k_{cat} , K_m and k_{cat}/K_m) of VWF96 proteolysis. Purified ADAMTS13 (0.4-11nM) (expressed and purified to homogeneity by my collaborator An-Sofie Schelpe) or concentrated MDTCS in *Drosophila* Schneider conditioned media (~1.2nM, as assessed qualitatively) were used instead of ADAMTS13 in conditioned media. The monoclonal antibodies (provided by my collaborator An-Sofie Schelpe) were incubated together with the enzyme prior to the initiation of the reaction, and were used at a final concentration of 10µg/ml to be in excess. Negative control reactions were set up using a non-activating, non-inhibitory anti-Spacer antibody (mAb 15D1).

3 RESULTS: GENERATION OF VWF96 AND DEVELOPMENT OF VWF96

ELISA

3.1 INTRODUCTION

The overarching aim of my project was to define the molecular mechanisms that dictate the proteolytic specificity exhibited by ADAMTS13. Despite apparently circulating in its proteolytically competent form, and in the absence of evidence to suggest on-demand activation/inhibition, ADAMTS13 cleaves only one substrate, VWF, at only one site, Y1605-M1606. I, therefore, formed the hypothesis that ADAMTS13 actually circulates in a latent conformation that is allosterically activated upon interaction of one or more of its functional exosites with their complementary binding sites in the VWF A2 domain. This induces a conformational transition within the active site in the MP domain, which enables scissile bond access and proteolysis. To test this hypothetical mechanism biochemically, I investigated the effect of ablating each entire ADAMTS13 exosite interaction on the kinetics of proteolysis of a VWF A2 domain fragment.

Circulating VWF in its globular conformation is resistant to proteolysis, unless there is shear-induced unfolding of the A2 domain to expose the exosite binding sites and scissile bond for ADAMTS13. For this reason, flow-based assays have been used to study the activity of ADAMTS13 against full-length VWF *in vitro*.^(125, 216, 267, 370) The use of denaturing agents, like Gdn-HCl and urea, can also unfold the VWF A2 domain, mimicking to some extent the shear-induced extended conformation, and allowing proteolysis of full-length VWF under static conditions.^(55, 87, 218) Alternatively, VWF A2 domain fragments have been used as substrates for ADAMTS13. Conveniently, these also mimic the unfolded conformation of the VWF A2 domain and they can be cleaved by ADAMTS13 in the absence of flow or chemical denaturants.^(242, 250, 251, 255, 266, 268, 360, 371, 372) The first aim of this project was to develop a new ELISA-based assay that monitors the kinetics of ADAMTS13-mediated proteolysis of a new VWF A2 domain-derived peptide substrate, VWF96. VWF96 spans the region G1573-R1668 in the VWF A2 domain, which possesses all functional complementary binding sites for ADAMTS13 exosites and the scissile bond (Figure 3-3A). The modular nature of the exosite binding sites within VWF96 allows for specifically targeting/ablating each one of them independently by mutation; this would allow me to generate VWF96 variants in order to characterise the contribution of

each exosite interaction to the kinetics of proteolysis, and, hence, probe the functionality of each exosite interaction.

Kokame *et al* were the first to show that peptides derived from the VWF A2 domain could be cleaved by ADAMTS13. Knowing that the scissile bond lies within the VWF A2 domain, they methodically sought for the minimal VWF peptide that could be cleaved by ADAMTS13. They generated VWF A2 domain fragments, of various lengths, and assessed the ability of ADAMTS13 to cleave these substrates. The 73aa region D1596-R1668 was identified as the minimal substrate for ADAMTS13; this peptide was termed VWF73.⁽³⁶⁰⁾ In a subsequent study, the same group created the fluorescent substrate FRETs-VWF73. In FRETs-VWF73, the P7 residue (Q1599) is substituted by a 2,3-diaminopropionic residue (A2pr) that contains a 2-(N-methylamino)benzoyl group (Nma), which, when excited at 340nm, fluoresces at 450nm. The P5' (N1610) residue in FRETs-VWF73 is substituted by A2pr that contains a 2,4-dinitrophenyl group (Dnp), which can quench the fluorescence resonance energy emitted by the Nma. Since the Nma and Dnp are on opposite sides of the scissile bond, quenching only occurs in the uncleaved FRETs-VWF73. Upon proteolysis, separation of the Nma and Dnp allows fluorescence emission, which can be monitored to quantify the rate of proteolysis in real-time.⁽³⁷²⁾ Despite having been used for kinetic analysis of ADAMTS13 activity in the past,^(235, 253, 373) and also currently being a standardized tool for measuring ADAMTS13 activity in plasma for the diagnosis of TTP,^(296, 374) FRETs-VWF73 was not an appropriate substrate for the purposes of this project. Firstly, it is a commercially available synthetic substrate. Multiple substrate variants would need to be generated for the purposes of this project, which justifies an adaptable and cost-effective in-house system for substrate production. Secondly, only a narrow range of substrate concentrations (0-65 μ M) has been used in the FRETs-VWF73 assay. I anticipated that ablating entire exosite interactions would substantially compromise the rate of proteolysis, which would necessitate performing reactions over a much wider range of substrate concentrations. The sensitivity and specificity of the FRETs-VWF73 assay at higher substrate concentrations have not been assessed/optimized; in fact inner filter effect was reported at FRETs-VWF73 concentrations >20 μ M, which can be problematic.⁽³⁷³⁾

Another VWF A2 domain-derived peptide, termed VWF115, that spans the region E1554-R1668, was also used as substrate for ADAMTS13 in prior studies.^(241, 242, 250, 252, 256, 266, 268, 362) Sub-samples from reactions between VWF115 and ADAMTS13 were stopped at multiple time points, and the percentage proteolysis in each sample was determined by HPLC; this was

plotted as a function of time to calculate the initial rate of proteolysis, and used to perform kinetic analysis. In theory, this approach would work for my project, since generating VWF115 variants could be performed in-house, and no further optimisation of the HPLC method should be required for working with high substrate concentrations. However, this technique is laborious and time-consuming, as it involves analysing one sub-sample at a time. Considering the number of substrate variants to be characterised, and the large number of reactions that would need to be performed, this approach was judged overly time-consuming.

To circumvent this issue, I chose to develop a new ELISA-based assay that allows quantitative analysis of ADAMTS13 activity. To do this, I aimed at fusing different tags to the two termini of VWF96 (N-terminal SUMO and C-terminal HSV peptides), which could then be exploited for specific detection of the full-length, uncleaved substrate in the ELISA. Assays could then be performed, in which reactions containing ADAMTS13 and VWF96 are stopped at multiple time points. The amount of uncleaved VWF96 at each time point could be measured to derive the percentage proteolysis by ELISA. The ELISA-based approach allows for analysis of multiple reaction sub-samples in parallel, and would therefore be a more efficient and less laborious way of performing kinetic analysis than the abovementioned alternative techniques. The N-terminal SUMO tag would also assist in both expression and solubility. Additionally, this method would allow reactions to be performed at high substrate concentrations, since remarkable reductions in the proteolytic rates were anticipated as a result of the mutations in the functional ADAMTS13 exosite binding sites in VWF96.

In this chapter, I describe the cloning, bacterial expression and purification of VWF96, its quantification by extinction coefficients and the qualitative assessment of its proteolysis by ADAMTS13. I also describe the development of the VWF96 ELISA, and assays showing how this ELISA can monitor VWF96 proteolysis and quantify the catalytic efficiency.

3.2 Cloning of VWF96 cDNA into the pET SUMO bacterial expression vector

Bacterial expression vectors expressing VWF96 were generated as described in section 2.1.1 and 2.1.2. The cDNA encoding VWF96 (G1573-R1668) was amplified by PCR (section 2.1.1.1), from a template cDNA encoding a longer VWF A2 domain fragment, VWF115 (E1554-R1668) ('VWF96 PCR amp' primers in Table A, Appendix I). The amplified VWF96 cDNA was separated from the rest of the PCR components by agarose gel electrophoresis on a 1.2% agarose gel (section 2.1.1.2), and the single predominant band migrating at the expected size (288bp)(Figure 3-1) was excised for gel DNA extraction (section 2.1.1.3).

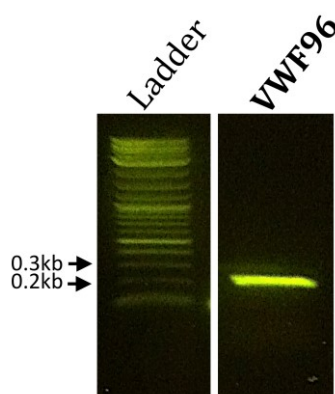


Figure 3-1 Agarose gel electrophoresis of VWF96 cDNA PCR amplification

The products of VWF96 cDNA PCR amplification were electrophoresed at 100V for 35 minutes, through a 1.2% agarose gel. Quick-Load 2-Log DNA ladder was used as a reference for the molecular size. A single predominant band was visible, which was consistent with the molecular size of VWF96 (288 bp).

The amplified VWF96 cDNA was cloned into the pET SUMO bacterial expression vector. *Taq* polymerase amplification added single 3' A overhangs to the VWF96 cDNA; this facilitated ligation into the TA cloning site of the linear pET SUMO vector, which possessed single 3' T overhangs (section 2.1.1.4). The VWF96-pET SUMO vectors were transformed into One Shot Mach-1 Chemically Competent *E. coli* (section 2.1.1.5), isolated using the QIAprep Spin Miniprep kit (section 2.1.1.6) and verified by sequencing ('Sequencing' primers in Table A, Appendix I).

The pET SUMO vector fused N-terminal HisG and SUMO tags to the VWF96 peptide. The SUMO tag would assist in expression and solubility, as well as be used for antibody detection of the N-terminus of VWF96 in the development of the ELISA-based assay that monitors VWF96 proteolysis. The HisG tag would be used for the purification of VWF96 on a Ni²⁺-charged

HiTrap Chelating HP column, and for alternative antibody detection of the N-terminus of VWF96 in the ELISA-based assay that monitors proteolysis. A C-terminal tag was needed for antibody detection in the VWF96 ELISA; I chose the short, 12aa HSV tag, which would be fused to VWF96 by subcloning the HisG-SUMO-VWF96 cDNA fragment into the pET-25b(+) vector.

3.3 Subcloning of HisG-SUMO-VWF96 cDNA into the pET-25b(+) by restriction digests

A restriction digest strategy was followed for subcloning the HisG-SUMO-VWF96 cDNA fragment into the pET-25b(+) vector. The NdeI restriction site was used, which was present both in the VWF96-pET SUMO vector (at the 5' end of the HisG-SUMO-VWF96 sequence), as well as in the multiple cloning site of the pET-25b(+). However, there was no second common restriction site in the two vectors that could facilitate the cloning at the 3' end of the HisG-SUMO-VWF96 cDNA. I introduced a 3' XhoI restriction site (already present in the pET-25b(+)) immediately downstream of the HisG-SUMO-VWF96 cDNA by PCR amplification, using the VWF96-pET SUMO vector as template (section 2.1.2.1)('VWF96-XhoI' primers in Table A, Appendix I). The PCR reaction was analysed by electrophoresis on a 1.2% agarose gel (section 2.1.1.2); the single DNA band (743bp) corresponded to the amplified HisG-SUMO-VWF96 cDNA flanked by 5' NdeI and 3' XhoI restriction sites (Figure 3-2A). Restriction digests were set up to generate complementary sticky ends in both the amplified HisG-SUMO-VWF96 cDNA fragment and the pET-25b(+) vector (section 2.1.2.2). The digested HisG-SUMO-VWF96 insert (~650bp) and pET-25b(+) vector (~5000bp) were isolated by agarose gel electrophoresis (section 2.1.1.2)(Figure 3-2B) and extracted from the gel (section 2.1.1.3). DNA ligation reactions were performed (section 2.1.2.3), which were used to transform NEB Turbo competent *E.coli*. pET-25b(+) possesses an ampicillin resistance gene, therefore, 100µg/ml ampicillin was used in the culture media for selecting successfully transformed bacteria. To distinguish colonies transformed with pET-25b(+) vectors that were successfully cloned with the HisG-SUMO-VWF96 insert, as opposed to empty, self-ligated pET-25b(+) vectors, multiple colonies were screened by PCR amplification (section 2.1.2.3)('PCR screening' primers in Table A, Appendix I) and analysed by agarose gel electrophoresis (section 2.1.1.2). Empty vectors produced bands of ~211bp, while successfully cloned pET-25b(+) vectors produced bands of ~950bp, due to the insertion of the HisG-SUMO-VWF96 cDNA (Figure 3-2C). The positively screened colonies were expanded and the plasmids isolated using 100µg/ml ampicillin in the culture media (section 2.1.1.6), and successful subcloning verified by sequencing ('Sequencing' primers in Table A, Appendix I).

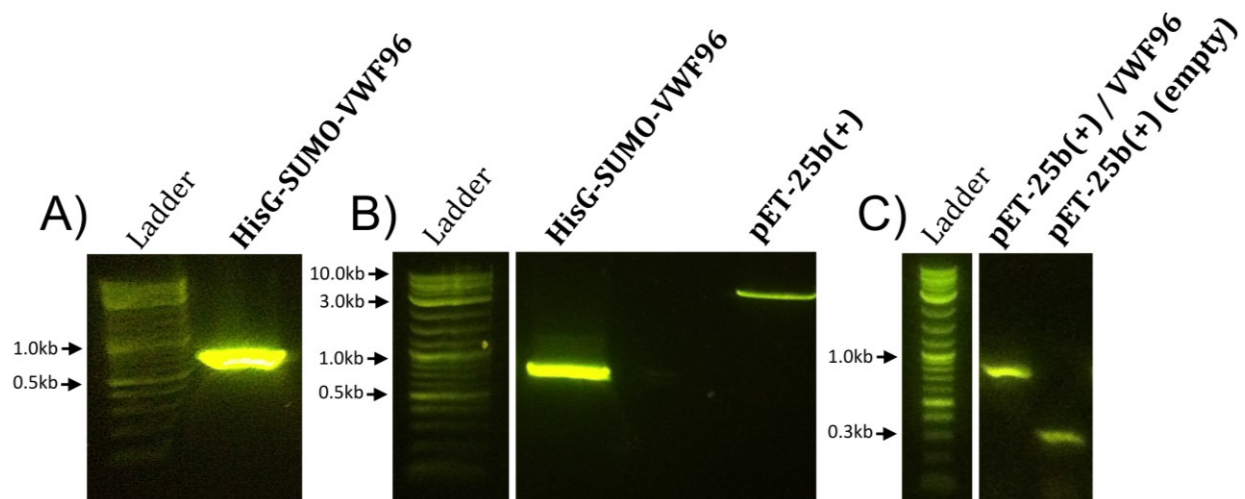


Figure 3-2 Subcloning of HisG-SUMO-VWF96 cDNA from the pET-SUMO vector to the pET-25b(+)

All samples were electrophoresed at 100V for 35 minutes, through a 1.2% agarose gel. Quick-Load 2-Log DNA ladder was used as a reference for the molecular size. (A) The cDNA encoding HisG-SUMO-VWF96 (~743bp) was amplified from the VWF96-pET SUMO vector. The PCR amplification introduced a 3' XhoI restriction site. (B) The amplified HisG-SUMO-VWF96 cDNA (~650bp) and the pET-25b(+) vector (~5000bp) were digested using XhoI and NdeI restriction enzymes. (C) Ligation reactions were set up between the digested HisG-SUMO-VWF96 cDNA and pET-25b(+) vector. The ligation products were transformed into NEB Turbo competent *E. coli*. Colonies were picked, and screened by PCR amplification. The PCR product from the successfully cloned pET-25b(+) vectors (~950bp) was distinguished from the PCR product from empty pET-25b(+) vectors (~211bp) based on size.

3.4 Expression and purification of VWF96

VWF96 was expressed in 2.5L of BL21 (DE3) *E. coli*. Bacteria were harvested and lysed using the BugBuster reagent mixed with Benzonase, Chicken Egg White Lysozyme and a Protease Inhibitor Cocktail (section 2.1.2.5).

VWF96 was purified from the soluble bacterial fraction in two steps (section 2.1.2.6). Firstly, immobilised metal ion affinity chromatography was used to purify 6xHis-tagged proteins. The sample was passed over a HiTrap Chelating HP column charged with Ni^{2+} . 6xHis-tagged proteins (VWF96 has an N-terminal HisG tag and a C-terminal 6xHis tag) bind to the Ni^{2+} on the column, while the rest of the soluble bacterial proteins remain in the flow-through. To elute the immobilised VWF96 from the column, 300mM Imidazole was used. The eluted material was analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8). The predominant band at ~28kDa corresponds to VWF96. The presence of both higher and lower molecular weight impurities in the eluted sample prompted me to use a second purification step in order to increase the purity of VWF96 (Figure 3-3B+C).

I purified the VWF96 further by anion exchange chromatography, using the HiTrap Capto Q Impres column (section 2.1.2.6). The column contains quaternary ammonium that is a strong anion exchanger (positively charged). Negatively charged proteins bind to the column with different affinities. Increasing NaCl concentrations (0.05-2M) were used to elute the proteins; the Cl^- in the salt solution displace the proteins on the column. The elution fractions were analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8). VWF96 was eluted with 540mM NaCl. Lower molecular weight impurities (than VWF96) were eluted from the column at lower salt concentrations, while higher molecular weight impurities were eluted from the column using 2M NaCl. The purity of the eluted VWF96 was estimated to be >95% (Figure 3-3D+E). This meant that its concentration could be determined by absorbance using extinction coefficients. The expression yield of VWF96 was predicted to be ~25mg per litre of bacterial culture, and the concentration of purified material was ~320 μM .

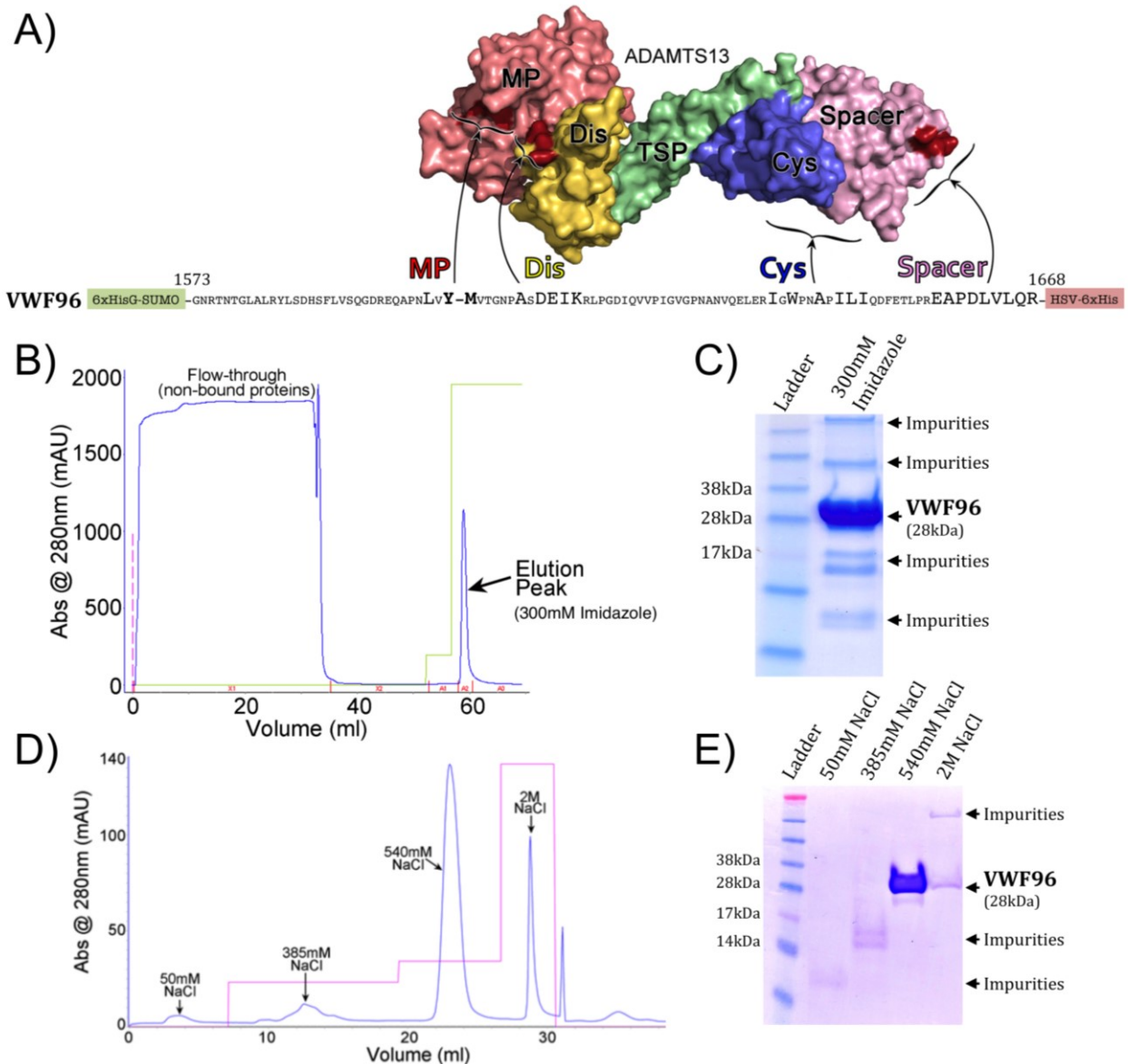


Figure 3-3 VWF96 purification

(A) The amino acid sequence of VWF96 (G1573-R1668). VWF96 possesses N-terminal HisG and SUMO tags, and C-terminal HSV and 6xHis tags. The scissile bond, Y1605-M1606, is shown in bold. Enlarged residues indicate the predicted binding sites for ADAMTS13 MP, Dis, Cys-rich and Spacer domain exosites. (B) Following expression, VWF96 was purified from the soluble fraction of bacterial cell lysates by immobilised metal ion affinity chromatography using HiTrap Chelating HP columns charged with NiSO₄. VWF96 had affinity for the column due to the possession of the HisG and 6xHis tags. Unbound bacterial proteins remained in the flow-through (labelled on the chromatogram). VWF96 was eluted using 300mM Imidazole (arrow on the chromatogram). (C) The eluted material was analysed by SDS-PAGE and Coomassie staining. The predominant band at ~28kDa corresponds to VWF96. Other bands correspond to impurities. (D) The partially purified VWF96 was purified further using the Capto Q Impres column (positively charged). Negatively charged proteins, including VWF96, bound to the column. Elution steps were performed using 0.5-2M NaCl (elution peaks labelled on the chromatogram). VWF96 was eluted using 540mM NaCl. (E) The analysis of the eluted fractions by SDS-PAGE and Coomassie staining showed that VWF96 (28kDa) was >95% pure in the 540mM eluted fractions, and that all impurities were successfully separated in the rest of the eluted fractions.

3.5 Qualitative assessment of VWF96 proteolysis by ADAMTS13

VWF96 is a new VWF A2 domain-derived peptide, and its suitability as a substrate for ADAMTS13 has not been validated. Both longer (VWF115, E1554-R1668) and shorter (VWF76, S1593-R1668) ADAMTS13 substrates had been proteolysed with similar catalytic efficiencies in prior studies.⁽²⁵⁶⁾ It was unlikely that the length of VWF96 would affect the rate of proteolysis, since the difference in length did not interfere with any known functionally important sites. However, VWF96 possesses N- and C-terminal tags, which could potentially influence proteolysis.

I performed parallel time course cleavage assays using 0.5nM ADAMTS13 in conditioned media and 3µM of either VWF96 or VWF115, to ensure that the specificity and rate of VWF96 proteolysis had not been affected (section 2.3.1). ADAMTS13 and the two substrates were preincubated separately in TBSC buffer, at 37°C for 15 minutes. Reactions were initiated by addition of the substrates to the enzyme, and stopped in 55mM EDTA between 0-90 minutes. Stopped sub-samples were analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8)(Figure 3-4). At 0 minutes, only single bands were visible at 28kDa (Lane 1) and 16.9kDa (Lane 6), representing uncleaved VWF96 and VWF115, respectively. The higher molecular weight of VWF96 (despite being a shorter VWF A2 domain fragment) than VWF115 is due to the possession of the N-terminal SUMO (~12kDa) tag. Specific cleavage was visible at 30 minutes, as the specific cleavage products for both substrates emerged. Cleavage of VWF115 produced the expected 10kDa N-terminal cleavage product (but not the 7kDa C-terminal cleavage product)⁽²⁶⁶⁾. Cleavage of VWF96 produced the expected 18kDa N-terminal cleavage product (but not the 10kDa C-terminal cleavage product). The C-terminal cleavage products of both substrates have the same primary structure (but different tags); the inability to detect any of the C-terminal cleavage products suggests inefficient staining of smaller peptides. The two substrates were proteolysed at similar rates, since the bands corresponding to the uncleaved substrates disappeared, and the bands corresponding to the cleavage products emerged, at similar rates. This assay showed that VWF96 could be cleaved specifically by ADAMTS13, and the possession of N- and C-terminal tags did not affect the rate of proteolysis.

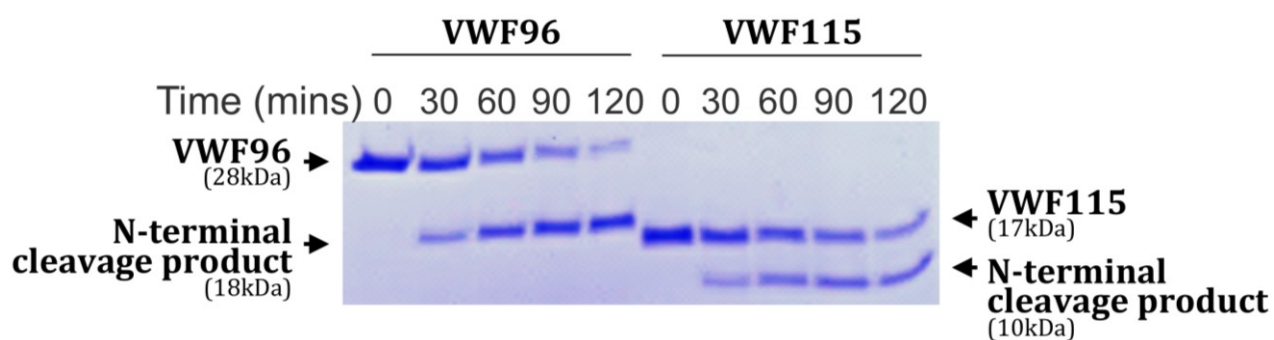


Figure 3-4 Qualitative assessment of VWF96 and VWF115 proteolysis by ADAMTS13

3 μ M VWF96 or VWF115 were incubated with 0.5nM ADAMTS13 (concentrated in conditioned medium). Reaction sub-samples were stopped in 55mM EDTA between 0-90 minutes. The sub-samples were analysed by SDS-PAGE and Coomassie staining. At 0 minutes, the single bands at 28kDa (Lane 1) and 17kDa (Lane 6) correspond to uncleaved VWF96 and VWF115, respectively. As the VWF96 and VWF115 are proteolysed with time, these bands diminish in intensity, at similar rates. Proteolysis of VWF96 produces 18kDa (N-terminal) and 10kDa (C-terminal) cleavage products. Proteolysis of VWF115 produces 10kDa (N-terminal) and 7kDa (C-terminal) cleavage products. In both reactions the C-terminal cleavage products are not visible on the gel. The rate of appearance of the N-terminal cleavage product is similar in both reactions. This assay shows that VWF96 and VWF115 are cleaved by ADAMTS13 with the same efficiency.

3.6 VWF96 ELISA development and optimisation

Multiple attempts were required to develop and optimise an ELISA protocol that would sensitively and accurately detect VWF96. To do this, the N- and C-terminal tags fused on VWF96 were exploited; VWF96 would be captured on a 96-well plate using antibodies against one of its tags and detected using antibodies against the tag on the opposite terminus. I used different commercially available antibodies against the SUMO and HSV tags, to find a combination with satisfactory affinity to the tags. I tried both orientations and different concentrations of the antibodies, and aimed at finding a range of VWF96 concentrations that was linearly associated with absorbance.

Certain conditions (coating conditions, washing buffer and conditions, blocking buffer and conditions, buffers used for antibody and VWF96 sample dilutions, detection) remained fixed during the optimization of the ELISA protocol (detailed in section 2.1.2.11).

Table 3-1 summarises all the conditions that were varied for the development and optimization of the VWF96 ELISA. The first antibody combination assessed was coating a rabbit pAb against the SUMO tag (A00640, GenScript) and detecting with a goat pAb antibody against the HSV tag (ab19354, abcam), followed by a secondary mAb anti-goat/sheep IgG conjugated with HRP (A9452-1VL, Sigma). I used 2.5µg/ml rabbit pAb anti-SUMO antibody (A00640, GenScript), 0.5µg/ml goat pAb anti-HSV antibody (ab19354, abcam) and 0-100nM VWF96, and allowed colour development for 20 minutes (**ELISA #1**, Table 3-1). There was a positive (non-linear) association between VWF96 concentration and absorbance up to ~10nM VWF96; after this point the signal plateaued. The maximum absorbance reached was ~0.25Au, which was too low for a sensitive ELISA assay. In subsequent attempts (**ELISA #2-3**, Table 3-1), I increased the concentration of rabbit pAb anti-SUMO antibody (A00640, GenScript) up to 15µg/ml, and the concentration of goat pAb anti-HSV antibody (ab19354, abcam) up to 1.33µg/ml. However, these conditions did not improve the sensitivity of the ELISA, as the maximum absorbance reached was still too low (<0.15 Au) for sensitively detecting VWF96 (Figure 3-5).

ELISA #3

Coating: Rabbit pAb anti-SUMO (GenScript)

Detection: Goat pAb anti-HSV (abcam)

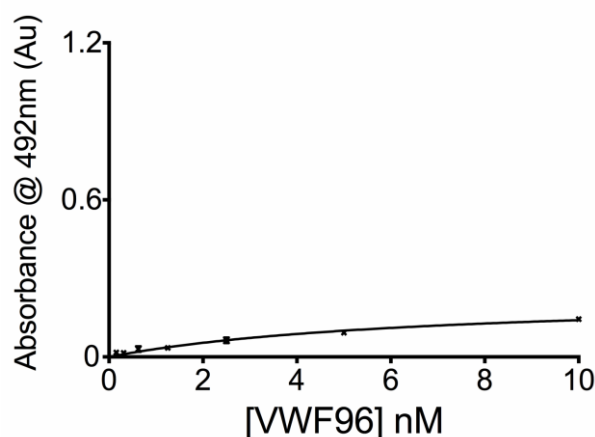


Figure 3-5 VWF96 ELISA optimisation – ELISA #3

15µg/ml rabbit pAb anti-SUMO antibody (A00640, GenScript) was coated on a 96-well plate to capture 0-10nM VWF96. 1.33µg/ml goat pAb anti-HSV antibody (ab19354, abcam) and mAb anti-goat/sheep IgG (HRP) were used to detect bound VWF96. After 20 minutes of substrate development, a poor signal <0.15Au indicated that an ELISA protocol using this antibody combination was insensitive.

I reversed the orientation of the antibodies (**ELISA #4-5**, Table 3-1), coating goat pAb anti-HSV antibody (ab19354, abcam)(up to 4µg/ml) and detecting with and rabbit pAb anti-SUMO antibody (A00640, GenScript)(up to 1µg/ml), followed by goat pAb anti-rabbit antibodies conjugated with HRP (P0448, Dako). This orientation did not improve the sensitivity of the ELISA, as saturation occurred at low absorbance (<0.35 Au), and a linear association between the assessed range of VWF96 concentrations and absorbance could not be established.

The lack of sensitivity indicated that the rabbit pAb anti-SUMO antibody (A00640, GenScript) and/or the goat pAb anti-HSV antibody (ab19354, abcam) had low affinity against the tags. To investigate this, I used a mouse monoclonal antibody against the C-terminal epitope (VWF1606-1665) of the human VWF A2 domain (anti-CtermA2)(MAB2764, R&D Systems) to capture VWF96, in combination with 1 μ g/ml rabbit pAb anti-SUMO antibody (A00640, GenScript)(**ELISA #6**, Table 3-1)(Figure 3-6A) or 1.33 μ g/ml goat pAb anti-HSV antibody (ab19354, abcam)(**ELISA #7**, Table 3-1)(Figure 3-6B), followed by their respective secondary antibodies, for detection. The sensitivity of the anti-CtermA2 in ELISA had been established previously. The two ELISA assays were performed in parallel using the same conditions, and the same range of VWF96 concentrations (0-10nM). Using the rabbit pAb anti-SUMO antibody (A00640, GenScript) for detection resulted in weak signal (<0.35Au), even after allowing colour development for 14 minutes. On the other hand, using the goat pAb anti-HSV antibody (ab19354, abcam) for detection produced a strong signal (up to 1Au) in only 3 minutes. This indicated that the low affinity of the rabbit pAb anti-SUMO antibody (A00640, GenScript) for the SUMO tag was probably the limiting factor in developing a sensitive VWF96 ELISA assay.

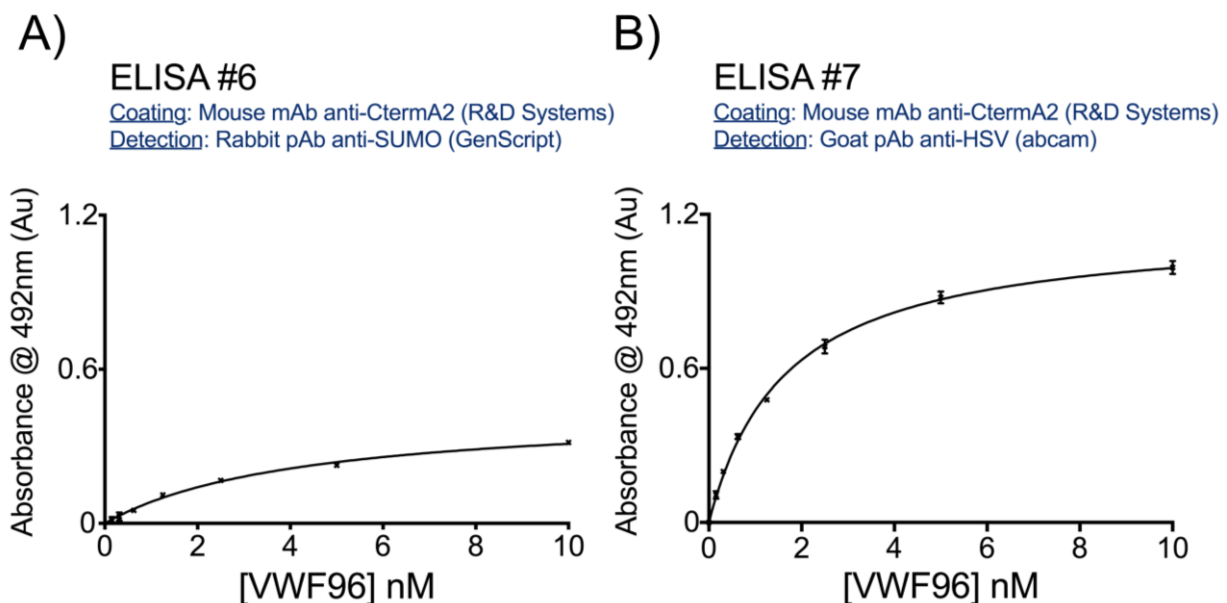


Figure 3-6 VWF96 ELISA optimisation – ELISA #6 and #7

The anti-CtermA2 was coated to capture 0-10nM VWF96. (A) When 1 μ g/ml rabbit pAb anti-SUMO antibody (A00640, GenScript) followed by goat pAb anti-rabbit (HRP), were used to detect VWF96, a weak signal (<0.35Au) was generated after 14 minutes of colour development. (B) In contrast, using 1.33 μ g/ml goat pAb anti-HSV antibody (ab19354, abcam) followed by mAb anti-goat/sheep IgG (HRP) to detect VWF96 produced a strong signal (~1Au) after only 3 minutes of colour development. These results indicate that the pAb anti-SUMO antibody (A00640, GenScript) had low affinity for the SUMO tag of VWF96, which compromised the sensitivity of the VWF96 ELISA.

I proceeded by changing the antibody against the SUMO tag. I used the rabbit pAb anti-Smt3 antibody (ab14405, abcam). I tried capturing 0-100nM VWF96 using 1.33µg/ml goat pAb anti-HSV antibody (ab19354, abcam) and detecting with 1µg/ml rabbit pAb anti-Smt3 antibody (ab14405, abcam), followed by goat pAb anti-rabbit antibodies conjugated with HRP (P0448, Dako) (**ELISA #8**, Table 3-1). Again, after allowing 20 minutes for colour development, the signal was poor (<0.2Au), which suggested that the rabbit pAb anti-Smt3 antibody (ab14405, abcam) had also low affinity against the SUMO tag. As previously, I confirmed this by using the anti-CtermA2 to capture VWF96, and detecting with either 1µg/ml rabbit pAb anti-Smt3 antibody (ab14405, abcam) or 1.33µg/ml goat pAb anti-HSV antibody (ab19354, abcam), followed by their respective secondary antibodies (**ELISA #9** and **#10**, respectively, Table 3-1). Again, the rabbit pAb anti-Smt3 antibody (ab14405, abcam) seemed to be the insensitive antibody, as, after allowing 20 minutes for colour development, the signal plateaued at ~0.3Au. In contrast, detecting with goat pAb anti-HSV antibody (ab19354, abcam) produced a strong signal (~1Au) in only 4 minutes, confirming the higher affinity of this antibody for the HSV tag.

I changed the antibody against the SUMO tag to chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors). Despite the previous experiments suggesting that no affinity issues were associated with the goat pAb anti-HSV antibody (ab19354, abcam), I also changed this antibody to a goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) for detection. This would reduce the washing steps in the ELISA protocol, as no secondary antibody would be required, which could minimise the loss of the VWF96 associated with washing, and, thus, increase the signal. Furthermore, one detection step would make a shorter ELISA assay, which would be more convenient.

I first tested 5µg/ml chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) for capturing 0-150nM VWF96, and 0.1µg/ml goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) for detection (**ELISA #11**, Table 3-1)(Figure 3-7A). Colour development was stopped at 3 minutes, and a signal up to 1Au was measured; this indicated that this antibody combination had significantly improved sensitivity in detecting VWF96, compared to all other antibody combinations tested. However, signal saturation was reached at ~10nM VWF96, after which point the absorbance stopped increasing with increasing concentrations of VWF96. I further optimized this protocol to identify the range of VWF96 concentrations that is linearly associated with absorbance.

I repeated the ELISA twice, using 0-5nM (**ELISA #12**, Table 3-1) and 0-2nM (**ELISA #13**, Table 3-1) VWF96. I also reduced the chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) concentration to 0.5µg/ml, as this would make this protocol more cost-effective. Colour development was stopped at 5 minutes, resulting in strong signals up to ~0.7Au. However, the association was still not linear, with signal saturation above 0.75nM VWF96.

In the final attempt, (**ELISA #14**, Table 3-1)(Figure 3-7B), I reduced the VWF96 concentration range (0-0.2nM), and also increased the concentration of the goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) by 5 times (0.5µg/ml). Colour development was stopped at 5 minutes. A strong signal was obtained (up to ~1.13Au), which was linearly associated with the VWF96 concentration. The antibody combination, the protocol conditions used, and the VWF96 concentration range made a sufficiently sensitive ELISA assay that detects VWF96. The full protocol outlining the optimal ELISA conditions is described in section 2.1.2.11.

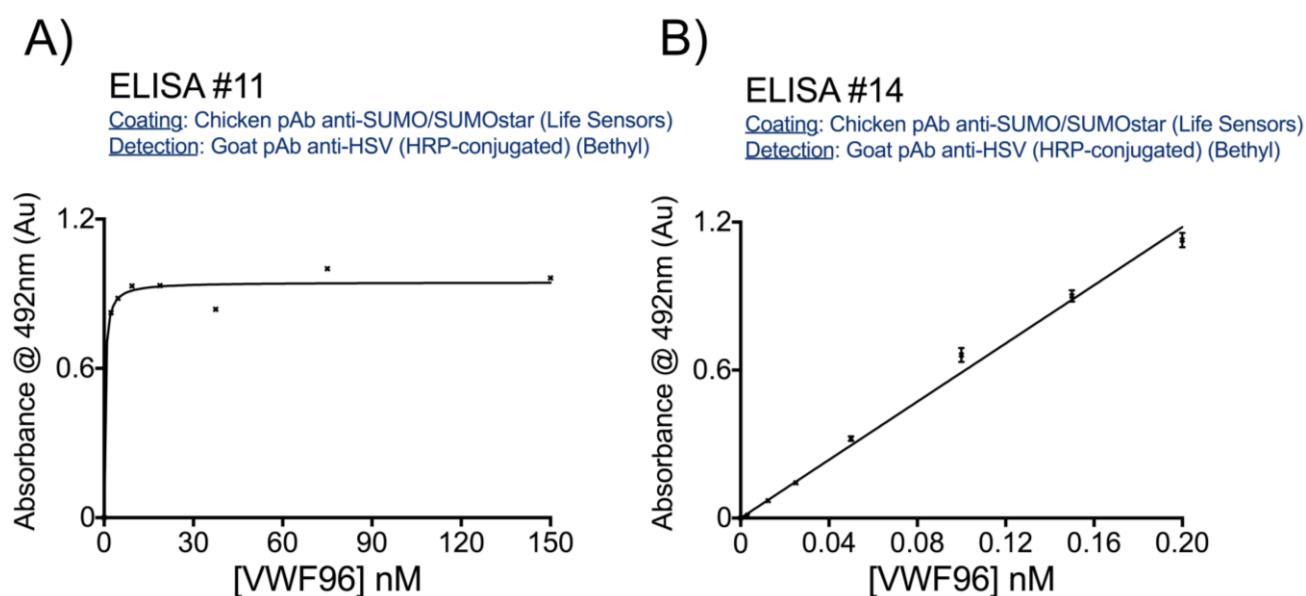


Figure 3-7 VWF96 ELISA optimisation – ELISA#11 and ELISA#14

(A) 5µg/ml chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) was used to capture 0-150nM VWF96, and 0.1µg/ml goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) was used for detection. A strong signal (~1Au) was generated after 5 minutes of substrate development, indicating that both antibodies bound VWF96 with sufficiently high affinities for a sensitive ELISA. The positive association between VWF96 concentration and absorbance is lost above ~10nM VWF96. (B) The ELISA conditions were optimized to obtain a linear positive association between VWF96 concentration and absorbance. 0.5µg/ml chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) was used to capture 0-0.2nM VWF96, and 0.5µg/ml goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) was used for detection. A strong signal was generated (>1Au) after ~5 minutes of substrate development. A linear positive association between VWF96 and absorbance was achieved.

Table 3-1 VWF96 ELISA optimisation (varied conditions)

CONDITIONS	ELISA #													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Coating antibody (µg/ml)	Rabbit pAb anti-SUMO (GenScript)			Goat pAb anti-HSV (abcam)		anti-CtermA2 (R&D Science)		Goat pAb anti-HSV (abcam)	anti-CtermA2 (R&D Science)		Chicken pAb α-SUMO/SUMOstar (Life Sensors)			
	2.5	4	15	2	4	1:200		1.33	1:200		5	0.5	0.5	0.5
Maximum [VWF96] (nM)	100	2	10	100	2	10		100			150	5	2	0.15
1° antibody (µg/ml)	Goat pAb α-HSV (abcam)			Rabbit pAb anti-SUMO (abcam)		Rabbit pAb anti-SUMO (abcam)	Goat pAb α-HSV (abcam)	Rabbit pAb anti-Smt3 (abcam)		Goat pAb α-HSV (abcam)	Goat pAb α-HSV (hrp) (Bethyl)			
	0.5	1	1.33	0.5	1	1	1.33	1		1.33	0.1			0.5
2° antibody	anti-goat/ sheep (hrp)			anti-rabbit (hrp)		anti-rabbit (hrp)	anti-goat/ sheep (hrp)	anti-rabbit (hrp)		anti-goat/ sheep (hrp)				
Develop time (min)	20	20	20	20	20	14	3	20	20	4	3	5	5	5
Maximum Absorbance (Au)	0.26	0.19	0.14	0.20	0.34	0.32	0.99	0.18	0.28	1.06	0.96	0.59	0.72	1.13

3.7 Analysis of VWF96 proteolysis by ELISA

A full-length VWF96 ELISA was developed to monitor the proteolysis of VWF96 by ADAMTS13, and ultimately, as a tool to characterise the kinetics of proteolysis. To verify that the VWF96 ELISA could reproducibly quantify VWF96 in cleavage assays, both sensitively and specifically, I incubated 0.5 μ M VWF96 with or without 0.75nM ADAMTS13, in 1% BSA/TBSC buffer, and stopped reaction sub-samples in 25mM EDTA between 0-90 minutes (section 2.3.2.1). The stopped sub-samples were diluted to 0.09nM VWF96 and analysed by VWF96 ELISA using the chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors). As the ELISA only detects full-length VWF96 (rather than cleaved), I predicted that, with time, cleavage of VWF96 would cause signal reduction. I used 0-0.2nM VWF96 to generate a standard curve, which was used to perform interpolation for quantifying uncleaved VWF96 at each time point. The concentration of VWF96 was plotted as a function of time, and one-phase exponential decay regression was performed in Graphpad Prism (Figure 3-8A). At 0 minutes, no proteolysis had occurred, therefore the concentration of VWF96 is the highest, at 0.09nM; this was expected as all sub-samples were diluted to 0.09nM VWF96. In the presence of ADAMTS13, the signal decreased with time, reaching ~0Au/0nM VWF96 at 90 minutes, indicating that the reaction had reached completion and all VWF96 had been proteolysed by ADAMTS13. The fact that the signal plateaued at 0Au/0nM VWF96 (and not above) verified that this ELISA only detected uncleaved VWF96 (and not any other assay component). In the absence of ADAMTS13, there was no reduction in the absorbance/VWF96 concentration over time, with the regression line converging at 0.09nM; this also verified the specificity of this ELISA-based assay for monitoring VWF96 proteolysis.

Using the same protocol, I performed 16 independent reactions using 0.75nM ADAMTS13 and VWF96 at 0.25 μ M (n=4), 0.5 μ M (n=4), 0.75 μ M (n=4) and 1 μ M (n=4), stopping eight sub-samples for each reaction (note: all VWF96 concentrations used were lower than the previously determined K_m for VWF fragment proteolysis, since the equation for deriving k_{cat}/K_m based on proteolytic time course analysis is only valid at substrate concentrations lower than the K_m of proteolysis). The concentration in each sub-sample was used to calculate the fraction of VWF96 proteolysed and was plotted against time (in seconds). As expected, the fraction of proteolysis increased to ~100% at 90 minutes (5400 seconds), indicating reaction completion (Figure 3-8B). I analysed the reaction time course to quantify the catalytic

efficiency, k_{cat}/K_m , of VWF96 proteolysis, by fitting the data in the equation $P = 1 - \exp(-1 \times [ADAMTS13] \times t \times k_{cat}/K_m)$, where P is the fraction of proteolysed substrate, $[ADAMTS13]$ is the concentration of ADAMTS13 in μM , t is the time in seconds, and k_{cat}/K_m is the catalytic efficiency in $\mu\text{M}^{-1}\text{s}^{-1}$. The k_{cat}/K_m of VWF96 proteolysis was $110 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=16$), which was in good agreement with previously published values for similar VWF A2 domain-derived peptide substrates.^(46, 242, 252, 267) These results demonstrated the suitability of the VWF96 ELISA as a means of quantitatively analysing the time course of VWF96 proteolysis.

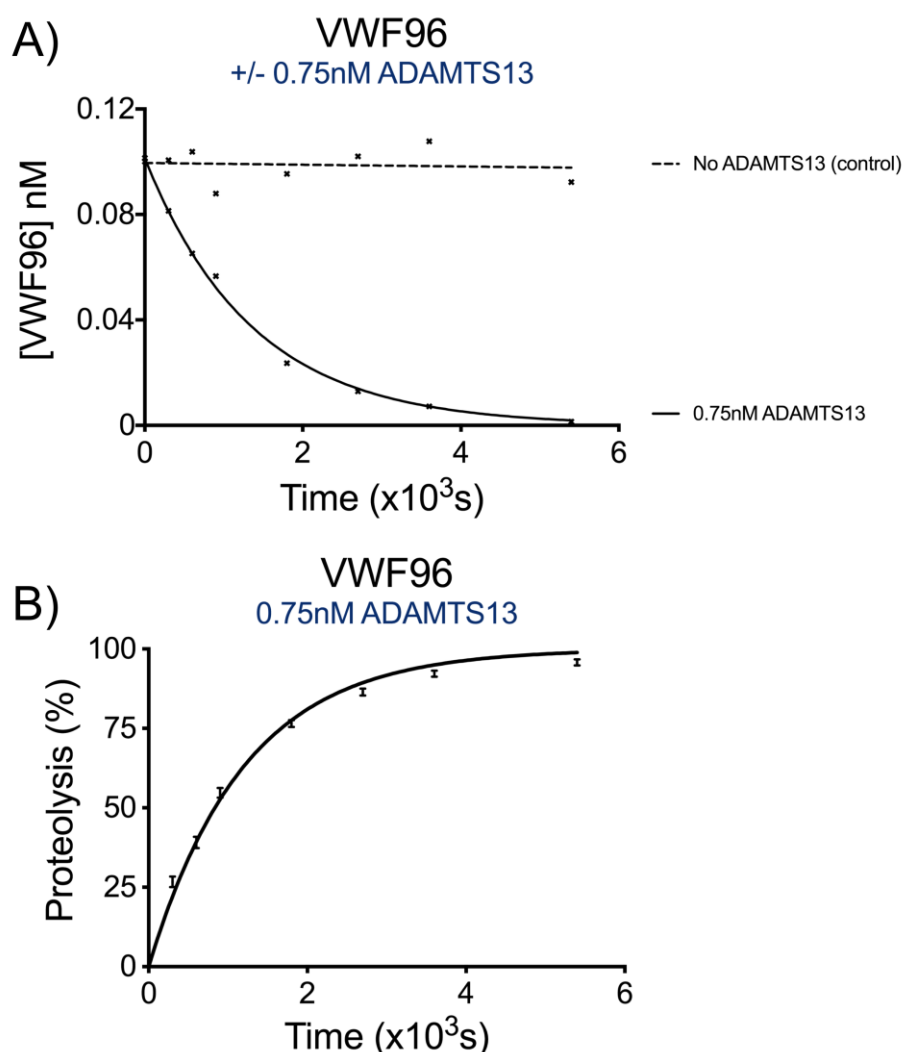


Figure 3-8 Analysis of VWF96 proteolysis using VWF96 ELISA

(A) VWF96 (0.5 μM) was incubated with or without ADAMTS13 (0.75nM). Reaction sub-samples were stopped between 0-90 minutes and analysed using the VWF96 ELISA. In the presence of ADAMTS13 (solid line), the concentration of VWF96 decreased with time, indicating proteolysis. The reaction is complete ($\sim 0\text{nM}$ VWF96) at 90 minutes. In the absence of ADAMTS13 (dotted line), there is no reduction in the concentration of VWF96 since no proteolysis occurred; this confirmed that the ELISA specifically monitors the proteolysis of VWF96. (B) VWF96 proteolysis (%) by ADAMTS13 was plotted against time and the data fitted using the equation $P = 1 - \exp(-1 \times [ADAMTS13] \times t \times k_{cat}/K_m)$. The catalytic efficiency (k_{cat}/K_m) of VWF96 proteolysis was $110 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=16$).

3.8 An alternative VWF96 ELISA protocol for monitoring VWF96 proteolysis

Although the ELISA conditions using the chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) and goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) were optimized, as described above, I observed variability in the sensitivity of the ELISA, which was associated with different batches of the chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors). I therefore optimized another VWF96 ELISA as a 'back-up' protocol, in case the chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors) was no longer available. To do this, I used an alternative method to specifically detect the N-terminus of VWF96. Apart from the SUMO tag, VWF96 also possesses a second N-terminal tag, the HisG; this is 6xHis tag followed by a Gly residue. I used the commercially available mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific), which was validated for ELISA.

A potential limitation would be that this antibody had affinity for the C-terminal 6xHis tag. Therefore, before optimizing the ELISA protocol using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific), I verified that it detected the N-terminal HisG tag specifically. I performed an activity assay between 0.4nM ADAMTS13 and 0.5 μ M VWF96. Sub-samples were stopped and analysed by Western blot using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific), followed by the rabbit pAb anti-mouse IgG conjugated with HRP (Dako)(section 2.1.2.9). As can be seen in Figure 3-9A, no proteolysis had occurred at 0 minutes, and only a single species at ~28kDa was detected, corresponding to uncleaved VWF96. With time, VWF96 was proteolysed, producing a single additional band at ~18kDa, corresponding to the N-terminal cleavage product. This suggested that the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) only recognized the N-terminal HisG tag specifically, and had no affinity for the C-terminal 6xHis tag.

I then optimized an ELISA protocol using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) (instead of chicken pAb anti-SUMO/SUMOstar IgY (AB7002, Life Sensors)) to capture VWF96, and the goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) for detection (section 2.1.2.11). I used 1.18 μ g/ml mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) to capture 0nM, 0.1nM, 0.2nM, 0.4nM, 0.6nM, 0.8nM, 1nM and 1.2nM VWF96, and 0.5 μ g/ml goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) for detection. These conditions detected VWF96 sensitively, with absorbance reaching ~0.9Au in up to 10 minutes, which was linearly associated with VWF96 concentration (Figure 3-9B).

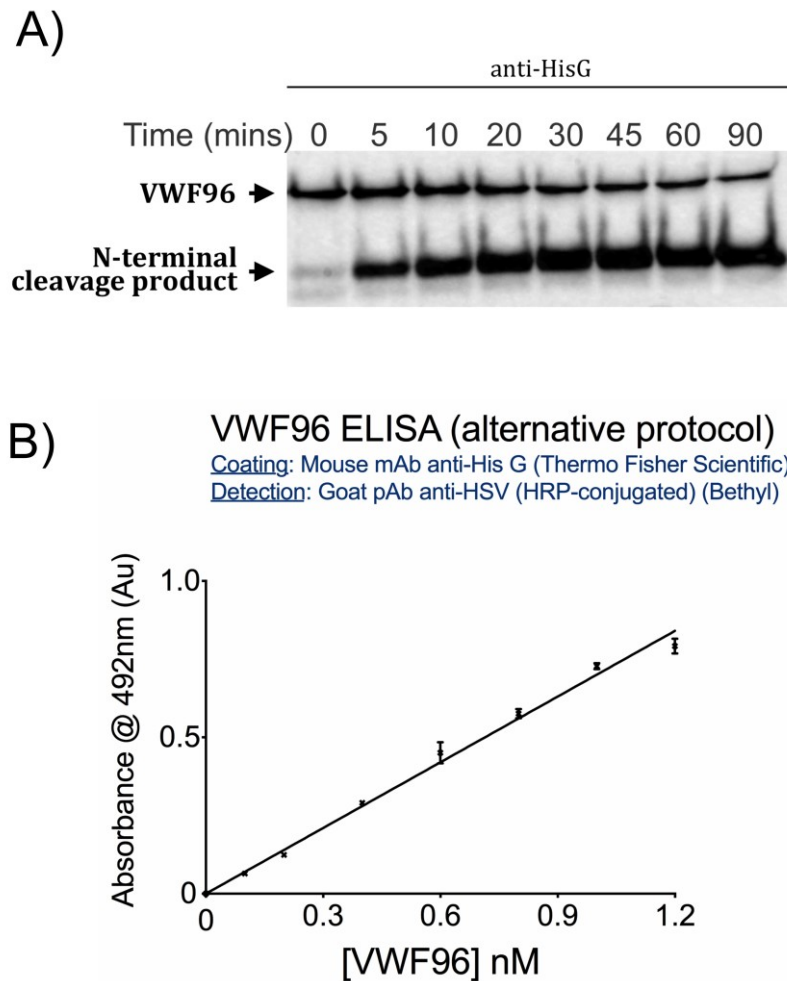


Figure 3-9 Optimisation of the VWF96 ELISA using anti-HisG antibody.

(A) Western blot analysis of VWF96 proteolysis using antibodies against the N-terminal HisG tag of VWF96. Stopped reaction sub-samples during VWF96 proteolysis by ADAMTS13 (0.4nM) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The HisG tag of VWF96 was detected using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) followed by HRP-conjugated rabbit pAb anti-mouse IgG antibody (Dako). Only the uncleaved VWF96 (~28kDa) and the N-terminal cleavage product (~18kDa) were detected, showing that the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) was specific for the HisG tag, and did not have affinity for the C-terminal 6xHis tag. (B) 1.18µg/ml mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) was used to capture 0-1.2nM VWF96, and 0.1µg/ml goat pAb anti-HSV antibody conjugated with HRP (A190-136P, Bethyl) was used for detection. A strong signal (~0.9Au) was generated within 10 minutes of colour development, which had a positive, linear association with VWF96 concentration, indicating the sensitivity and specificity of this ELISA protocol for VWF96 detection.

To confirm that VWF96 proteolysis can be monitored using this VWF96 ELISA protocol, activity assays were set up between 0.4nM ADAMTS13 and 0.5 μ M VWF96 (section 2.3.2.1). Sub-samples were stopped in EDTA between 0-90 minutes, diluted to 0.75nM VWF96 and analysed by ELISA (in duplicate) using the mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) for capturing VWF96. The concentration of VWF96 in each sub-sample was plotted against time and analysed by one-phase exponential decay regression (Figure 3-10A). The profile of proteolysis was similar to that seen previously, with reaction completion at ~90 minutes. Duplicate analysis of each sub-sample generated very similar absorbance readings, showing that no significant intra-assay variability was associated with this VWF96 ELISA protocol.

To quantify the rate of proteolysis using this VWF96 ELISA protocol, I repeated the assay three times, using 0.4nM ADAMTS13 and 0.5 μ M VWF96. The mean fraction of proteolysed substrate in each sub-sample was plotted as a function of time, and fitted as before (Figure 3-10B). The k_{cat}/K_m of $155 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ was very similar to the value obtained when the VWF96 ELISA (anti-SUMO protocol) was used for analysis ($110 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$), showing that the two ELISA protocols could be used interchangeably to monitor VWF96 proteolysis.

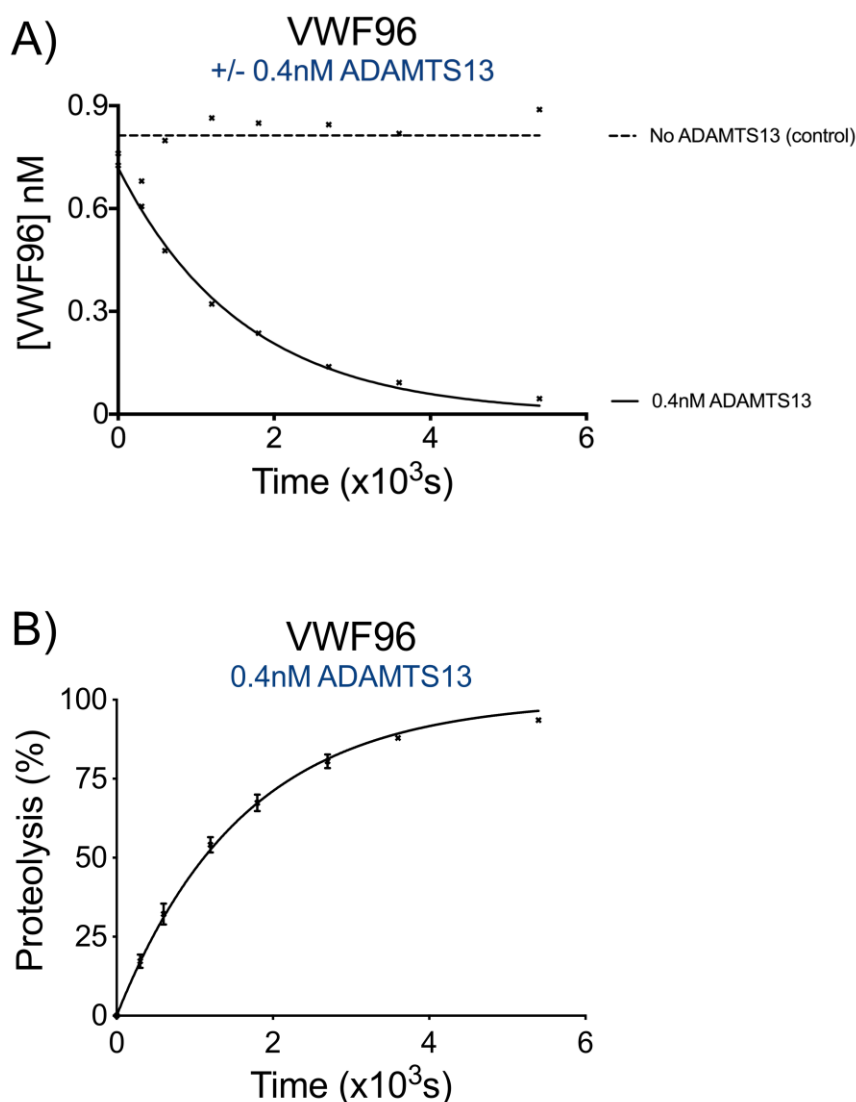


Figure 3-10 Analysis of VWF96 proteolysis using the VWF96 ELISA (anti-HisG)

(A) VWF96 (0.5 μ M) was incubated with or without ADAMTS13 (0.4nM). Reaction sub-samples were stopped in EDTA between 0-90 minutes, which were diluted to 0.75nM VWF96 and analysed using the VWF96 ELISA. The mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) was used to capture VWF96. In the presence of ADAMTS13 (solid line), the concentration of VWF96 decreased with time, indicating proteolysis. The reaction is complete ($\sim$ 0nM VWF96) at $\sim$ 90 minutes. In the absence of ADAMTS13 (dotted line), there is no reduction in the concentration of VWF96 since no proteolysis occurred; this confirmed that the ELISA specifically monitors the proteolysis of VWF96. (B) The fraction of VWF96 substrate proteolysed was plotted against time and the data fitted using the equation $P = 1 - \exp(-1 \times [\text{ADAMTS13}] \times t \times k_{\text{cat}}/K_m)$ (see section 3.7). The catalytic efficiency (k_{cat}/K_m) of VWF96 proteolysis was $155 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$).

3.9 DISCUSSION

The first aim of my project was to design and develop an ELISA-based assay that would allow me to characterise the kinetics of ADAMTS13 activity. In this chapter, I described how I generated VWF96, a new substrate for ADAMTS13, and the development of an ELISA assay that can specifically and sensitively detect VWF96 and monitor its proteolysis.

VWF96 (G1573-R1668) was derived from the A2 domain of VWF and contains the scissile bond and binding sites for ADAMTS13 exosites (Figure 3-3). This is similar to other VWF A2 domain-derived substrates for ADAMTS13, including VWF73 (D1596-R1668)^(235, 253, 296, 360, 372-374) and VWF115 (E1554-R1668),^(241, 242, 250, 252, 256, 266, 268, 362) which had been used in prior studies. VWF96 was cloned in bacterial expression vectors in two steps, in order to fuse the N-terminal HisG and SUMO tags and the C-terminal HSV and 6xHis tags. I successfully expressed VWF96 in 2.5L of *E. coli*, which was harvested from the soluble bacterial fraction (Figure 3-3B-E). One reason the SUMO tag was chosen was its ability to increase the expression yield and solubility of peptides; this would be of interest as high VWF96 concentrations would be required later for performing kinetic analysis. The affinity of the 6xHis tags for Ni²⁺ was exploited to purify the expressed VWF96 from the bacterial lysate by performing immobilised metal ion affinity chromatography. VWF96 bound to the Ni²⁺-charged column to separate it from the rest of the proteins in the bacterial lysate, and was then eluted using a high concentration of Imidazole. The presence of impurities in the eluted fractions (likely other bacterial proteins with affinity for Ni²⁺ and/or VWF96 cleavage products by bacterial proteases), suggested the need for a second purification step; therefore, VWF96 was purified to homogeneity using anion exchange chromatography. Due to its high purity (>95%), VWF96 could be quantified by measuring absorbance at 280nm and using extinction coefficients; based on this, the yield (~25mg per litre of bacterial culture) and molar concentration (~320μM) of VWF96 were satisfactorily high.

The first assessment of VWF96 as a suitable substrate for ADAMTS13 was performed qualitatively (Figure 3-4). I predicted that VWF96 would be proteolysed normally as it is longer than the minimal substrate for ADAMTS13, VWF73, by 23 N-terminal amino acids. This means that VWF96 possesses all functionally important sites for proteolysis, and the extra residues were not expected to induce any structural changes. In fact, even longer substrates than VWF96, like VWF115, retain their capacity to be cleaved specifically by ADAMTS13.

Perhaps my only concern with respect to VWF96 proteolysis was the fused tags at the two termini. In particular, the N-terminal SUMO tag is a relatively large polypeptide, ~12kDa, which could potentially interfere sterically with functional sites in VWF96; this was the reason for choosing to include the extra 23 amino acids at the N-terminus, and not fusing the SUMO tag on the minimal substrate, VWF73. The proteolysis of VWF96 and VWF115 (already available in the lab) by ADAMTS13, stopped at multiple time points, was analysed by SDS-PAGE and Coomassie staining. The assay was not sensitive enough to accurately quantify the rate of proteolysis. However, it could identify gross differences in the rates of proteolysis of different reactions, qualitatively. No major differences in the rates of VWF96 and VWF115 proteolysis were evident. This assay could inform on the specificity of VWF96 proteolysis reactions (ie. whether proteolysis occurred at the scissile bond). There was only one cleavage product, consistent with the molecular weight of the specific N-terminal cleavage product, which suggested that cleavage occurred at the scissile bond of VWF96. The absence of the C-terminal cleavage product was not concerning, since this was also absent in the case of VWF115 proteolysis; perhaps the sequence of this fragment is stained inefficiently or washed out of the gel, which had also been observed previously.

I developed an ELISA assay, which detects VWF96 specifically. To do this, I had to find a combination of antibodies with sufficiently high affinities for the SUMO and HSV tags of VWF96. I varied the antibody combinations, orientations and concentrations, as well as the concentration range of VWF96. This ELISA would be used to detect VWF96 during the time course of proteolysis; therefore, it needed to be equally sensitive in detecting both high (0% proteolysis) and low (>95% proteolysis) VWF96 concentrations in the same assay. For this, a strong signal was important, that exploits the entire absorbance range that the spectrophotometer could detect sensitively (~0.1-1.5Au). The chicken pAb anti-SUMO/SUMOstar (AB7002, Life Sensors) and the HRP-conjugated goat pAb anti-HSV (A190-136P, Bethyl) produced a signal above 1.0Au in less than 10 minutes, and were chosen for the optimal ELISA protocol. Having achieved a strong signal, I then attempted to find a range of VWF96 concentrations that were linearly associated with absorbance; this was important to minimise any intra-assay variability. 0-0.2nM VWF96 produced a linear ELISA standard curve, still retaining a strong signal, and were therefore chosen for the optimal ELISA protocol.

I attempted to use the VWF96 ELISA to analyse the time course of VWF96 proteolysis. Initially, I incubated 0.5 μ M VWF96 with 0.75nM ADAMTS13 and stopped multiple sub-samples

between 0-90 minutes. Prior to analysis, the sub-samples needed to be diluted to a concentration that the ELISA can detect sensitively; I chose 0.09nM, which is in the middle of the standard curve range. As VWF96 was proteolysed, a reduction in the signal was obtained. Interpolation on the standard curve showed that there was indeed 0.09nM VWF96 at 0 minutes, which, with time, decreased to 0nM. The fact that the curve did not plateau above 0nM shows that this ELISA specifically detects only VWF96, and not any other reaction component, and that the reaction proceeded to completion, unaffected by potential limitations like enzyme inactivation. I included a control reaction, where no ADAMTS13 was added. As expected, at all time points, the concentration of VWF96 remained constant around 0.09nM, without any significant variability. This was a proof-of-principle, that the VWF96 ELISA can monitor VWF96 proteolysis sensitively and specifically. To ensure that I minimized the effect of variability (associated with the assay itself or human error), I chose to analyse eight time points for each reaction, and to analyse each sub-sample in duplicate, taking the mean absorbance. The raw data suggested that no major variability was associated with the ELISA, as the absorbance readings of the duplicates were very similar. Repeating the assay 16 times, at four different concentrations of VWF96, produced essentially the same reaction profile, with reaction completion at ~90 minutes. What was striking was that the fraction of proteolysis in all sub-samples collected at the same time points of independent assays was very similar, confirming that there was no major inter-assay variability associated with this method. The k_{cat}/K_m ($110 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) was consistent with previously published values,^(46, 242, 252, 267) further validating VWF96 as a suitable substrate for ADAMTS13, and the VWF96 ELISA as an accurate method of monitoring the proteolysis.

Finally, I optimised a second VWF96 ELISA, using mouse mAb anti-HisG IgG2a (R940-25, Thermo Fisher Scientific) for capturing VWF96 instead of the chicken pAb anti-SUMO/SUMOstar (AB7002, Life Sensors), which proved to be an equally sensitive and specific method of detecting VWF96 and quantifying the rate of proteolysis ($k_{cat}/K_m = 155 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). The small difference between the k_{cat}/K_m values obtained with the two ELISA protocols was most likely associated with inter-batch variability in the specific activity of ADAMTS13, as well as with quantification differences between the different batches of ADAMTS13 and VWF96 used in the two assays, and not with the ELISA protocol itself.

4 RESULTS: GENERATION AND KINETIC CHARACTERISATION OF VWF96 VARIANTS

4.1 INTRODUCTION

In the previous chapter, I described the generation of VWF96, a new VWF A2 domain-derived substrate for ADAMTS13. I optimised expression and purification strategies, which achieve high yields of purified VWF96. I verified, qualitatively, that VWF96 is cleaved with similar efficiency and specificity as VWF115 and that the possession of tags does not influence proteolysis. I also developed and optimised ELISA assays, which sensitively and specifically detect VWF96, and can be used to quantify VWF96 proteolysis kinetically.

My next aim was to generate a panel of VWF96 variants, with the intention of ablating the binding interaction of each ADAMTS13 functional exosite (in the MP, Dis, Cys-rich and Spacer domains). To do this, I targeted all the residues for which there was evidence for involvement in a functional interaction with ADAMTS13 exosites (Figure 4-1A). I created amino acid substitutions with amino acids of similar sizes, with uncharged side-chains, which did not predict changes in the secondary structure (if any) of VWF96. By endeavouring to ablate the entire exosite binding interaction I hoped to ascertain the individual contribution of each interaction to both binding and proteolysis.

For the MP domain exosite interaction, the L1603 (P3), Y1605 (P1) and M1606 (P1') residues are all functionally important.⁽³⁶²⁾ I chose not to target the Y1605-M1606 residues that correspond to the scissile bond, as this would severely impair proteolysis. The L1603 (P3) residue was proposed to interact with the L198 in the MP domain of ADAMTS13.⁽²⁵²⁾ I only targeted the L1603 (L1603N), termed VWF96-MP variant, to ascertain the exosite rather than the cleavage site function.

In the ADAMTS13 Dis domain, the residues R349, L350 and V352 are all important for ADAMTS13 activity. An interaction was definitively demonstrated between R349 in the ADAMTS13 Dis domain and the D1614 in the VWF A2 domain. Other residues in the proximity of D1614 could be involved in an exosite interaction with the ADAMTS13 Dis domain. It was proposed that the A1612 in the VWF A2 domain could form a hydrophobic interaction with the L350 in the ADAMTS13 Dis domain.⁽²⁵⁰⁾ Furthermore, mutations at the E1615 and K1617 residues of the VWF A2 domain were previously shown to reduce the rate of proteolysis.⁽²⁵⁶⁾ I

hypothesized that the entire region A1612-K1617 in the VWF A2 domain served as the complementary binding site for the Dis domain exosite of ADAMTS13, therefore, I created the VWF96-Dis1 variant with the substitutions ADEIK1612/1614-1617SNQQQ (in light of additional data published during the course of this project, this variant was later modified to the VWF96-Dis2 variant DIKRD1614/1616-1618/1622AQEET, see sections 4.4 and 4.5).

A hydrophobic exosite interaction exists between the residues G471-V474 in the ADAMTS13 Cys-rich domain and the residues I1642/W1644/I1649/L1650/I1651.⁽²⁶⁶⁾ I substituted all hydrophobic residues in this region of VWF96 (IWAILI1642/1644/1647/1649-1651QYSQQQ) to generate the VWF96-Cys variant.

The precise residues that are involved in the ADAMTS13 Spacer domain exosite interaction are not known. In the Spacer domain of ADAMTS13, the R659, R660, Y661, Y665 have been proposed to be part of the exosite, although this has never been formally demonstrated.^(235, 267, 268) In the VWFA2 domain, the E1660-R1668 region is important for the functionality of the Spacer domain exosite.^(251, 255, 360) Previous data suggested that the charged residues in the E1660-R1668 have little influence on the binding interaction with the Spacer domain.⁽³⁶¹⁾ Therefore, I chose to substitute the hydrophobic residues in this region for hydrophilic (LVL1664-1666TNQ) to create the VWF96-Spacer variant.

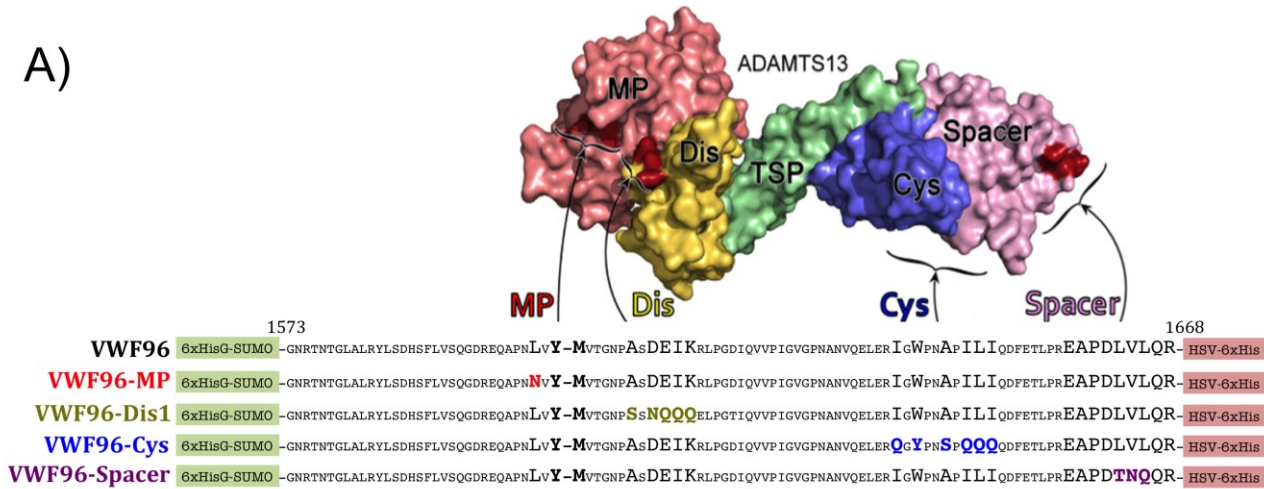
In this chapter, I describe the generation of the VWF96 variants. The VWF96 variants were used as substrates for ADAMTS13 to assess the effect of ablating each exosite interaction on the rate of proteolysis. The VWF96 ELISA-based assay (described in Chapter 3) was used to characterise the kinetics of proteolysis of each VWF96 variant. The equilibrium binding affinity between ADAMTS13 and the VWF96 variants was measured using plate-based binding assays.

4.2 Generation of VWF96 variants

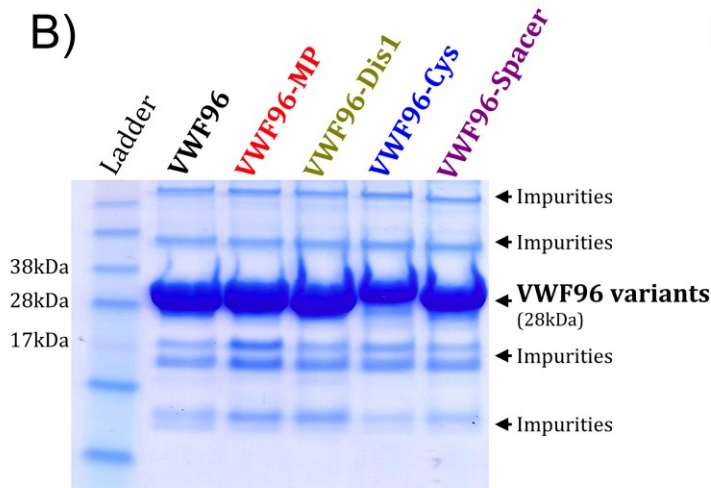
I performed inverse PCR (section 2.1.2.4) to generate vectors expressing VWF96 variants. I used primers that introduce mutations to the VWF96 cDNA sequence and amplify the entire VWF96 pET-25b(+) vector inversely.

The VWF96 variants were expressed in BL21(DE3) *E. coli* (section 2.1.2.5), and purified in two steps in the same way as described for VWF96 (section 2.1.2.6). Figure 4-1B shows the eluted fractions after the first purification step of the VWF96 variants, analysed by Coomassie staining. The predominant bands at ~28kDa correspond to the VWF96 variants. The VWF96-Cys variant migrated slightly slower; this effect was likely to be a result of the specific amino acid composition of the mutated area in this variant. The main impurities in all partially purified VWF96 variants are the same as those seen for VWF96. As shown in Figure 4-1C, all VWF96 variants were purified to homogeneity after the second purification step (estimated >95% pure). All VWF96 variants were expressed with comparable yields to VWF96 described in Chapter 3 (section 3.4).

A)



B)



C)

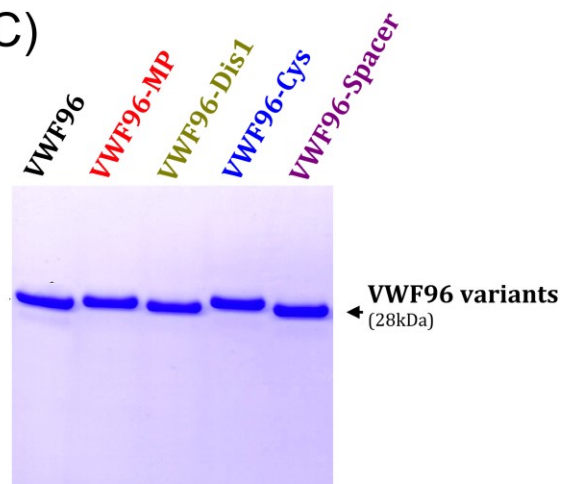


Figure 4-1 Generation of VWF96 variants

(A) VWF96 (G1573-R1668) possesses N-terminal HisG and SUMO tags, and C-terminal HSV and 6xHis tags. Mutations were introduced in VWF96 to create the VWF96-MP (L1603N), VWF96-Dis1 (ADEIK1612/1614-1617SNQQQ), VWF96-Cys (IWAILI1642/1644/1647/1649-1651QYSQQQ) and VWF96-Spacer (LVL1664-1666TNQ) variants that ablate the interaction of VWF96 with each ADAMTS13 exosite independently (in the MP, Dis, Cys-rich and Spacer domains, respectively). (B) The VWF96 variants were partially purified by immobilised metal ion affinity chromatography, using Ni²⁺-charged HiTrap Chelating HP columns. The eluted proteins were analysed by SDS-PAGE and Coomassie staining. (C) The VWF96 variants were further purified by anion exchange chromatography, using Capto Q Impres columns. Analysis by SDS-PAGE and Coomassie staining indicated that all variants were >95% pure.

4.3 Qualitative assessment of VWF96 variant proteolysis by ADAMTS13

I performed cleavage assays to qualitatively assess the ability of ADAMTS13 to specifically proteolyse the VWF96 variants. I initially incubated 3 μ M of each VWF96 variant with the same concentration of ADAMTS13 (4nM), and stopped the reactions between 0-120 minutes (section 2.3.1). The proteolysis of VWF96 at each time point was analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8)(Figure 4-2A). At the start of the reaction (0 minutes), no VWF96 was proteolysed, as seen by the single band at 28kDa, corresponding to the uncleaved substrate. Both specific cleavage products were visible at 30 minutes (N-terminal cleavage product at 18kDa and C-terminal cleavage product at 10kDa), and the reaction was complete between 60-90 minutes. The rate of proteolysis of all VWF96 variants by ADAMTS13 was appreciably slower than that of VWF96, since very little proteolysis had occurred even after 120 minutes. The VWF96-MP and VWF96-Dis1 variants were proteolysed least efficiently, while the mutations in VWF96-Cys and VWF96-Spacer were less detrimental to the rate of proteolysis. Nevertheless, none of the mutations seemed to impair the specificity of ADAMTS13-mediated proteolysis, since, for all VWF96 variants, the specific N-terminal cleavage products at 18kDa were visible (squared in Figure 4-2 Left).

I adjusted the concentration of ADAMTS13 in each reaction to better estimate the relative effect of the mutations upon the rate of proteolysis, and to improve the detection of the specific cleavage products for those variants that were proteolysed very inefficiently (Figure 4-2 Right). VWF96 was incubated with 3nM ADAMTS13, and the reaction profile was essentially the same as previously (complete reaction between 60-90 minutes). VWF96-MP and VWF96-Dis1 were incubated with 20 times more ADAMTS13 (60nM); this increased the rate of proteolysis, such that both specific cleavage products were clearly visible. However, the reactions were still not complete, with a significant proportion of the substrate still uncleaved after 120 minutes. Apart from the three predominant bands corresponding to the uncleaved/cleaved substrates, additional bands were visible on the gel. These were proteins present in the ADAMTS13 expression media. The VWF96-Cys and VWF96-Spacer variants were incubated with 20nM and 15nM ADAMTS13, respectively. Both specific cleavage products were visible after 30 minutes, but neither of the reactions was complete after 120 minutes. The VWF96-Cys reaction, despite containing more ADAMTS13, proceeded slower than the VWF96-Spacer reaction, indicating that the mutations in the Cys-rich domain exosite binding site

caused a greater reduction in the rate of proteolysis than the mutations in the Spacer domain exosite binding site.

The qualitative analysis of the proteolysis of VWF96 variants showed that the MP and Dis domain exosite interactions have the most important role to play in the proteolysis of VWF by ADAMTS13. The next most important exosite is found in the Cys-rich domain. The Spacer domain exosite interaction has the smallest contribution to the rate of proteolysis in relation to the other exosite interactions. Nevertheless, it enhances the rate of proteolysis considerably (by ~20-fold, as predicted based on qualitative analysis).

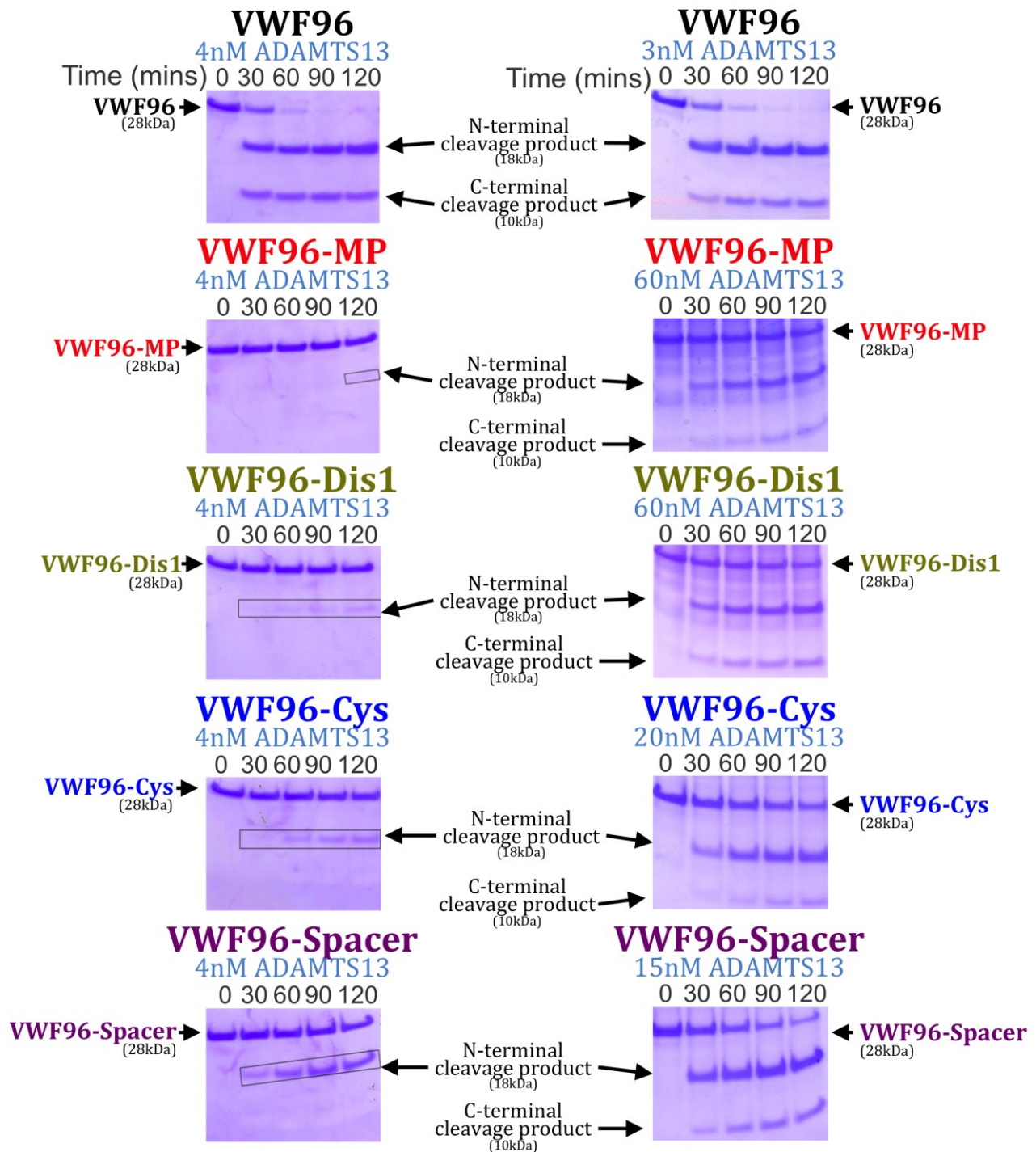


Figure 4-2 Qualitative assessment of the proteolysis of VWF96 variants by ADAMTS13

(Left) The VWF96 variants (3 μ M) were incubated with 4nM ADAMTS13, and proteolysis between 0-120 minutes was analysed by SDS-PAGE and Coomassie staining. VWF96 proteolysis was complete between 60-90 minutes. All VWF96 variants were cleaved specifically, as seen by the emergence of the specific N- and C-terminal cleavage products (18kDa and 10kDa, respectively). The rates of proteolysis of all VWF96 variants were significantly lower than VWF96. (Right) The ADAMTS13 concentrations were adjusted for all VWF96 variants to estimate the relative reduction in the rate of proteolysis associated with the ablation of the different exosite interactions by mutation.

4.4 'Massively parallel' kinetic analysis of VWF73 proteolysis by ADAMTS13

After the VWF96 variants introduced above were generated, a study by Kretz *et al* was published,⁽³⁶³⁾ which reported a method for multi-parallel kinetic analysis of VWF73 proteolysis, and the effect of every possible residue substitution at each position in VWF73 on the rate of proteolysis by ADAMTS13. Briefly, they created a mutagenized VWF73 phage display library. The phage displaying VWF73 variant peptides were incubated with ADAMTS13, and uncleaved variants were selected at multiple time points and sequenced by high throughput DNA sequencing. The enrichment and reduction in complexity of the library identified the amino acid residues that, on average, reduce the rate of proteolysis when mutated. Analysis of the sequenced library at multiple time points during the reaction with ADAMTS13 provided information on the relative effect of all single amino acid substitutions at each position on the rate of proteolysis. This allowed generation of the heat map below (Figure 4-3), where red colour signifies mutations that caused the biggest reductions in the rate of proteolysis, yellow signifies mutations that had no influence on the rate of proteolysis and blue signifies mutations that enhanced the rate of proteolysis.

The results of this study, to a great extent, corroborated my mutation strategy for the different exosite binding sites in VWF96. In other words, most of the residues targeted for my project were present in the red regions on the heat map. However, some residues that appeared to be important for proteolysis based on the heat map were not targeted for my project. To ablate the Dis domain exosite interaction, I originally targeted the A1612-K1617 region. Based on the heat map, it is clear that the region targeted does not fully cover the entire Dis domain exosite binding site. The region immediately downstream of the targeted residues, R1618-D1622, was also likely to be involved in the interaction with the Dis domain exosite. Therefore, I generated the variants VWF47 (G1573-L1619), VWF57 (G1573-G1629) and VWF96-Dis2 (DIKRD1614/1616-1618/1622AQEET) to investigate the functionality of this region (see section 4.5).

To ablate the Spacer domain exosite interaction, I originally hypothesized that the L1664, V1665 and L1666 residues were involved in a hydrophobic interaction with the ADAMTS13 Spacer domain. However, based on the heat map, more residues in the region E1660-R1668 (which includes the targeted L1664, V1665 and L1666 residues) were likely to be involved in the Spacer domain exosite interaction. Therefore, I generated a further variant that lacks the

entire region E1660-R1668, termed VWF87(Δ Spacer) variant, to investigate any additional functional deficit (see section 4.6).

I decided not to change the VWF96 variants that ablate the MP and Cys-rich domain exosite interactions. The residues P1601-V1606 appear red on the heat map and could be included as part of the targeted residues in the VWF96-MP variant. I wanted to avoid interfering with the Y1605-M1606 of the scissile bond, as this would reduce proteolysis in an exosite independent manner. I avoided mutating P1601 as this could alter the secondary structure of VWF96 and also impair the rate of proteolysis in an exosite independent manner. Substitutions of the N1602 and V1604 were performed in a prior study,⁽²⁵²⁾ and did not have major effects on the rate of proteolysis. Substituting the L1603, as seen by the qualitative analysis of the VWF96-MP variant (L1603N)(section 4.3) and previously,⁽²⁵²⁾ is detrimental to the rate of proteolysis, but still retaining some quantifiable specific proteolysis. I expect the reduction in the rate of proteolysis as a result of this mutation to be manifest through a reduction in the functionality of the active site, which could act as a control for the kinetic studies. In the VWF96-Cys variant, the residues I1642, W1644, A1647, I1649, L1650 and I1651 were targeted. In the heat map, the most important residues in this region for proteolysis were likely to be the I1649, L1650 and I1651. Nevertheless, I decided to keep the set of mutations in the VWF96-Cys variant unchanged.

In the following sections, I describe the revision of the panel of VWF96 variants, in order to include new variants with mutations that more completely ablate the Dis and Spacer domain exosite interactions.

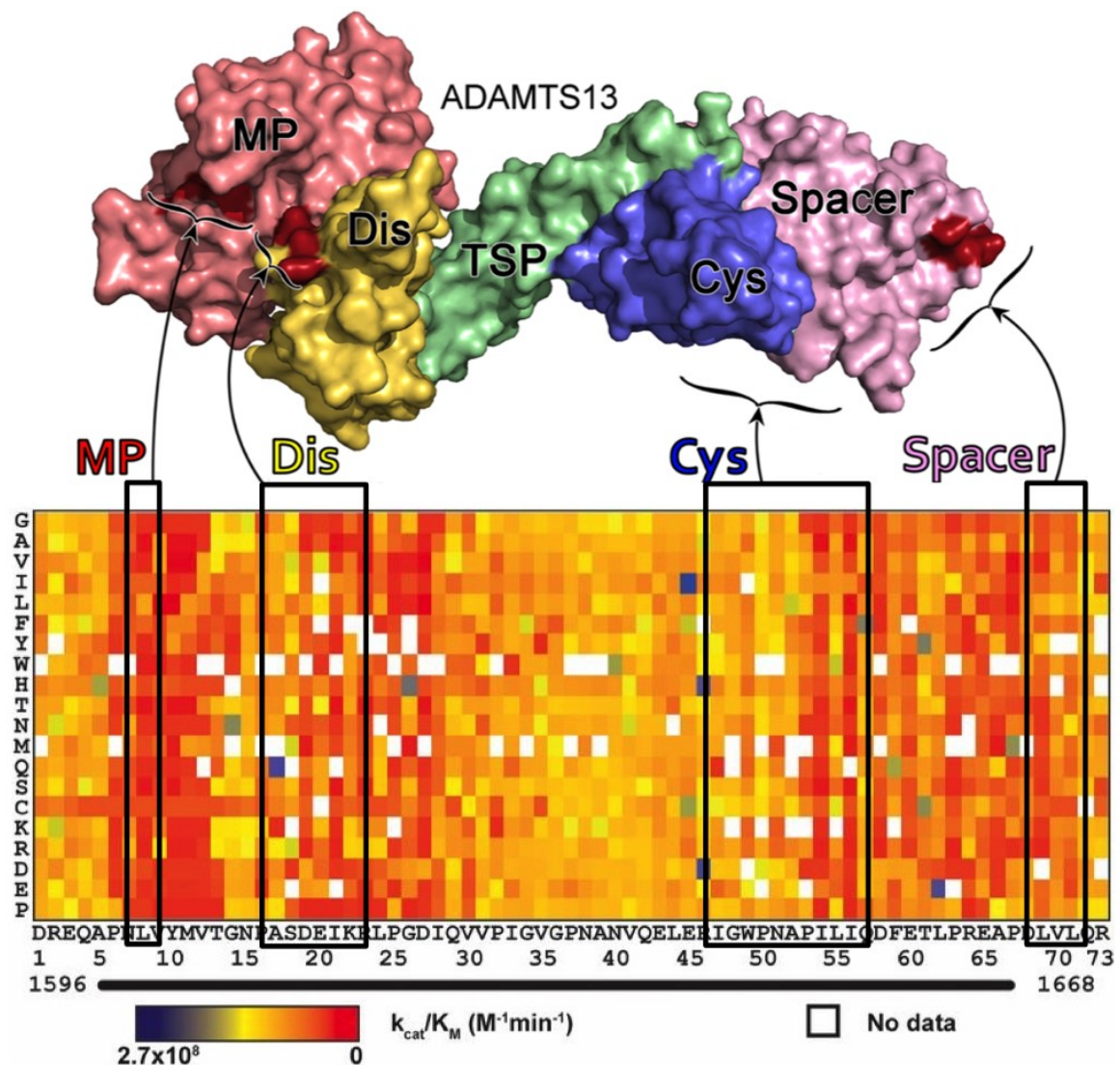


Figure 4-3 Heat map illustrating the effect on the k_{cat}/K_m of substituting each residue in the VWF73 sequence.

A mutagenized VWF73 phage display library was incubated with ADAMTS13. At multiple time points, phage displaying uncleaved VWF73 peptides were sequenced and the enrichment and reduction in complexity of the library quantified and normalised to the k_{cat}/K_m of wild type VWF73 proteolysis. This study by Kretz *et al*⁽³⁶³⁾ provides information on the effect on the k_{cat}/K_m of every possible substitution at every amino acid residue in the VWF73 sequence. Red indicates substitutions that most severely affected the rate of proteolysis. Yellow indicates that substitutions did not have a major influence on the k_{cat}/K_m . Curiously, some substitutions, shown in blue, enhance the rate of proteolysis (discussed in section 4.13). The regions targeted by mutagenesis to generate the VWF96 variants are squared, and the arrows indicate the domains in ADAMTS13 these regions were predicted to interact with. The heat map was obtained from⁽³⁶³⁾ and adapted (for permission, see Table B, Appendix II).

4.5 Generation of the VWF47, VWF57 and VWF96-Dis2 variants

To corroborate the findings of Kretz *et al.*⁽³⁶³⁾ I used a different strategy to investigate the functionality of the region R1618-D1622, which is immediately downstream of the targeted region in VWF96-Dis1. I generated two peptides, VWF47 and VWF57, which had the same N-terminus as the VWF96 variants but are C-terminally truncated at different positions (Figure 4-4A). VWF47 spanned the region G1573-L1619, including only two additional residues downstream of the targeted region in the VWF96-Dis1 variant. VWF57 spanned the region G1573-G1629, including 12 additional residues immediately downstream of the targeted region in the VWF96-Dis1 variant, some of which were predicted to have a functional role in the ADAMTS13 Dis domain exosite binding and proteolysis. If the additional region in VWF57 was implicated in proteolysis, then VWF57 should be proteolysed faster than VWF47.

The cDNA encoding HisG-SUMO-VWF47 and HisG-SUMO-VWF57 (both with a 3' XhoI restriction site introduced) were amplified by PCR using the VWF96-pET SUMO vector as template ('VWF47-XhoI' and 'VWF57-XhoI' primers in Table A, Appendix I). The bacterial pET-25b(+) vectors for the expression of VWF47 and VWF57 were created using the same cloning strategy already described for VWF96 (section 3.3). VWF47 and VWF57 were expressed and purified, as previously described for VWF96 (section 3.4) Figure 4-4B+C show Coomassie staining analysis of the eluted VWF47 and VWF57 after each purification step, migrating on the gels at ~22kDa and ~23kDa, respectively. VWF47 and VWF57 were also specifically detected by Western blotting (section 2.1.2.9), using antibodies against the HSV tag (Figure 4-4D).

To investigate any differences in the rates of VWF47 and VWF57 proteolysis, activity assays were set up between an ADAMTS13 variant truncated after the Dis domain (MP-Dis, 266nM, purified and already available) and 3μM of each substrate (section 2.3.1). Reactions were stopped between 0-16 hours, and analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8)(Figure 4-4E). Proteolysis produced the same N-terminal cleavage product at ~18kDa for both VWF47 and VWF57, and ~4kDa and ~5kDa C-terminal cleavage products, respectively. The proteolysis of VWF57 was estimated to be ~5-fold more efficient than the proteolysis of VWF47; this demonstrates that the additional region in VWF57 (P1620-G1629) is, at least partly, implicated in proteolysis. The use of the MP-Dis variant in the reaction, as opposed to full-length ADAMTS13, showed that the additional region in VWF57 interacts with the Dis domain of ADAMTS13, and not with an exosite in another domain.

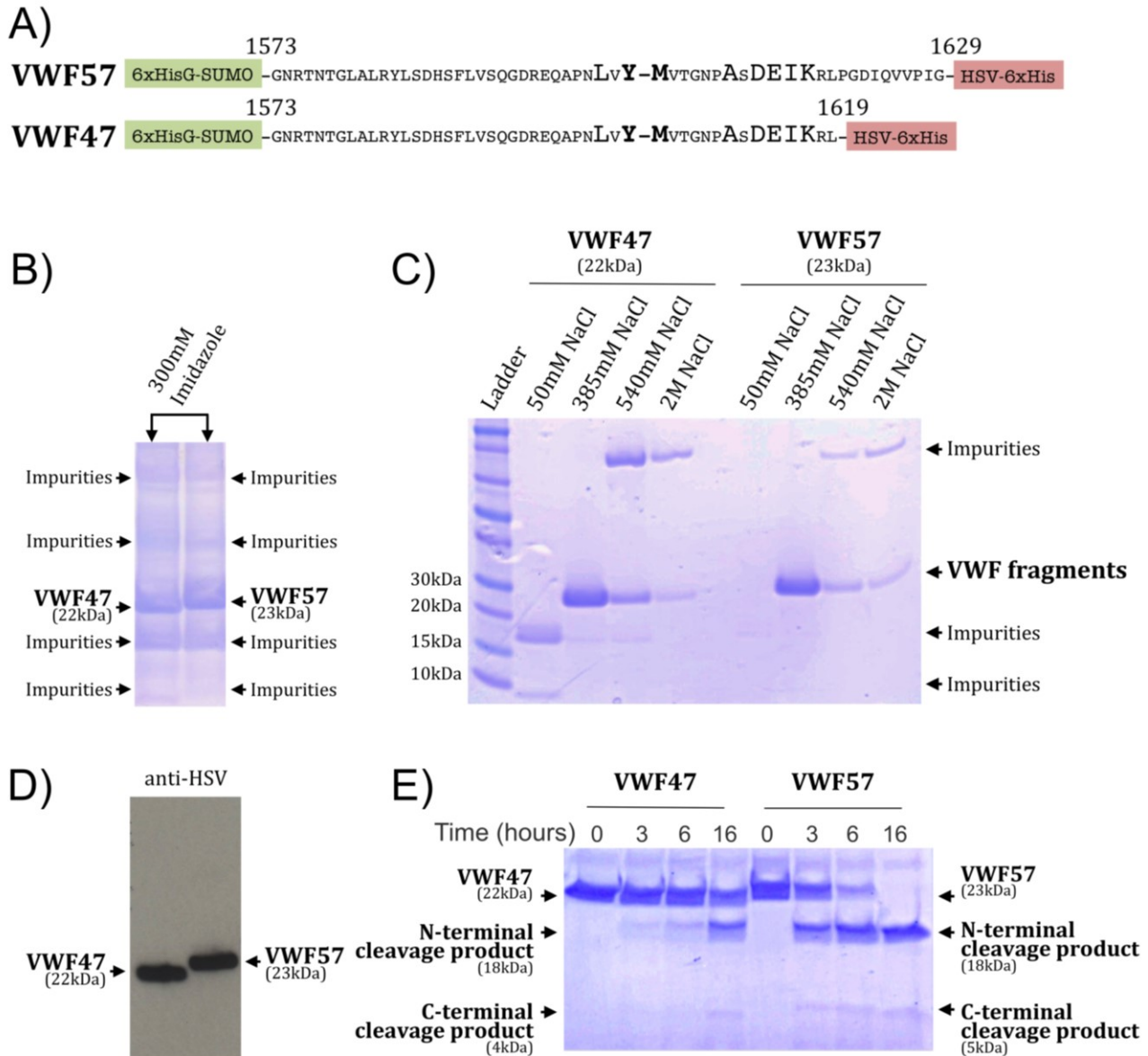


Figure 4-4 Generation and proteolysis of VWF47 and VWF57

(A) The amino acid sequence of VWF57 (G1573-G1629) and VWF47 (G1573-L1619). Both variants possess N-terminal HisG and SUMO tags, and C-terminal HSV and 6xHis tags. The enlarged residues correspond to the proposed MP (L1603) and Dis (ADEIK1612/1614-1617) domain exosite binding sites. The scissile bond, Y1605-M1606, is enlarged and in bold. (B) VWF47 (22kDa) and VWF57 (23kDa) were expressed in BL21 (DE3) *E. coli*, partially purified by immobilised metal ion affinity chromatography using Ni²⁺-charged HiTrap Chelating HP columns, and elution fractions at 300mM were analysed by SDS-PAGE and Coomassie staining. (C) VWF47 and VWF57 were further purified by anion exchange chromatography on CaptoQ Impres columns and analysed by SDS PAGE and Coomassie staining. (D) Western blot of VWF47 and VWF57 using goat pAb anti-HSV conjugated with HRP (A190-136P, Bethyl). (E) Qualitative analysis of VWF47 and VWF57 (3μM) proteolysis by MP-Dis (~266nM). Reaction sub-samples were stopped between 0-16 hours and analysed by SDS-PAGE and Coomassie staining. VWF47 and VWF57 were specifically proteolysed by MP-Dis for 16 hours producing the specific N-terminal (18kDa) and C-terminal (4kDa and 5kDa, respectively) cleavage products. VWF57 was proteolysed more efficiently by MP-Dis than VWF47.

I used the VWF96 ELISA to compare the rates of proteolysis of VWF47 and VWF57 quantitatively (section 2.3.2.1). These fragments possessed the same tags as VWF96, and could therefore be analysed using the same ELISA protocol (section 2.1.2.11). I normalised the concentrations of the two fragments using ELISA (Figure 4-5A), to ensure that both fragments could be detected sensitively, and that the same amount of substrate was used in both reactions. I then performed activity assays (n=2) between 100nM ADAMTS13 and 1 μ M of each substrate. The reactions were stopped between 0-120 minutes and analysed by VWF96 ELISA. The Figure 4-5B+C shows the percentage of substrate proteolysis plotted as a function of time. VWF57 was cleaved with k_{cat}/K_m of $0.72 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, while VWF47 was cleaved 8.4-fold slower, with k_{cat}/K_m of $0.086 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$.

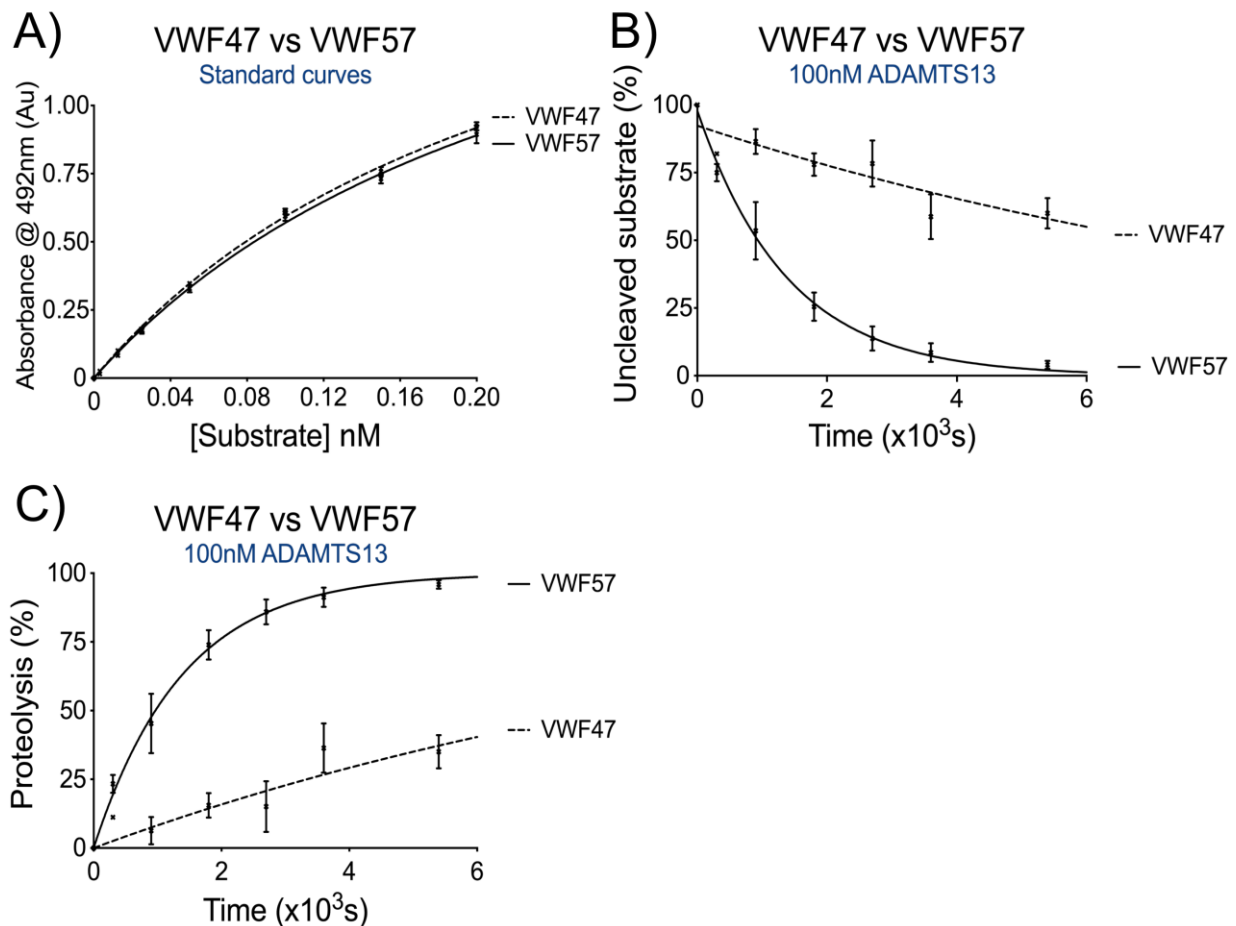


Figure 4-5 Quantitative analysis of VWF57 and VWF47 proteolysis by ADAMTS13 using the VWF96 ELISA.

(A) VWF47 and VWF57 concentrations were normalised using the VWF96 ELISA. (B-C) The proteolysis of VWF47 and VWF57 (1 μ M) by 100nM ADAMTS13 was monitored for 120 minutes, using the VWF96 ELISA and fitted to the equation $P = 1 - \exp(-1 \times [\text{ADAMTS13}] \times t \times k_{cat}/K_m)$ (see section 3.7). ADAMTS13 cleaved VWF57 ($k_{cat}/K_m = 0.72 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) 8.4 times more efficiently than VWF47 ($k_{cat}/K_m = 0.086 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (n=2).

The experiments using the truncated variants VWF47 and VWF57 suggested that the P1620-G1629 region in the VWF A2 domain is functionally important for VWF proteolysis and is involved in interactions with the Dis domain exosite of ADAMTS13. Therefore, I decided to generate a new VWF96 variant with mutations that ablate the entire Dis domain exosite binding site (Figure 4-8A) and chose the residues that are most likely to be involved in the interaction based on the findings of Kretz *et al*⁽³⁶³⁾(Figure 4-3). I introduced the substitutions DIKRD1614/1616-1618/1622AQEET (section 4.2) and successful mutagenesis was verified by sequencing. The new variant, termed VWF96-Dis2, was expressed and purified as already described for VWF96 (section 3.4).

To confirm that there was indeed an additional deficit in the rate of proteolysis of the VWF96-Dis2 variant, when compared to the VWF96-Dis1 variant, associated with the new set of mutations, I performed activity assays between 100nM ADAMTS13 and 3 μ M of either substrate (section 2.3.1). The reaction was stopped using EDTA between 0-120 minutes and analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8). Figure 4-6 shows that the new mutations introduced in the VWF96-Dis2 variant further reduce the rate of proteolysis compared to the VWF96-Dis1 variant and confirm the involvement of these residues in the Dis domain exosite binding site. The VWF96-Dis1 and VWF96-Dis2 variants have the same molecular weight, ~28kDa, and produced the same ~18kDa and ~10kDa specific cleavage products upon proteolysis. At 0 minutes, neither of the variants were proteolysed. The VWF96-Dis1 variant was completely proteolysed after 120 minutes, while the VWF96-Dis2 variant was only partially proteolysed. I estimated that the VWF96-Dis2 variant was cleaved ~5 times slower than the VWF96-Dis1 variant. Due to contaminants in the ADAMTS13 preparation, the gel is not clear.

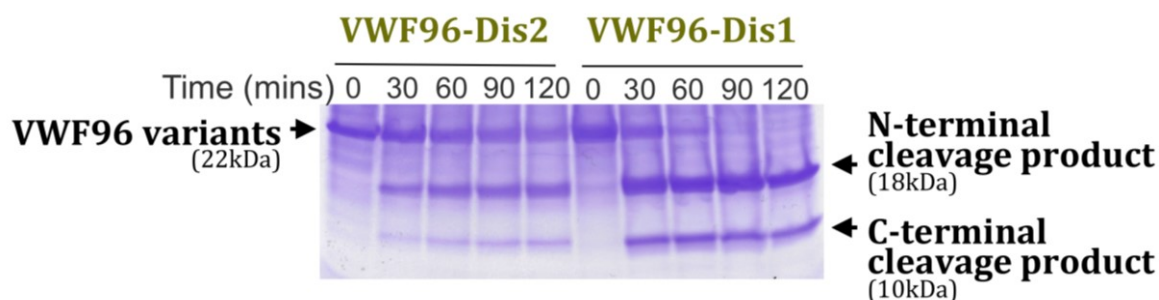


Figure 4-6 Qualitative analysis of the proteolysis of the VWF96-Dis1 and VWF96-Dis2 variants by ADAMTS13.

3 μ M of the VWF96-Dis1 (ADEIK1612/1614-1617SNQQQ) or the VWF96-Dis2 (DIKRD1614/1616-1618/1622AQEET) variants were incubated with 100nM ADAMTS13. The reactions were stopped between 0-120 minutes and analysed by SDS-PAGE and Coomassie staining. The VWF96-Dis1 and VWF96-Dis2 variants (~28kDa) are cleaved over time, producing the specific cleavage products of ADAMTS13 proteolysis (~18kDa and ~10kDa). The proteolysis of the VWF96-Dis2 variant was estimated to be approximately 5 times less efficient than the proteolysis of the VWF96-Dis1 variant.

4.6 Generation and proteolysis of the VWF87(Δ Spacer) variant

Based on the results of Kretz *et al*⁽³⁶³⁾(Figure 4-3), the entire VWF region E1660-R1668 is likely to be implicated in the interaction with the ADAMTS13 Spacer domain exosite, as opposed to only the L1664, V1665 and L1666 residues that were initially targeted by mutation to create the VWF96-Spacer variant. I decided to generate a truncated variant, VWF87(Δ Spacer), which lacks the entire E1660-R1668 region (Figure 4-8A); if other residues in this region are indeed involved in the Spacer domain exosite interaction, the VWF87(Δ Spacer) variant should be cleaved less efficiently than the VWF96-Spacer variant.

The VWF87(Δ Spacer)-expressing pET-25b(+) vector was generated using the same cloning strategy described for the VWF47 and VWF57 variants (section 4.5)(‘VWF87 XhoI’ primers in Table A, Appendix I), and expressed and purified as already described for VWF96 (section 3.4).

To qualitatively assess whether there was any additional functional deficit associated with deleting the entire E1660-R1668 (VWF87(Δ Spacer) variant), as opposed to only introducing the LVL1664-1666TNQ mutations in the Spacer domain exosite binding site of VWF96 (VWF96-Spacer variant), I performed cleavage assays (section 2.3.1) between 15nM ADAMTS13 and 3 μ M of either substrate. The reaction was stopped in EDTA between 0-120 minutes. Figure 4-7 shows the profile of proteolysis of both substrates. The ~27kDa and

~28kDa bands corresponding to the uncleaved VWF87(Δ Spacer) and VWF96-Spacer substrates, respectively, disappeared at a very similar rate, and both reactions were complete at 120 minutes. Proteolysis of the two substrates produced the same N-terminal cleavage products (~18kDa), while the C-terminal cleavage product of the VWF87(Δ Spacer) variant (~9kDa) was slightly smaller than the C-terminal cleavage product of VWF96-Spacer variant (~10kDa). At each time point, the intensity of the bands corresponding to the cleavage products was comparable between the two reactions. This qualitative assay could not identify subtle differences in the rate of proteolysis between the VWF87(Δ Spacer) and VWF96-Spacer variants. This could mean that, as initially hypothesized, only the hydrophobic residues L1664, V1665 and L1666 in the VWF A2 domain are implicated in the functional interaction with the Spacer domain exosite of ADAMTS13. However, due to the lack of sensitivity of this assay, I decided to investigate this further, by including both variants in the revised panel of ADAMTS13 substrates that would be characterized kinetically.

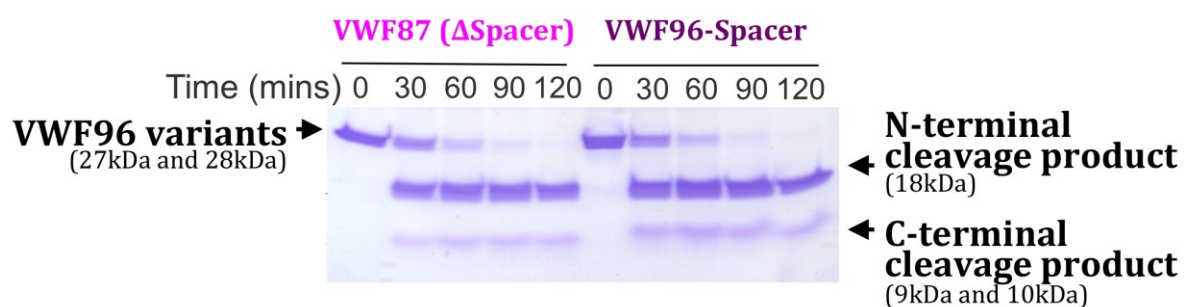


Figure 4-7 Qualitative analysis of the proteolysis of the VWF96-Spacer and VWF87(Δ Spacer) variants by ADAMTS13.

3 μ M of the VWF96-Spacer (LVL1664-1666TNQ) or VWF87(Δ Spacer)(delE1660-R1668) variants were incubated with 15nM ADAMTS13. The reactions were stopped between 0-120 minutes and analysed by SDS-PAGE and Coomassie staining. The VWF96-Spacer and VWF87(Δ Spacer) variants (~28kDa and ~27kDa, respectively) were cleaved over time, producing the same N-terminal cleavage products of ADAMTS13 proteolysis (~18kDa) and slightly different C-terminal cleavage products (~10kDa and ~9kDa, respectively). Based on this assay, the two substrates were proteolysed at apparently indistinguishable rates.

4.7 The revised panel of VWF96 variants

Figure 4-8A shows the revised panel of VWF96 variants that would be used as substrates for ADAMTS13 in this project. These are VWF96, VWF96-MP (L1603N), VWF96-Dis2 (DIKRD1614/1616-1618/1622AQEET), VWF96-Cys (IWAILI1642/1644/1647/1649-1651QYSQQQ), VWF96-Spacer (LVL1664-1666TNQ) and VWF87(Δ Spacer) (delE1660-R1668). As shown in Figure 4-8B, all VWF96 variants were purified to homogeneity and their concentrations were normalised. Western blotting (section 2.1.2.9) using the chicken pAb anti-SUMO/SUMOstar (AB7002, Life Sensors)(Figure 4-8C, Left) and the HRP-conjugated goat pAb anti-HSV (A190-136P, Bethyl)(Figure 4-8C, Right) antibodies verified that all the variants possessed functional N-terminal SUMO and C-terminal HSV tags, respectively.

I qualitatively assessed the relative reduction in the rate of proteolysis associated with the revised set of mutations by performing parallel cleavage assays between each of the VWF96 variants at 3 μ M and ADAMTS13 at adjusted concentrations (section 2.3.1). VWF96 was incubated with 2nM of ADAMTS13. The VWF96-MP and VWF96-Dis2 variants were incubated with 53nM ADAMTS13 because these mutations were predicted to cause the most dramatic reductions in the rate of proteolysis. Smaller reductions in the rates of proteolysis were predicted for the VWF96-Cys, VWF96-Spacer and VWF87(Δ Spacer) variants, therefore 20nM ADAMTS13 was used in these reactions. All reactions were stopped between 0-120 minutes using EDTA, and analysed by SDS-PAGE (section 2.1.2.7) and Coomassie staining (section 2.1.2.8)(Figure 4-9).

For all VWF96 variants, the single ~28kDa (or ~27kDa) bands at 0 minutes, corresponding to the uncleaved substrate, were proteolysed to the specific ~18kDa and ~10kDa (or ~9kDa) cleavage products in later time points. VWF96 was proteolysed with the same efficiency as seen earlier (section 4.3), and the reaction was complete between 60-90 minutes. The reaction profiles for the VWF96-Spacer and VWF87(Δ Spacer) variants were essentially identical to that of VWF96; based on the differences in the ADAMTS13 concentrations, I estimated that both sets of mutations in the Spacer domain exosite binding site of VWF96 caused a ~10-fold reduction in the rate of proteolysis. The VWF96-Cys variant was proteolysed slower, which allowed me to estimate that the interaction with ADAMTS13 mediated by the mutated residues in this variant contributes ~20-fold to the rate of proteolysis. Neither of the VWF96-MP and VWF96-Dis2 variants were completely proteolysed after 120 minutes as both reactions

proceeded very slowly. Interestingly, as a result of the new set of mutations ablating the Dis domain exosite interaction, the VWF96-Dis2 variant is proteolysed slower (<50% proteolysis in 120 minutes) than the VWF96-MP variant (>50% proteolysis in 120 minutes); in contrast, the VWF96-Dis1 variant was proteolysed more efficiently than the VWF96-MP variant in section 4.3. Considering the relative amounts of substrates proteolysed at different time points, as well as the ADAMTS13 concentration used in these reactions, I estimated that the mutations in the VWF96-MP and VWF96-Dis2 variants reduce the rate of proteolysis by ~150-fold and >500-fold, respectively. These results reveal the importance of the interaction mediated by the ADAMTS13 Dis domain exosite as a whole (a remote exosite) for VWF proteolysis, which had not been reported prior to this project.

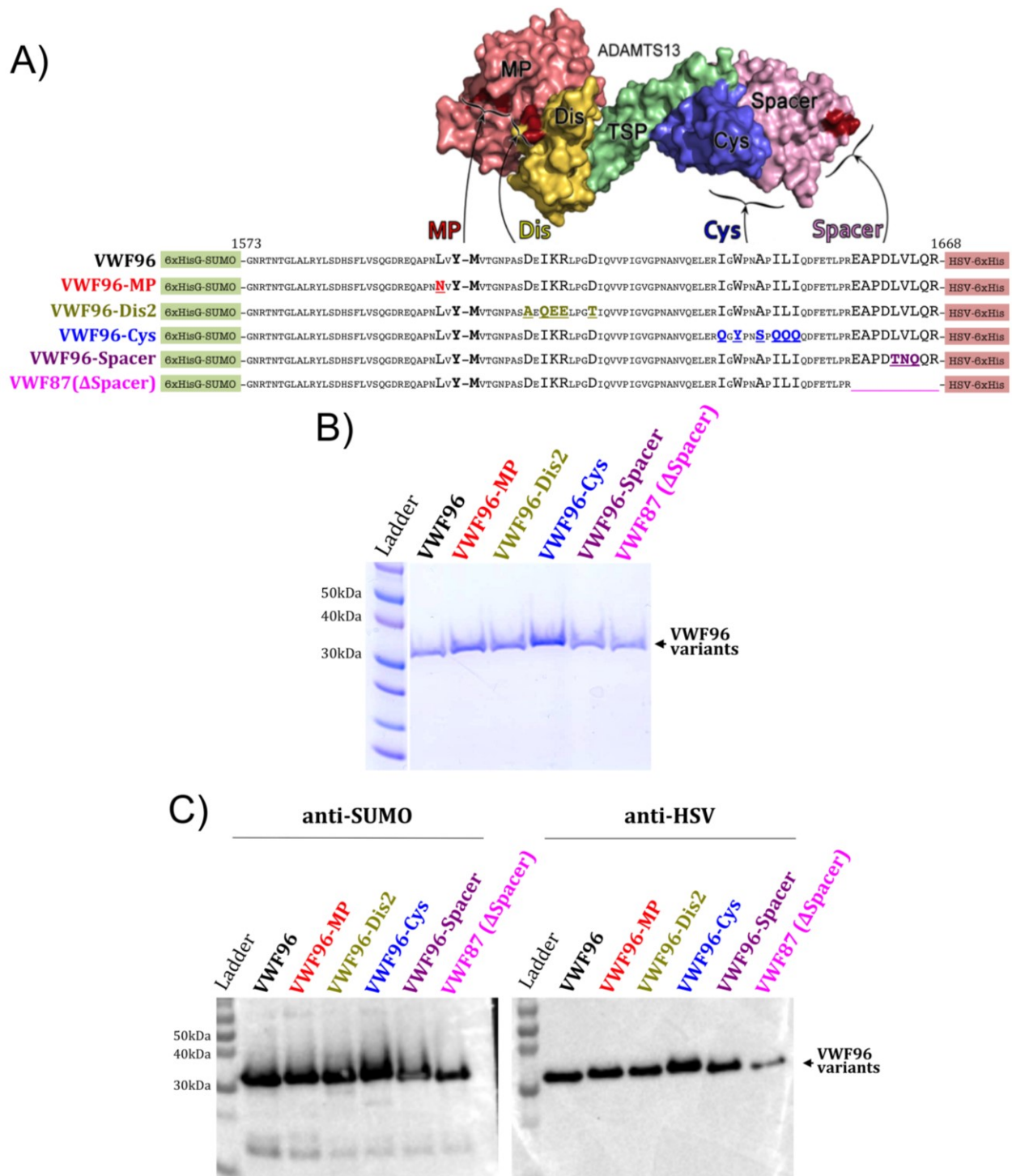


Figure 4-8 The revised panel of VWF96 variants.

(A) The amino acid sequence of the revised panel of VWF96 variants. These are VWF96, VWF96-MP (L1603N), VWF96-Dis2 (DIKRD1614/1616-1618/1622AQEET), VWF96-Cys (IWAILI1642/1644/1647/1649-1651QYSQQQ), VWF96-Spacer (LVL1664-1666TNQ) and VWF87(Δ Spacer) (delE1660-R1668). (B) SDS-PAGE and Coomassie staining of the purified VWF96 variants. All variants were purified to homogeneity and their concentrations normalized. (C) Western blot analysis of all VWF96 variants using antibodies against the SUMO (left) and HSV (right) tags.

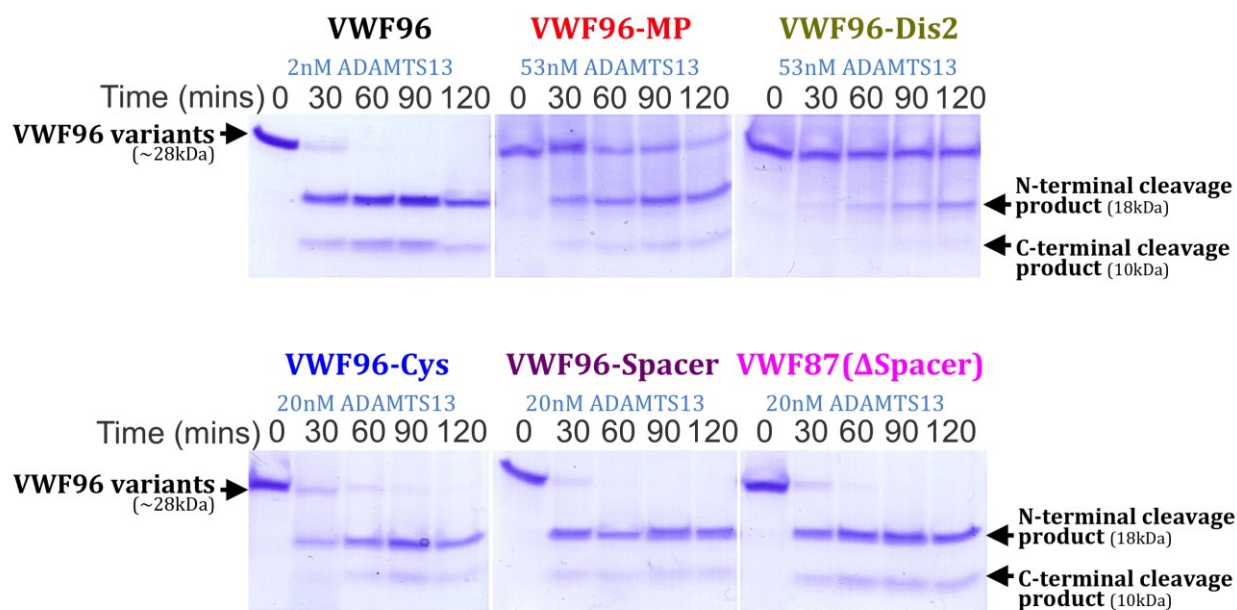


Figure 4-9 Qualitative analysis of the proteolysis of the revised panel of VWF96 variants by ADAMTS13.

3 μ M of all VWF96 variants were incubated with adjusted concentrations of ADAMTS13. Reaction sub-samples were stopped between 0-120 minutes and analysed by SDS-PAGE and Coomassie staining. All variants were specifically proteolysed. The VWF96-MP, VWF96-Dis2, VWF96-Cys, VWF96-Spacer and VWF87(Δ Spacer) variants were predicted to be cleaved ~150-fold, >500-fold, ~20-fold, ~10-fold and ~10-fold, respectively, less efficiently than wild-type VWF96.

4.8 VWF96 ELISA for VWF96 variant quantification

Prior to quantitatively analysing the rate of proteolysis of the VWF96 variants by ADAMTS13, it was necessary to ensure that the VWF96 ELISA (section 2.1.2.11) detected all variants with the same sensitivity and specificity as VWF96. As demonstrated earlier, absorbance and VWF96 concentrations were linearly associated between 0-0.2nM VWF96. I generated standard curves for all VWF96 variants, between 0-0.2nM (as established by absorbance at 280nm and extinction coefficients)(section 2.1.2.10).

Figure 4-10 shows that all variants were detected with similar sensitivity to VWF96, maintaining the linear association between concentration and absorbance. The ability of the VWF96 ELISA to detect all VWF96 variants in a similar fashion indicated that the mutations/truncations do not influence the detection by the antibodies. The ELISA was repeated in order to normalise the concentrations of all VWF96 variants, using the concentration of VWF96 (as established by absorbance at 280nm and extinction coefficients) as the baseline.

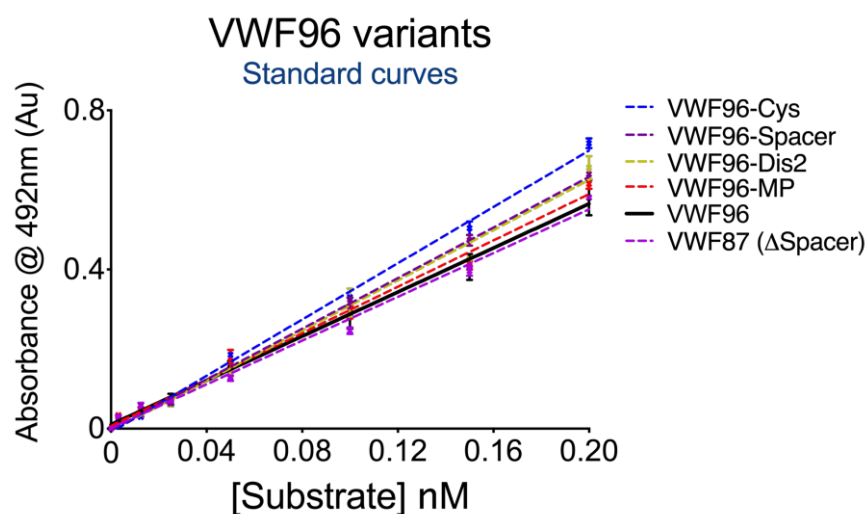


Figure 4-10 Normalisation of VWF96 variant concentration by VWF96 ELISA

The concentrations of VWF variants were previously estimated using predicted extinction coefficients and absorbance at 280nm. The VWF96 ELISA was used to further normalise the concentrations of all VWF96 variants. Linear standard curves (0-0.2nM) were generated for all variants, reaching similar absorbance levels; this indicates that all VWF96 variants were detected with similar specificity and sensitivity as VWF96.

4.9 Catalytic efficiency of VWF96 variants

The VWF96 ELISA was used to quantitatively analyse the rate of VWF96 variant proteolysis by ADAMTS13, as described for VWF96 in Chapter 3 (sections 3.7 and 3.8). Briefly, activity assays were set up using different concentrations of ADAMTS13 and the VWF96 variants. Sub-samples were stopped at different time points using EDTA, diluted such that the final concentration of VWF96 variants was 0.09nM or 0.75nM and analysed by VWF96 ELISA (using the anti-SUMO or anti-HisG protocols, respectively). The fraction of substrate proteolysed in each sub-sample was plotted against time, and the time course data were fitted as before (section 2.3.2.1).

Figure 4-11 shows the fitted time course data for each VWF96 variant. VWF96 (<1 μ M) was incubated with 0.75nM ADAMTS13. The reaction approached completion at 90 minutes/5400 seconds, giving a k_{cat}/K_m of $110 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=16). The VWF96-Spacer variant (<13 μ M) was incubated with 3nM ADAMTS13 and proteolysed with $k_{cat}/K_m = 13.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3), which was 8-fold slower than the proteolysis of VWF96. Similarly, the VWF87(Δ Spacer) variant (<17.5 μ M) was incubated with 4nM ADAMTS13, and was proteolysed with $k_{cat}/K_m = 7.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=4), which was 15-fold slower than the proteolysis of VWF96. Incubating the VWF96-Cys variant (<25 μ M) with 5nM ADAMTS13, resulted in a k_{cat}/K_m of $7.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=7), which was ~16 times slower than the proteolysis of VWF96. The mutations in the VWF96-MP and VWF96-Dis2 variants caused the biggest reductions in the rate of proteolysis by ADAMTS13, as predicted by the qualitative analysis (section 4.7). 75nM ADAMTS13 cleaved the VWF96-MP variant (<4 μ M) with a k_{cat}/K_m of $0.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=4), which was ~138-fold less efficient than the proteolysis of VWF96. Finally, the most detrimental effect on the rate of proteolysis was seen with the VWF96-Dis2 variant. ADAMTS13 (188nM) cleaved the VWF96-Dis2 variant (<30 μ M) with a k_{cat}/K_m of $0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=8); the ~527-fold reduction in the rate of proteolysis corroborated the qualitative analysis of the proteolysis of this variant, which showed that the ADAMTS13 Dis domain exosite is the most important remote exosite interaction for proteolysis. The catalytic efficiencies measured in this section are in excellent agreement with the qualitative analysis of proteolysis described in section 4.7. The results are summarised in Table 4-1, including standard error.

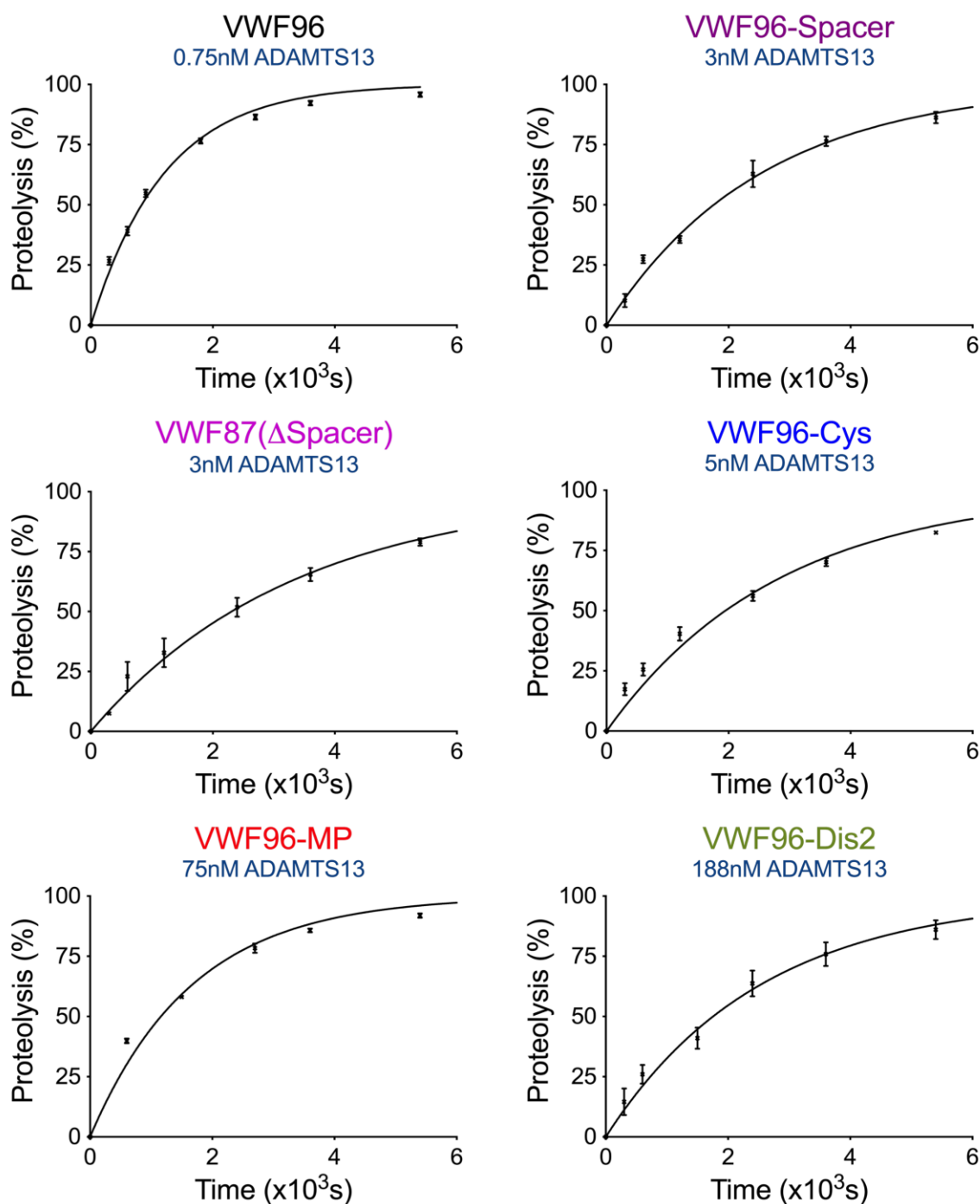


Figure 4-11 The catalytic efficiency of VWF96 variant proteolysis by ADAMTS13, as assessed by analysing the proteolytic time course of the reactions.

VWF96 variants (concentration $< K_m$) were incubated with adjusted concentrations (0.75-188nM) of ADAMTS13. Reaction sub-samples were stopped between 0-90 minutes and analysed by VWF96 ELISA. The percentage of substrate proteolysed was plotted as a function of time (seconds) and fitted to the equation $P = 1 - \exp(-1 \times [ADAMTS13] \times t \times k_{cat}/K_m)$ (see section 3.7) to estimate the k_{cat}/K_m of proteolysis of each variant: VWF96 $k_{cat}/K_m = 110 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=16). VWF96-Spacer $k_{cat}/K_m = 13.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3). VWF87(Δ Spacer) $k_{cat}/K_m = 7.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=4). VWF96-Cys $k_{cat}/K_m = 7.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=7). VWF96-MP $k_{cat}/K_m = 0.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=4). VWF96-Dis2 $k_{cat}/K_m = 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=8). Results are summarised in Table 4-1.

4.10 Michaelis Menten kinetic analysis of VWF96 variant proteolysis

Analysing the proteolytic time course of single reactions (section 4.9) allowed comparison of the relative effect of ablating each entire exosite interaction on the catalytic efficiency, ie. the k_{cat}/K_m . This, however, does not inform on the precise contribution of each exosite interaction to the proteolytic event; whether an exosite is implicated in a high affinity interaction with the substrate that simply facilitates approximation of the two molecules, or whether it is implicated in the functionality of the active site. Further information on the precise contribution of each exosite interaction can be gained by calculating the effect of ablating each exosite interaction on the separate components of the catalytic efficiency, the K_m and k_{cat} . The K_m refers to the Michaelis constant, and measures the functional binding affinity between the substrate and the enzyme. The k_{cat} is a measure of the substrate turnover, which relates to the functionality of the active site. If an exosite is involved in simply approximating the enzyme and substrate, ablation of the exosite interaction would only increase the K_m . If, on the other hand, an exosite only mediates accommodation of the cleavage site in the active site, ablation of the exosite interaction would only reduce the k_{cat} . I hypothesized that ablation of an exosite interaction that allosterically activates ADAMTS13 would cause both a significant reduction in the k_{cat} and a significant increase in the K_m , because the exosite in binding VWF is also important in allosterically activating the enzyme by transducing a structural change in the active site.

I used the VWF96 ELISA to perform Michaelis Menten analysis of the proteolysis of each VWF96 variant (section 2.3.2.2). Reactions were performed between ADAMTS13 and multiple concentrations of each variant, and analysed by VWF96 ELISA. The initial rates of proteolysis were plotted as a function of substrate concentration and fitted to the Michaelis Menten equation $V_i = \frac{V_{max} \times [substrate]}{K_m + [substrate]}$, where V_i is the initial rate of proteolysis (nMs^{-1}), V_{max} is the maximum rate of proteolysis that can be achieved (nMs^{-1}), $[substrate]$ is the substrate concentration (μM), K_m is the concentration of substrate (μM) that achieves half maximum rate of proteolysis ($\frac{V_{max}}{2}$). The k_{cat} (s^{-1}) can be calculated by dividing the maximum rate of proteolysis V_{max} by the enzyme concentration ($k_{cat} = \frac{V_{max}}{[ADAMTS13]}$).

The Michaelis Menten plots for the proteolysis of each VWF96 variant by ADAMTS13 are shown in Figure 4-12 and the analysis is summarised in Table 4-1, including standard error.

Incubating 0-140 μ M VWF96 with ADAMTS13 resulted in a k_{cat} of 5.65s⁻¹ and a K_m of 3.99 μ M (n=113). Using these values, I calculated a k_{cat}/K_m of 142x10⁴ M⁻¹s⁻¹, which is in good agreement with the value independently obtained from time course analysis ($k_{cat}/K_m=110\times 10^4$ M⁻¹s⁻¹)(section 4.9).

For the VWF96-Spacer variant (0-135 μ M, n=41), the k_{cat} was only modestly reduced (1.4-fold) to 4.1s⁻¹. However, the K_m was 8-fold increased to 33 μ M, giving a k_{cat}/K_m of 12.6x10⁴ M⁻¹s⁻¹, which, again, is very similar to the value obtained by time course analysis ($k_{cat}/K_m=13.1\times 10^4$ M⁻¹s⁻¹)(section 4.9). Analysis of the VWF87(Δ Spacer) variant (0-350 μ M, n=58) revealed the same slight reduction in k_{cat} (1.4-fold) to 4.0s⁻¹, a 12-fold increase in the K_m to 46 μ M, and a k_{cat}/K_m of 8.6x10⁴ M⁻¹s⁻¹, which was again consistent with the time course data ($k_{cat}/K_m=7.5\times 10^4$ M⁻¹s⁻¹)(section 4.9). These data suggest that the reduction in the rate of proteolysis associated with ablating the Spacer domain exosite interaction is primarily due to reduced substrate binding affinity (up to 12-fold). Notably, the small difference ($\sim$ 1.4-fold) between the K_m of VWF96-Spacer and VWF87(Δ Spacer) proteolysis, suggests that the reduced binding of the VWF87(Δ Spacer) was primarily due to the deletion of the residues L1664, V1665 and L1666.

I incubated 0-230 μ M of the VWF96-Cys variant (n=41) with ADAMTS13 and obtained a slightly increased ($\sim$ 1.5-fold) k_{cat} of 8.3s⁻¹ and a K_m of 123 μ M, which is 31-fold increased compared to VWF96. The k_{cat}/K_m based on the Michaelis Menten analysis was 6.7x10⁴ M⁻¹s⁻¹, in accord with the value obtained by time course analysis ($k_{cat}/K_m=7.1\times 10^4$ M⁻¹s⁻¹)(section 4.9). The fact that the mutations in the Cys-rich domain exosite binding site of VWF96 primarily influenced the K_m of the reaction again shows that the Cys-rich domain exosite interaction, like the Spacer domain exosite interaction, is also primarily involved in the substrate binding affinity.

The analysis of the VWF96-MP variant (0-65 μ M, n=28) suggested that the nature of the contribution of the MP domain exosite interaction to proteolysis is quite different from that of the Spacer and Cys-rich domain exosite interactions. The L1603N mutation in the VWF96-MP variant caused a large reduction (66-fold) in the k_{cat} to 0.086s⁻¹ and a relatively small increase (3-fold) in the K_m to 13 μ M. The calculated k_{cat}/K_m of 0.65x10⁴ M⁻¹s⁻¹ is again similar to the value obtained by time course analysis ($k_{cat}/K_m=0.8\times 10^4$ M⁻¹s⁻¹)(section 4.9). This result shows that the interaction between the MP domain of ADAMTS13 and the P3 (L1603) residue in the VWF A2 domain mainly contributes to the k_{cat} of the reaction and has implications in the functionality of the catalytic centre and the precise 'fit' of the cleavage site in the active site.

The VWF96-Dis2 variant analysis (0-350 μ M, n=84) gave, potentially, the most interesting result. Ablating the Dis domain exosite interaction remarkably reduced both the substrate turnover, as seen by a 56-fold drop in the k_{cat} to 0.1s⁻¹, as well as the functional substrate binding, as seen by the 14-fold increase in the K_m to 56 μ M. Using these values I calculated the smallest k_{cat}/K_m of 0.18x10⁴ M⁻¹s⁻¹, which was 787-fold lower than the k_{cat}/K_m of VWF96, and was consistent with the predicted catalytic efficiency based on time course analysis ($k_{cat}/K_m=0.2 \times 10^4$ M⁻¹s⁻¹)(section 4.9).

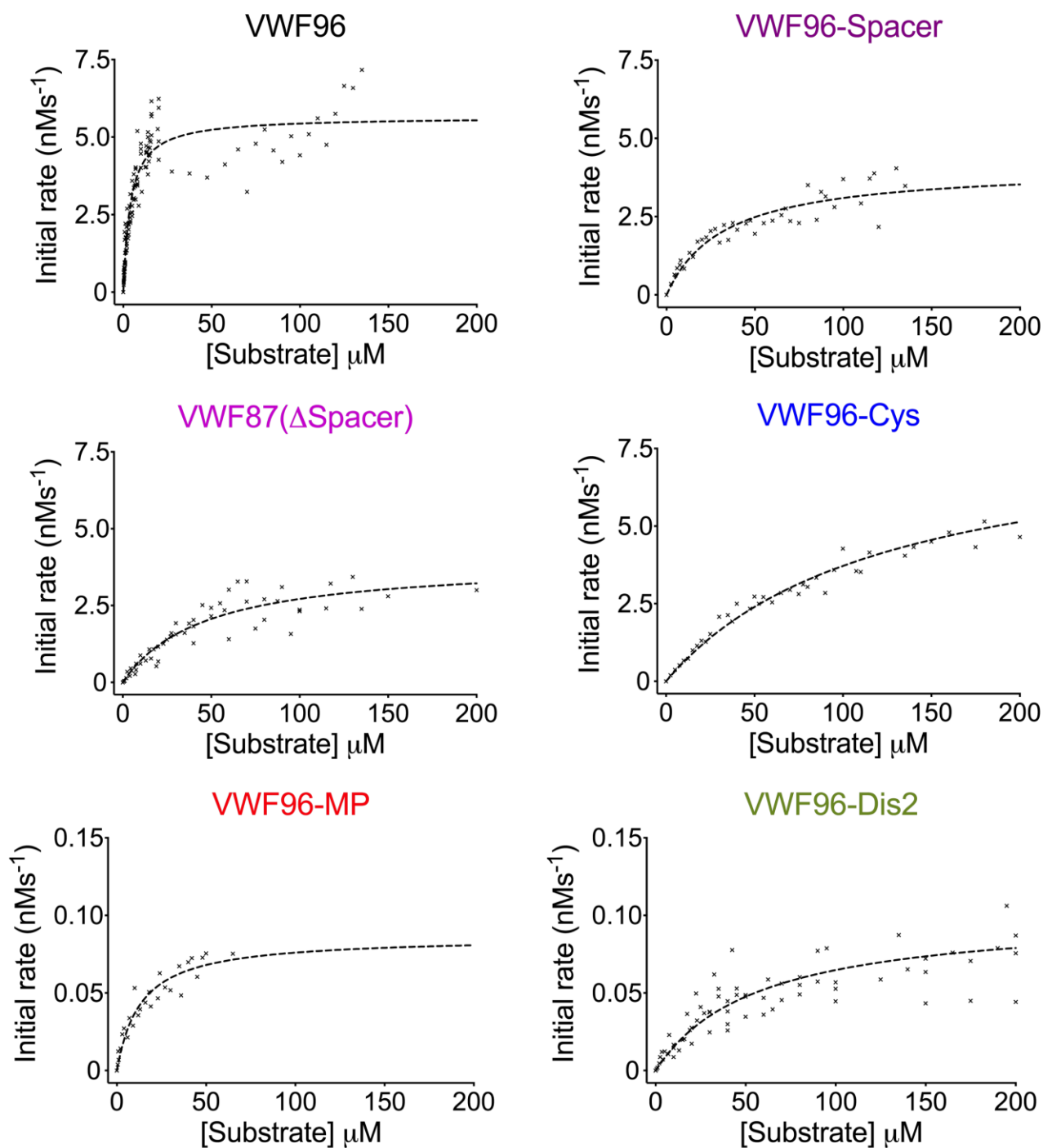


Figure 4-12 Michaelis Menten kinetic analysis of the proteolysis of the VWF96 variants by ADAMTS13

Initial rates (nMs⁻¹) of proteolysis by ADAMTS13 at multiple VWF96 concentrations were analysed by VWF96 ELISA and plotted as a function of substrate concentration (μM). These were fitted to the Michaelis Menten equation (see section 2.3.2.2). The derived k_{cat} and K_m values for the proteolysis of each variant are shown in Table 4-1.

Due to the fact that the data were plotted on axes with different scales in Figure 4-12, the relative effects of the different sets of mutations on the k_{cat} of proteolysis may not be obvious at first glance. To make it easier to understand the magnitude of the differences in the k_{cat} of proteolysis between the different VWF96 variants, all data were plotted on the same axes in Figure 4-13.

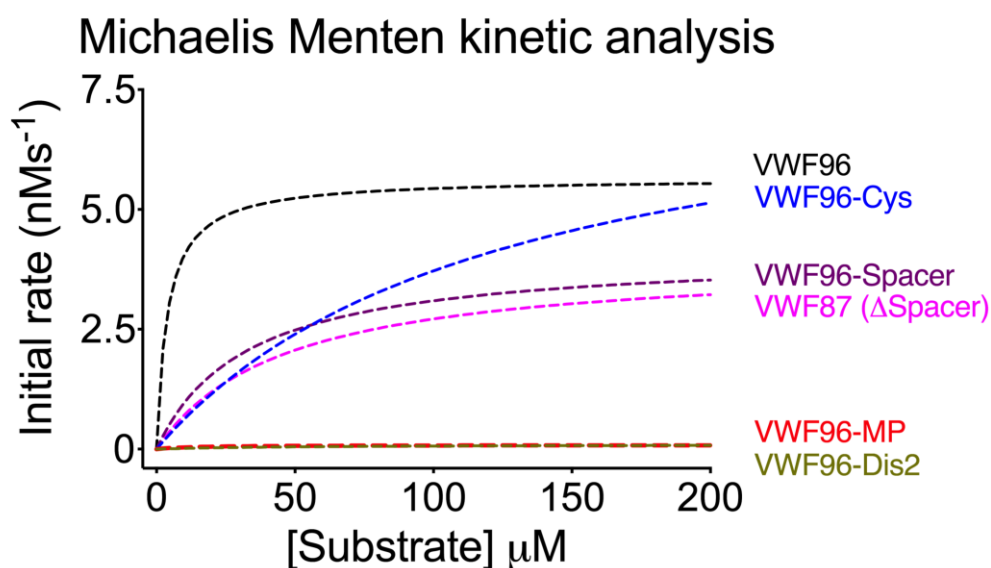


Figure 4-13 Michaelis-Menten kinetic analysis of the proteolysis of the VWF96 variants by ADAMTS13 plotted on the same graph

The fitted initial rates (nMs⁻¹) of VWF96 (variants) proteolysis as a function of substrate concentration (μM) were plotted on the same axes. This was to highlight the striking differences in the k_{cat} of proteolysis between the VWF96-MP (~66-fold reduction) and/or VWF96-Dis2 (~56-fold reduction) variants and the rest of the VWF96 variants (up to ~1.5-fold influence).

Table 4-1 Summary of the effect of ablating the ADAMTS13-VWF exosite interactions on the kinetics of proteolysis

SUBSTRATE	TIME COURSE		MICHAELIS-MENTEN KINETICS					
	$k_{cat}/K_m \pm \text{SEM}$ ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)	Fold change	$k_{cat} \pm \text{SEM}$ (s^{-1})	Fold change	$K_m \pm \text{SEM}$ (μM)	Fold change	$k_{cat}/K_m \pm \text{SEM}$ ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)	Fold change
VWF96	111 $\pm$ 2.28	-	5.7 $\pm$ 0.2	-	4.0 $\pm$ 0.4	-	142 $\pm$ 15.2	-
VWF87 (ΔSpacer)	7.5 $\pm$ 0.39	15 $\downarrow$	4.0 $\pm$ 0.3	1.4 $\downarrow$	46 $\pm$ 7.4	12 $\uparrow$	8.6 $\pm$ 1.5	16 $\downarrow$
VWF96-Spacer	13.1 $\pm$ 0.12	8 $\downarrow$	4.1 $\pm$ 0.3	1.4 $\downarrow$	33 $\pm$ 6.2	8 $\uparrow$	12.6 $\pm$ 2.5	11 $\downarrow$
VWF96-Cys	7.1 $\pm$ 0.26	16 $\downarrow$	8.3 $\pm$ 0.5	1.5 $\uparrow$	123 $\pm$ 15.0	31 $\uparrow$	6.7 $\pm$ 0.9	21 $\downarrow$
VWF96-Dis2	0.2 $\pm$ 0.01	527 $\downarrow$	0.1 $\pm$ 0.006	56 $\downarrow$	56 $\pm$ 10.1	14 $\uparrow$	0.18 $\pm$ 0.03	787 $\downarrow$
VWF96-MP	0.8 $\pm$ 0.03	138 $\downarrow$	0.09 $\pm$ 0.006	66 $\downarrow$	13 $\pm$ 2.8	3 $\uparrow$	0.65 $\pm$ 0.14	219 $\downarrow$

4.11 The equilibrium binding affinity (K_D) between ADAMTS13 and the VWF96 variants

I performed ELISA-based equilibrium binding assays as an alternative way of measuring the effect of ablating each exosite interaction on the binding affinity between the VWF96 variants and ADAMTS13. These involve immobilising one of the two interacting proteins on an ELISA plate, titrating the other interacting protein (ligand) and measuring the amount of the ligand bound. With increasing concentrations, the amount of ligand bound to the immobilised protein increases until all binding sites of the immobilised protein are saturated/occupied (termed B_{max}). The surface-phase equilibrium binding affinity can be determined by measuring the dissociation constant (K_D), which is the concentration of the ligand that achieves half maximum binding ($\frac{B_{max}}{2}$). Similar assays had been used previously to establish the K_D between ADAMTS13 and other VWF A2 domain-derived substrates, like VWF115 and VWF73.^(42, 46, 248, 266, 267) I expected that the reduction in the equilibrium binding affinity (measured as increased K_D) associated with ablating each exosite interaction would be analogous to the reduction in functional substrate binding (increased K_m) measured in the Michaelis Menten studies.

I initially immobilised 100nM of each VWF96 variant directly on a 96 well microtiter plate and titrated 0-600nM ADAMTS13 (section 2.4.1). EDTA was included in all buffers to inactivate ADAMTS13 in order to prevent proteolysis of the immobilised VWF96 variants. ADAMTS13 that had bound to the VWF96 variants was detected using antibodies against the TSP2-4 domains. The data were fitted to the one site binding equation in Graphpad Prism software, to obtain the B_{max} and K_D between ADAMTS13 and each VWF96 variant. Figure 4-14 shows the absorbance expressed as a percentage of the B_{max} ('% binding') and plotted as a function of ADAMTS13 concentration. For all VWF96 variants immobilised, there was a positive association between the concentration of ADAMTS13 and binding. Ideally, high enough concentrations of ADAMTS13 would be used to achieve the B_{max} . However, due to limiting ADAMTS13 concentrations available, none of the VWF96 variants reached the B_{max} at 600nM ADAMTS13; therefore, the extrapolated B_{max} values obtained from the regression were used for analysis. For VWF96 (n=5), the K_D was 40.2nM, which was very similar to published values for VWF115, VWF73, VWF A2 domain and full-length VWF.^(42, 46, 248, 266, 267) ADAMTS13 bound to the VWF96-MP variant with a K_D of 65.4nM, ~1.6-fold higher than VWF96. This small change in the binding affinity as a result of the L1603N mutation in the VWF96-MP variant was in agreement with the Michaelis Menten analysis, where a small (~3-fold) increase in the K_m was

also seen. The mutations in the rest of the variants also reduced the binding affinity in line with the Michaelis Menten analysis. ADAMTS13 bound to the VWF96-Spacer variant with a K_D of 241nM ($n=3$), which was 6-fold higher than VWF96. ADAMTS13 bound to the VWF87(Δ Spacer) variant less efficiently, with a K_D of 475nM ($n=3$), which was 12-fold higher than VWF96. As expected, the mutations in the VWF96-Cys and VWF96-Dis2 variants caused the biggest reductions in the binding affinity to ADAMTS13; the K_D values were very similar, 615nM ($n=3$) and 632nM ($n=3$), respectively, which were $\sim 15/16$ -fold higher than VWF96. The results are summarized in Table 4-2 including standard error.

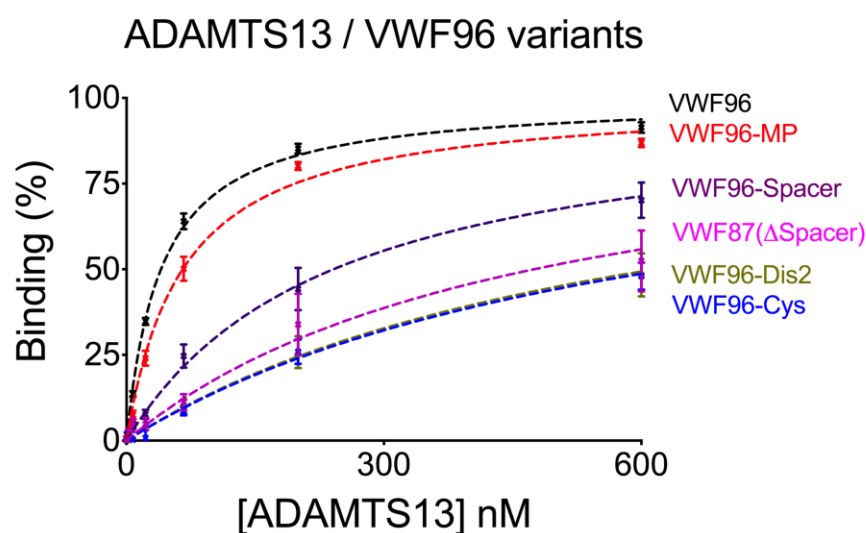


Figure 4-14 Equilibrium binding affinity between immobilised VWF96 variants and ADAMTS13 in solution

100nM VWF96 variants were immobilised on ELISA plates. ADAMTS13 (0-600nM) was titrated, in the presence of EDTA to inhibit proteolysis. Bound ADAMTS13 was detected using antibodies against the TSP2-4 domains. The absorbance was expressed as percentage of the absorbance at B_{max} and plotted as a function of ADAMTS13 concentration. The data were fitted using one site binding non-linear regression in Graphpad Prism software. The K_D values for the binding of the VWF96 variants to ADAMTS13 are summarised in Table 4-2.

4.12 The equilibrium binding affinity (K_D) between MDTCS(E225Q) and the VWF96 variants

As already mentioned, the limiting concentrations of ADAMTS13 used for the binding assays in section 4.11 did not allow accurate determination of the B_{max} with any of the VWF96 variants, although, theoretically at least, they should all have the same B_{max} at saturation, given that the same VWF96 variant quantities were immobilised. In an alternative attempt, I replaced ADAMTS13 with MDTCS(E225Q), an inactive, active site variant of ADAMTS13 (as a result of the E225Q substitution), which is truncated after the Spacer domain. MDTCS has been reported to bind to the VWF A2 domain fragments with higher affinity.⁽⁴³⁾ Moreover, MDTCS(E225Q) was expressed and purified in high amounts for structural studies (by Dr Frank Xu), therefore, its concentration was not a limiting factor.

The same protocol was followed as described in section 4.11, but using 0-600nM MDTCS(E225Q) instead of ADAMTS13, and using a polyclonal antibody that was raised in-house against ADAMTS13 (depleted of the anti-TSP2-4 antibodies)(section 2.4.2). Figure 4-15 shows ‘% binding’ ($\frac{Absorbance}{B_{max}} \times 100$) plotted as a function of MDTCS(E225Q) concentration. The relative affinities between the VWF96 variants and MDTCS(E225Q) were similar to those obtained in the assays with ADAMTS13 (section 4.11); however, as expected due to the higher affinity to VWF associated with the truncation of the C-terminal domains of ADAMTS13, the absolute K_D values were generally lower. MDTCS(E225Q) bound to VWF96 with a K_D of 7.1nM. Using the VWF96-MP variant, I measured a K_D of 22nM, which was ~3-fold higher than VWF96. MDTCS(E225Q) bound to the VWF96-Spacer variant with a K_D of 60nM (~8.4-fold higher than VWF96), and to the VWF87(Δ Spacer) variant with a K_D of 107nM (~15-fold higher than VWF96). The lowest affinities were measured with the VWF96-Dis2 and VWF96-Cys variants again. Using the VWF96-Dis2 variant resulted in K_D of 124nM (~17-fold higher than VWF96), and using the VWF96-Cys variant resulted in K_D of 150nM (~21-fold higher than VWF96). The results are summarized in Table 4-2 including standard error.

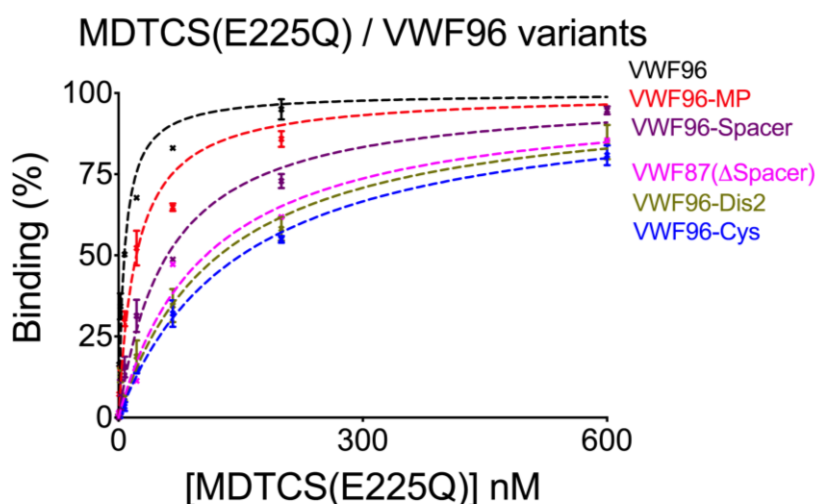


Figure 4-15 Equilibrium binding affinity between immobilised VWF96 variants and MDTCS(E225Q) in solution

100nM VWF96 variants were immobilised on ELISA plates. MDTCS(E225Q) (0-600nM) was titrated. No EDTA was used, as the E225Q mutation in the active site inactivates MDTCS. Bound MDTCS(E225Q) was detected by rabbit pAb anti-ADAMTS13 (depleted of the antibodies against TSP2-4 domains). The absorbance was expressed as percentage of absorbance at $B_{\max}$ and plotted as a function of MDTCS(E225Q) concentration. The data were fitted using one site binding non-linear regression in Graphpad Prism software. The K_D values for the binding of the VWF96 variants to MDTCS(E225Q) are summarised in Table 4-2.

Table 4-2 Summary of the equilibrium binding affinity (K_D) between immobilised VWF96 variants and ADAMTS13 or MDTCS(E225Q) in solution

SUBSTRATE (Immobilised)	LIGAND			
	ADAMTS13 (n=3)		MDTCS(E225Q) (n=1)	
	$K_D \pm \text{SEM}$ (nM)	Fold change	$K_D \pm \text{SEM}$ (nM)	Fold change
VWF96	40.2 $\pm$ 1.3	-	7.1 $\pm$ 0.96	-
VWF87 (Δ Spacer)	475 $\pm$ 61.3	12 $\uparrow$	107 $\pm$ 15.7	15 $\uparrow$
VWF96-Spacer	241 $\pm$ 19.3	6 $\uparrow$	60 $\pm$ 5.1	8.4 $\uparrow$
VWF96-Cys	632 $\pm$ 40.6	16 $\uparrow$	150 $\pm$ 7.7	21 $\uparrow$
VWF96-Dis2	615 $\pm$ 51.7	15 $\uparrow$	124 $\pm$ 13.3	17 $\uparrow$
VWF96-MP	65.4 $\pm$ 3.1	1.6 $\uparrow$	22 $\pm$ 2.7	3.1 $\uparrow$

4.13 DISCUSSION

In this Chapter, I described how I generated VWF96 variants possessing mutations that independently ablate each entire exosite interaction. These were used as substrates in cleavage and binding assays to study the biochemistry of each exosite's contribution to the recognition and proteolysis of VWF by ADAMTS13. For the first time all functional exosites in the ADAMTS13-VWF axis were ablated in parallel, which enabled direct comparison of the relative contribution of each exosite to binding and proteolysis. I made the assumption that the exosite interactions were ablated by the mutations. This was based on targeting the most important residues in the regions of the VWF A2 domain that had been linked to functional exosite binding. It is naturally possible that the exosite interactions were not fully ablated. Nevertheless, the effects of each set of mutations were specific to one exosite.

I chose to ablate the exosite interactions by introducing composite substitutions to all the ADAMTS13 exosite binding sites in VWF96. An alternative way to ablate exosite interactions is to generate ADAMTS13 variants by directly targeting the exosites for mutagenesis, as performed in a number of prior studies.^(242, 250, 266-268) I elected not to use this approach for various reasons. First, ADAMTS13 is a large multidomain protein with complex secondary/tertiary structure. Targeting entire exosites in ADAMTS13 could have structural implications that could potentially influence the rate of proteolysis in an exosite independent manner or lead to misfolding and poor secretion. Secondly, ADAMTS13 variants would need to be expressed in mammalian cells; this would be relatively laborious and time consuming, particularly if substitutions caused secretion defects. Thirdly, purification (by immobilised metal ion affinity chromatography) can impair the enzymatic activity of ADAMTS13 in an exosite independent manner, which would complicate determining the relative contribution of the targeted exosites. In contrast, VWF96 is a short polypeptide without a complex structure. Therefore, mutations are less likely to induce structural modifications that would impact upon the rate of proteolysis of the VWF96 variants. Furthermore, bacterial expression and purification of the VWF96 variants is relatively easy and quick; conveniently, this allowed me to modify my mutagenesis strategy (after having generated an initial panel of variants) to generate a revised panel of VWF96 variants (see sections 4.4 to 4.7).

To explore the relative importance of the exosite interactions, the proteolysis of VWF96 variants was initially analysed qualitatively (section 4.7). The qualitative experiments showed

that individual ablation of all exosite interactions reduced the rate of proteolysis significantly. Despite their significant contribution, the Cys-rich and Spacer domain exosite interactions were, relatively, less important for proteolysis than the Dis and MP domain exosite interactions. The VWF96-MP variant was cleaved very inefficiently. This was expected as substitution of the L1603 residue had been reported previously to cause substantial reductions in the rate of proteolysis (probably due to its proximity to the active/cleavage site).⁽²⁵²⁾ It was clear that the mutations ablating the Dis domain exosite interactions caused the most detrimental reduction to, and nearly abolished, the rate of proteolysis. This was surprising given that, to date, no major differences between the contribution of the Dis, Cys-rich and Spacer domains to the rate of VWF proteolysis had been inferred. However, it must be pointed out that direct comparison of the functionality of these exosites had not been performed prior to this project.

The VWF96 ELISA was used to make quantitative comparisons of the reduction in the rate of proteolysis associated with ablating each exosite interaction. The rate of proteolysis was calculated for each variant in two ways. First, by analysing the time course of proteolysis to measure the catalytic efficiency, k_{cat}/K_m (section 4.9). For accurately calculating the k_{cat}/K_m from single time course analysis, it is important that the VWF96 variant concentrations used are below the K_m of proteolysis. This is because substrate concentrations lower than the K_m are positively and linearly associated with the rate of proteolysis; therefore, at any time point, the same proportion of substrate will be cleaved by the same amount of enzyme, resulting in the same k_{cat}/K_m . For some VWF96 variants, reactions at relatively higher substrate concentrations were used in calculating the k_{cat}/K_m ; these were added to the dataset retrospectively, after the Michaelis Menten kinetic analysis had established higher K_m of proteolysis for those variants. Secondly, the separate k_{cat} and K_m constants were measured by Michaelis Menten analysis of the initial rates of proteolysis at different VWF96 variant concentrations; these were used to manually calculate the k_{cat}/K_m for each variant (section 4.10).

I predicted that an exosite interaction that facilitates allosteric activation of ADAMTS13 would exhibit the greatest contribution towards the rate of proteolysis when compared to exosites involved only in substrate binding; therefore, ablating that exosite would most severely impair proteolysis. Based on the quantitative analysis, the Spacer and Cys-rich domain exosites contribute ~15-to-20-fold towards the k_{cat}/K_m . Contributions of similar magnitude had been assigned to the Spacer^(248, 251, 255) and Cys-rich^(255, 266) domain exosites in prior studies.

However, it was the first time that the importance of these exosites was directly compared to each other as well as to the importance of the Dis domain exosite. It was intriguing to observe such a big difference between the contribution of the Dis domain exosite interaction (up to ~787-fold) and the contribution of the Spacer and Cys-rich domain exosite interactions (~15-to-20-fold), which had not been obvious previously. This was a first indication that the Dis domain exosite interaction could contain a remote exosite that allosterically activates ADAMTS13.

It was necessary to further understand the biochemical nature of the exosite interactions, to determine whether the exosites contribute solely to the binding affinity between ADAMTS13 and VWF, or have a functional role to play in the activity of the active site and substrate turnover. I used Michaelis Menten analysis of the proteolysis and ELISA-based equilibrium-binding assays to obtain the kinetic constants (k_{cat} , K_m and K_D) of proteolysis/binding between all VWF96 variants and ADAMTS13/MDTCS(E225Q). This was the first time that the effect of ablating all exosite interactions on the rate of proteolysis and binding between ADAMTS13 and VWF was analysed and directly compared kinetically. I expected that an exosite that allosterically activates ADAMTS13 would influence the k_{cat} (substrate turnover) as well as the K_m and K_D (substrate binding affinity) significantly. On the other hand, exosites that mainly contribute to the binding affinity between ADAMTS13 and VWF were expected to primarily influence the K_m and the K_D .

I studied the binding affinity between the VWF96 variants and ADAMTS13 in two ways (hence, quantifying two binding affinity constants for each variant, the K_m and K_D). K_m is a measure of the functional substrate binding that contributes to proteolysis. As this was obtained from kinetic analysis of proteolytic reactions, it reflects the solution-phase binding affinity. K_D measures the equilibrium binding affinity, and, as it was calculated from ELISA-based plate-binding assays with the VWF96 variants immobilised on the plate, it reflects the surface-phase binding affinity. Plate binding equilibrium assays are associated with limitations, particularly when measuring lower affinity interactions. This is because of the multiple washing steps involved, which tend to remove unbound ligand, shifting the binding equilibrium. Nevertheless, similar reductions in the binding affinity were measured with both methods, giving confidence that the measured effects of the mutations on the binding affinity are valid. As it had been observed previously,^(46, 251, 256, 267) the absolute K_m and K_D values are very different. For example, while the K_m of VWF96 proteolysis by ADAMTS13 is ~4 μ M, the K_D of ADAMTS13-

VWF96 binding is $\sim 40.2\text{nM}$, showing major differences between the solution-phase binding affinity and the affinity when VWF96 variants are immobilized. An explanation for this phenomenon has never been speculated. It is possible that VWF96 or similar VWF A2 domain fragments are not perfectly linear, as widely considered, but they possess tertiary structure. If this holds true, then conformational differences between adsorbed and soluble VWF96 might explain the differences in the affinities; immobilization on a plate could promote uniform exposure of all the binding sites of VWF96, while only $<10\%$ of soluble VWF96 might be in a conformation that is permissive to binding at any one time. Kretz *et al* report substitutions of single residues in the VWF73 sequence that can enhance the rate of proteolysis.⁽³⁶³⁾ This might be manifest by disruption of the tertiary structure of the VWF73, enhancing the affinity for ADAMTS13. Further discussion is provided in Chapter 6. I also made further attempts to study the equilibrium affinity of solution-phase binding, with ELISA protocols that use antibodies to capture ADAMTS13 or VWF96 variants. These assays were not sensitive and/or reproducible, and, as predicted, suggested lower binding affinities. Equilibrium binding assays lose sensitivity when affinities are low.

The reductions in the rate of proteolysis associated with the ablation of the Spacer and Cys-rich domain exosites were primarily attributed to reductions in binding affinity. In all assays, the contribution of the Cys-rich domain in the binding affinity (~ 31 -fold contribution to the K_m and ~ 16 -to- 22 -fold contribution to the K_D) was consistently higher than the contribution of the Spacer domain (~ 12 -fold contribution to the K_m and ~ 12 -to- 15 -fold contribution to the K_D). The relatively modest influence (<2 -fold) of the Spacer and Cys-rich domain exosites to the k_{cat} indicates that these exosite interactions do not contribute to the functionality of the active site. Therefore, my data are not consistent with the existence of an allosteric link between the Spacer or the Cys-rich domain exosites and the MP domain.

Based on epitope mapping using anti-ADAMTS13 autoantibodies from aTTP patients, followed by mutagenesis studies, the solvent exposed residues R659, R660, Y661, Y665 were proposed to form the exosite in ADAMTS13 Spacer domain.^(235, 267, 268) Mutagenesis/deletion studies also showed that the E1660-R1668 region in the VWF A2 domain forms the binding site for the Spacer domain of ADAMTS13.^(251, 255, 360) However, the precise residues that facilitate the Spacer domain exosite interaction have not been definitively demonstrated. Mutating the charged residues located in the E1660-R1668 region of the VWF A2 domain did not influence the rate of proteolysis (apart from a modest effect associated with mutating the D1663).⁽³⁶¹⁾ It

was, therefore, likely that hydrophobic residues in the E1660-R1668 interact with a hydrophobic pocket in the Spacer domain. For this reason, I generated two VWF96 variants with mutations in the E1660-R1668 region. The VWF87(Δ Spacer) variant had the entire E1660-R1668 region deleted, while the VWF96-Spacer variant had the hydrophobic residues L1664, V1665 and L1666 substituted (LVL1664-1666TNQ). Notably, my results are in excellent agreement with the data of Gao *et al*, who showed that deleting the E1660-R1668 region in VWF73 increased the K_m by ~ 22 -fold without affecting the k_{cat} .⁽²⁵¹⁾ This verifies the importance (and magnitude) of the Spacer domain exosite interaction towards ADAMTS13 binding, and further validates my approach as a sensitive and accurate way of characterising the kinetics of ADAMTS13 proteolysis.

Comparing the results from the experiments using the VWF96-Spacer and VWF87(Δ Spacer) variants, the functional deficit associated with mutating just the L1664, V1665 and L1666 residues explained most of the functional deficit associated with removing the entire E1660-R1668 region. The results from the Michaelis Menten analysis show that deleting the entire E1660-R1668 region in the VWF87(Δ Spacer) variant reduces the k_{cat}/K_m by 16-fold, while the LVL1664-1666TNQ substitutions in the VWF96-Spacer variant reduce the k_{cat}/K_m by 11-fold; this modest 1.5-fold difference between the two variants is negligible compared to the functional deficit associated with ablating the Spacer exosite interaction. One possibility is that the functional difference between the VWF96-Spacer and VWF87(Δ Spacer) variants is artefactual, and associated with variability or protein quantification errors. Wu *et al*⁽³⁶¹⁾ showed that the mutation D1663A causes a modest reduction in the rate of proteolysis; the absence (due to deletion) of this residue in the VWF87(Δ Spacer) variant could account for the 1.5-fold lower rate of proteolysis and binding affinity compared to the VWF96-Spacer, where this residue is present. My data strongly suggest that primarily hydrophobic residues facilitate the interaction between the Spacer domain of ADAMTS13 and the VWF A2 domain; whether they interact with the proposed Y661 and Y665 residues, or with another proximal hydrophobic site, remains to be definitively concluded.

The P3 residue, L1603, in the VWF A2 domain had been known to be functionally important. P3 substitutions (L1603S and L1603A) reduced the rate of proteolysis by up to >400 -fold. The L1603N mutation (also introduced in the VWF96-MP variant) had also been shown, qualitatively, to markedly reduce the rate of VWF115 proteolysis.⁽²⁵²⁾ My results corroborate the critical importance of this residue. I showed that the L1603N substitution reduced the rate

of proteolysis of the VWF96-MP variant by ~138-to-219-fold. Based on the fact that a single residue that is in close proximity to the cleavage site has such a major influence on the rate of proteolysis, I expected that the interaction with the MP domain of ADAMTS13 mainly influenced the catalytic capacity of the active site and not so much the binding affinity to ADAMTS13. Indeed, this mutation caused the biggest reduction (~66-fold) in the substrate turnover (k_{cat}), while only a relatively small (up to ~3-fold) contribution to the binding affinity (K_m and K_D) was recorded. Despite a relatively small reduction in binding compared to the effects of the mutations in the rest of the exosites, this is still a somewhat large reduction in the binding affinity that would not be expected from mutating only a Leu residue. One possibility for this effect could be that the influence of the L1603N mutation on the binding affinity is an artefact, due to variability in the amounts of VWF96 variants immobilised, or due to the mutation affecting immobilisation of the VWF96-MP variant on the plate, but in my opinion, the contribution of these is likely negligible. It is also possible that the L1603N had structural implications affecting an exosite; in this case, the influence on the binding affinity recorded is real, but not dependent on the MP domain exosite interaction. The VWF96-MP variant acted as a good control in this project, showing the sensitivity of the assays performed in differentiating between changes in the k_{cat} and the K_m .

The most insightful finding of this project was the magnitude and nature of the contribution of the Dis domain exosite towards proteolysis by ADAMTS13. Ablating the Dis domain exosite interaction markedly reduced the rate of proteolysis by up to ~787-fold. This very large reduction in the rate of proteolysis far outcompeted the effect of ablating any other exosite interaction, which ranged between ~11-to-219-fold. Notably, the influence of the Dis domain exosite interaction on the rate of proteolysis is even greater than that of the P3 residue, which, when mutated, caused the biggest reported reduction in the rate of proteolysis.

To date, individually mutating residues hypothesized to be involved in the Dis exosite interaction has only reduced the rate of proteolysis by up to ~20-fold^(250, 256). This was clearly an underestimate, compared to the ~787-fold reduction in the rate of proteolysis as a result of the complete ablation of the exosite interaction in the VWF96-Dis2 variant. My results indicate that what had been considered to be the Dis domain exosite interaction was incomplete. I showed that the Dis domain exosite binding site covers the region D1614-D1622. This also suggests that, apart from the R349, more residues in the ADAMTS13 Dis domain are involved in functional interactions with the VWF A2 domain, which are yet to be identified.

The exosite in the ADAMTS13 Dis domain is a remote exosite, as it is not present in the catalytic MP domain. As expected, this interaction is indeed important for substrate binding, as seen by the ~14-to-17-fold contribution to the K_m and K_D . In fact, this exosite is as important as the rest of the remote exosites (Spacer and Cys-rich domain exosites) in substrate binding. Surprisingly, the interaction of the Dis exosite also dictates the substrate turnover, to a much greater extent, as seen by the 56-fold contribution to the k_{cat} . These results are consistent with an allosteric activation mechanism that is dependent on the Dis domain exosite interaction. For this to occur, binding of VWF to the Dis domain must induce a conformational change in the MP domain. That the Dis domain is adjacent to, and is predicted to share a large interface with, the MP domain further supports the possibility of an allosteric linkage. This will be further discussed in Chapter 6.

The VWF96 ELISA assay was not associated with major intra-assay variability; therefore, assay replicates that were performed during the same period of time and with the same reagent preparations generated very reproducible results. This is obvious considering the small error when analysing the catalytic efficiency from proteolytic time course experiments (see section 4.9 and Figure 4-11). The main source of variability in the kinetic analysis of VWF96 (variant) proteolysis by ADAMTS13 was the variation in the specific activity (up to ~2-3-fold) of different preparations of ADAMTS13. This somewhat complicated combining datasets that were generated from experiments performed over long periods of time. To minimise the error in my results, I performed a large number of reactions ($n=113$ for VWF96 and $n=84$ for the VWF96-Dis2 variant) at multiple substrate concentrations so that the calculated fitted initial rate values at any concentration were as close to the true values. However, this variability could not have significantly affected the conclusions of this study. The magnitude of the influence of ablating the exosites on the rate of proteolysis ranged from ~8-fold to ~787-fold, which cannot be explained by the modest (~2-to-3-fold) variability in the specific activity of the enzyme preparations. What is more, the excellent agreement of the results obtained from independent and different sets of experiments (catalytic efficiency from proteolytic time course, Michaelis Menten analysis and ELISA-based plate binding assays) gives confidence about the validity and quality of my data.

5 RESULTS: KINETIC CHARACTERISATION OF THE ACTIVATING EFFECT OF ANTI-SPACER AND ANTI-CUB ANTIBODIES ON VWF96 VARIANT PROTEOLYSIS BY ADAMTS13

5.1 INTRODUCTION

As introduced in Chapter 1 (see section 1.8.1), ADAMTS13 circulates in what is thought to be a globular fold, in which the C-terminal tail folds back over the N-terminal domains of the molecule (section 1.8.1). This folded conformation is maintained by intra-molecular forces between different domains of the molecule. Disruption of these forces causes a structural transition into an unfolded conformation, which enhances the activity of ADAMTS13 ~2-to-10-fold.^(43, 44, 46)

The first evidence of the conformational activation mechanism was the unexpected finding that anti-ADAMTS13 autoantibodies from one TTP patient appeared to enhance the activity of ADAMTS13 but not MDTCS, indicating a functional role associated with the C-terminal domains of the enzyme.⁽⁴³⁾ Various conditions were shown to induce the conformational extension that enhances the activity of ADAMTS13 *in vitro*. Lowering the pH (to ~pH 6) enhances the activity of ADAMTS13 but not the activity of MDTCS.⁽⁴³⁾ Furthermore, mutations in the Spacer domain (R568K/F592Y/R660K/Y661F/Y665F) impair the ability of ADAMTS13 to adopt the folded conformation (as assessed by transmission electron microscopy and multiple functional assays), and, therefore, enhance its activity (hence termed gain-of-function ADAMTS13).^(46, 375)

Three linker regions in the TSP2-CUB2 domains give the flexibility to the C-terminal tail of ADAMTS13 to fold and unfold.⁽²⁶⁵⁾ According to the proposed model, intra-molecular interactions between the Spacer and C-terminal CUB domains facilitate the adoption of the folded conformation of ADAMTS13, although this has not been definitively demonstrated. The CUB1 domain has been reported to interact with the Y661/Y665 residues of the Spacer domain.⁽⁴⁴⁾ In this model, the Spacer-CUB domain interaction imposes steric hindrance to the exosite in the Spacer domain (normally involved in a functional interaction with the VWF A2 domain that is important for proteolysis), and in so doing accounts for the reduced activity of ADAMTS13 in the folded conformation.

The isolated D4 can also enhance the activity of ADAMTS13.⁽⁴³⁾ The D4-CK domains were shown to interact with the TSP8 domain of ADAMTS13, and this interaction also enhances the activity of the protease.^(44, 46) These observations were used to attribute physiological relevance to the conformational activation of ADAMTS13. It was proposed that binding to the VWF D4(-CK) in circulation interferes with the Spacer-CUB interaction and unfolds ADAMTS13, making it more active in cleaving VWF.

Monoclonal antibodies with specific affinities to the C-terminal domains of ADAMTS13 have been proposed to mimic the conformational activation of ADAMTS13, enhancing its activity against VWF. These include the anti-TSP6/7 domain mAb 7C4,⁽⁴³⁾ anti-TSP8 domain mAb 14D2 and mAb 19H4,⁽⁴³⁾ anti-CUB1 domain mAb 12D4⁽⁴³⁾ and mAb 17G2,⁽²⁶⁵⁾ and anti-CUB2 domain mAb 20E9.⁽⁴⁶⁾ These monoclonal antibodies were produced in hybridoma cells by immunisation of mice with recombinant MDTCS, followed by fusion of spleen and SP2/0 myeloma cells.⁽²⁸⁴⁾ Epitope mapping was performed by ELISA, as previously described.^(43, 46, 265, 284)

Recently, a novel anti-Spacer mAb, 3E4, with similar activity-enhancing properties to the abovementioned monoclonal antibodies was also generated, following the same standard procedures. However, the precise mechanism by which this antibody activates ADAMTS13 has not been characterised. Revealing the precise mechanism by which these antibodies act upon ADAMTS13 and enhance its function is potentially interesting, as it may shed light on the biochemistry of ADAMTS13 unfolding and its functional implications. To further understand the mechanism by which the proposed antibody-mediated unfolding of ADAMTS13 enhances its activity, I performed kinetic analysis on the activating effect of the anti-Spacer mAb 3E4 and anti-CUB1 mAb 17G2 on VWF96 proteolysis using the VWF96 ELISA. I hypothesized that, if the activity enhancement of ADAMTS13 is attributed to the unfolding of the C-terminal tail to fully expose the Spacer exosite, the activation should be dependent on the presence of the C-terminal tail of ADAMTS13 and the Spacer exosite interaction. Furthermore, I hypothesized that if the conformational activation of ADAMTS13 is dependent on promoting exposure of the Spacer exosite to enhance the binding affinity to VWF96, the presence of the activating antibodies should reduce the K_m of the proteolysis reaction, and not affect the k_{cat} . The work described in this chapter is a collaboration with An-Sofie Schelpe (KULAK, Belgium). An-Sofie generated the mAb 3E4 and mAb 15D1 antibodies as part of her PhD, provided the mAb 17G2 and wild-type ADAMTS13, and contributed to the experiments that follow.

5.2 Measuring the activating effect of the mAb 3E4 and mAb 17G2 antibodies on the catalytic efficiency of VWF96 proteolysis by ADAMTS13

To specifically quantify the effect of the anti-Spacer (mAb 3E4) and anti-CUB (mAb 17G2) monoclonal antibodies upon the activity of ADAMTS13, I measured the catalytic efficiency of VWF96 proteolysis by ADAMTS13 in the presence and absence of the activating antibodies. For this, I pre-incubated the antibodies (10 μ g/ml) with ADAMTS13 (0.6nM) for 10 minutes, and VWF96 (0.5 μ M) was added to initiate the reaction. Sub-samples were stopped between 0-90 minutes and analysed by VWF96 ELISA (section 2.5). As a negative control, I used a non-activating (and non-inhibitory) antibody against the Spacer domain of ADAMTS13 (mAb 15D1), which was generated and characterised in the same way as mAb 3E4 and mAb 17G2.

Figure 5-1 shows the fitted time course data of VWF96 proteolysis in the presence and absence of the different antibodies tested. In the absence of antibodies, VWF96 was proteolysed with a k_{cat}/K_m of $127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=7). This is in excellent agreement with my previous estimate (k_{cat}/K_m of $110 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) reported in Chapter 4 (section 4.9). Essentially the same k_{cat}/K_m ($127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (n=7) was obtained when VWF96 was proteolysed in the presence of the non-inhibitory, non-activating mAb 15D1, justifying the use of this antibody as a negative control. As expected, both mAb 3E4 and mAb 17G2 had an activating effect on the catalytic efficiency of VWF96 proteolysis by ADAMTS13. The mAb 3E4 enhanced the catalytic efficiency ~2.0-fold ($k_{cat}/K_m = 249 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, n=7), while the mAb 17G2 enhanced the catalytic efficiency ~1.8-fold ($k_{cat}/K_m = 229 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, n=7). The magnitude of the activating effect of both antibodies was entirely consistent with the activating effect measured by An-Sofie Schelpe previously, using FRETs-VWF73 (1.7-fold, unpublished). To understand whether the activating effects of the two antibodies are dependent on the same activating mechanism, I used a cocktail of both mAb 3E4 and mAb 17G2 in the same reaction. For these, I obtained a k_{cat}/K_m of $274 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3), which was 2.2-fold higher than the catalytic efficiency of VWF96 proteolysis by ADAMTS13 in the absence of antibodies. The absence of any appreciable synergism suggested that the mAb 3E4 and mAb 17G2 activate ADAMTS13, at least partly, through the same mechanism. The results are summarised in Table 5-1 including standard error.

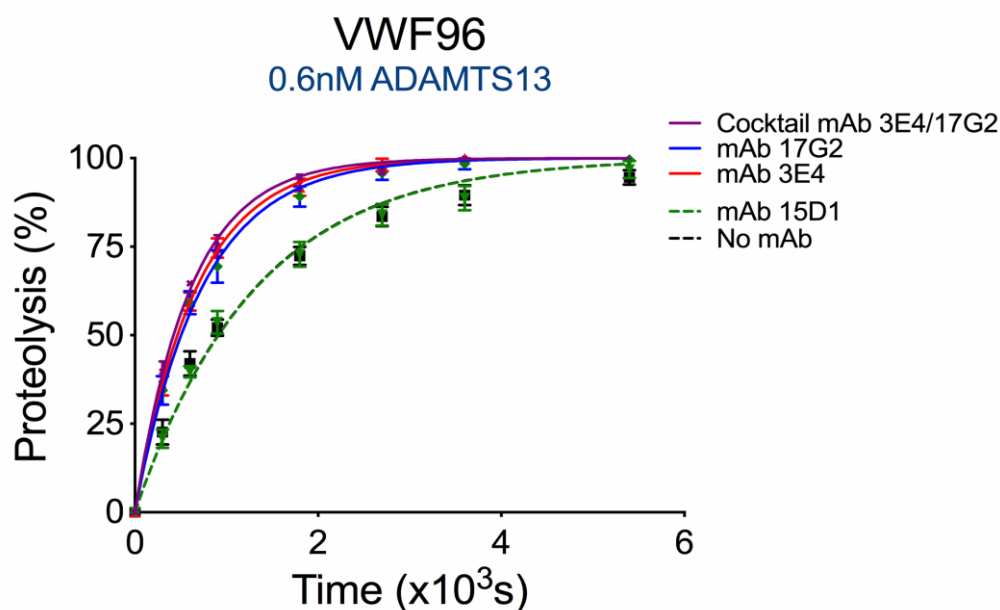


Figure 5-1 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96 by ADAMTS13.

VWF96 (0.5 μ M) was incubated with purified ADAMTS13 (0.6nM) in the presence and absence of 10 μ g/ml monoclonal antibodies. Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. In the absence of antibodies, VWF96 was proteolysed with k_{cat}/K_m of $127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=7$). The negative control, mAb 15D1, did not enhance the rate of proteolysis ($k_{cat}/K_m=127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$)($n=7$).The presence of mAb 3E4 and mAb 17G2 enhanced the activity of ADAMTS13, separately, by ~ 2.0 - and ~ 1.8 -fold, respectively ($k_{cat}/K_m=249 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{cat}/K_m=229 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively, $n=7$). Together, the antibodies induced a ~ 2.2 -fold enhancement in the activity of ADAMTS13 ($k_{cat}/K_m=274 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). Results are summarised in Table 5-1.

5.3 The activating effect of mAb 3E4 and mAb 17G2 is dependent on the unfolding of ADAMTS13 C-terminal tail

The hypothetical activating mechanism of the mAb 3E4 and mAb 17G2 involves disruption of the inferred CUB-Spacer interaction that unfolds ADAMTS13 from a folded to an extended conformation; this removes the steric hindrance imposed by the C-terminal tail to an exosite interaction (presumably the Spacer domain exosite interaction), enhancing the binding affinity between ADAMTS13 and VWF96. To test whether the antibody-mediated activation was dependent on the C-terminal tail, I used the same approach described in section 5.2, but substituting ADAMTS13 with the enzyme variant truncated after the Spacer domain (MDTCS). The mAb 17G2 served as an additional negative control in these experiments, since its ligand (CUB domains) was not available on the MDTCS variant for binding.

As shown in Figure 5-2, the monoclonal antibodies had no effect upon the catalytic efficiency of VWF96 proteolysis by MDTCS (1.2nM). VWF96 was cleaved with a k_{cat}/K_m of $91.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3). In the presence of the mAb 3E4, mAb 17G2, mAb 15D1 and mAb 3E4/17G2 (cocktail), VWF96 was proteolysed at very similar rates ($k_{cat}/K_m = 78.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3), $k_{cat}/K_m = 89.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3), $k_{cat}/K_m = 78.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3) and $k_{cat}/K_m = 84.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3), respectively). These results suggest that the activating effect of the mAb 3E4 and mAb 17G2 is indeed dependent on the presence of the C-terminal tail of ADAMTS13. The results are summarised in Table 5-1 including standard error.

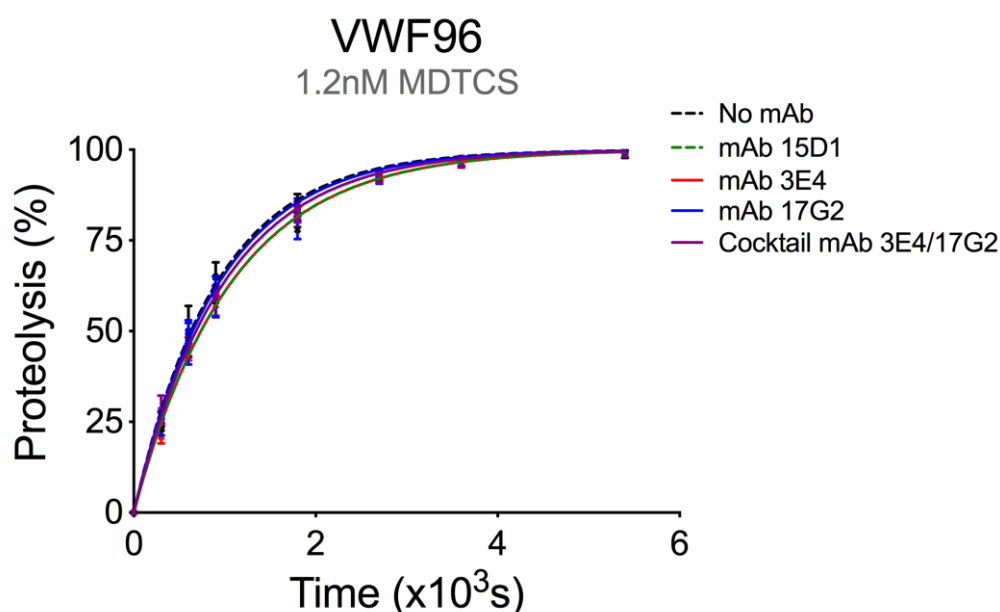


Figure 5-2 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96 by MDTCS
VWF96 (0.5 μM) was incubated with MDTCS (1.2nM) concentrated in conditioned media, in the presence and absence of 10 $\mu\text{g}/\text{ml}$ monoclonal antibodies. Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. In the absence of antibodies, VWF96 was proteolysed with k_{cat}/K_m of $91.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (n=3). No major difference in the catalytic efficiency was observed with any of the different antibody conditions. The results are summarised in Table 5-1.

5.4 The activating effect of the mAb 3E4 and mAb 17G2 is independent of the ADAMTS13 Spacer and Cys-rich domain exosite interactions

In section 5.3, I demonstrated that the mAb 3E4 and mAb 17G2 enhance the activity of ADAMTS13 via a mechanism that is dependent on the C-terminal tail of the enzyme. My hypothesis was that binding of the antibodies to the Spacer and CUB domains unfolds the C-terminal tail of ADAMTS13, sterically unmasking ADAMTS13 exosites to enable their interaction with their complementary binding sites in VWF96. In Chapter 4, I showed that the Spacer domain exosite interaction is mediated by the residues E1660-R1668 in the VWF A2 domain. I, therefore, used the VWF87(Δ Spacer) variant (delE1660-R1668), which has the Spacer domain exosite interaction ablated, in reactions with ADAMTS13 (9nM). If the hypothesis holds true, no difference in the rate of ADAMTS13 proteolysis of this variant should be associated with any of the activating antibodies, since the Spacer domain exosite interaction is already ablated.

Surprisingly, the activating effect of the mAb 3E4 and mAb 17G2 on the proteolysis of the VWF87(Δ Spacer) variant was still evident (Figure 5-3). In the absence of antibodies, the VWF87(Δ Spacer) variant was cleaved with a k_{cat}/K_m of $4.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$); the ~ 26 -fold lower k_{cat}/K_m , compared to the proteolysis of VWF96 measured in section 5.2 ($k_{cat}/K_m = 127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) was expected, due to the ablation of the Spacer domain exosite interaction. The presence of mAb 15D1 did not exert any influence on the rate of the VWF87(Δ Spacer) variant proteolysis ($k_{cat}/K_m = 5.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). In contrast, both mAb 3E4 and mAb 17G2 increased the rate of proteolysis by ~ 2.2 -fold ($k_{cat}/K_m = 10.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). This showed that, contrary to the hypothesis, the antibody-mediated activation of ADAMTS13 was independent of the Spacer domain exosite interaction. The results are summarised in Table 5-1 including standard error.

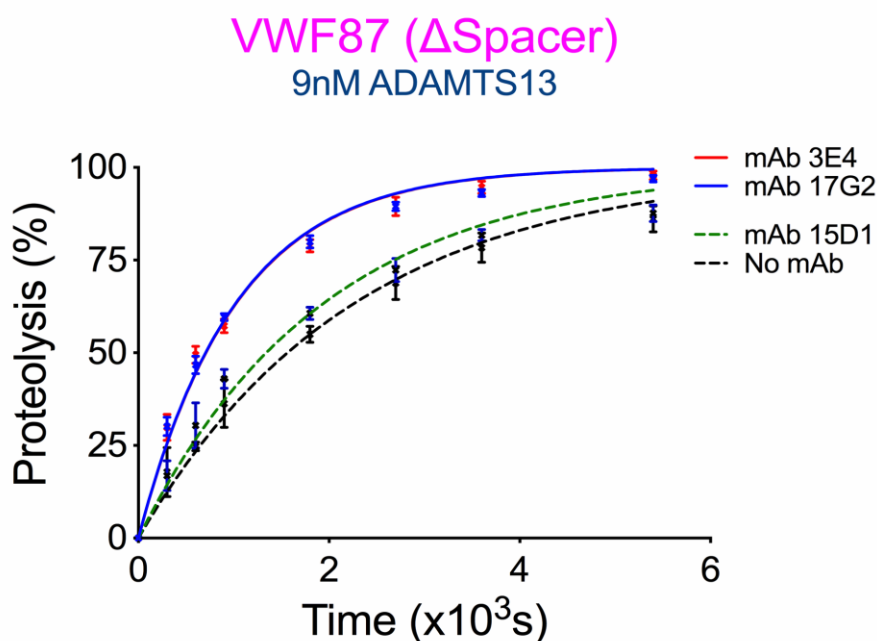


Figure 5-3 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF87(Δ Spacer) by ADAMTS13

VWF87(Δ Spacer)(0.5 μ M) was incubated with purified ADAMTS13 (9nM), in the presence and absence of 10 μ g/ml monoclonal antibodies. Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. In the absence of antibodies, VWF96 was proteolysed with k_{cat}/K_m of $4.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$). Both mAb 3E4 and mAb 17G2, separately, enhanced the catalytic efficiency of ADAMTS13 by ~ 2.2 -fold ($k_{\text{cat}}/K_m = 10.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). The results are summarised in Table 5-1.

The apparent lack of dependence of the antibody-mediated activity enhancement of ADAMTS13 on the Spacer domain exosite interaction urged me to investigate the potential importance of the adjacent Cys-rich domain exosite interaction on this mechanism. The Cys-rich and Spacer domains are neighbouring domains, and their interaction sites in the VWF A2 domain are proximal; it would not be unlikely that the Spacer-CUB interaction that maintains the folded, and relatively less active, conformation of ADAMTS13 sterically hindered the Cys-rich domain exosite interaction and not the Spacer domain exosite interaction. I, therefore, performed reactions between the VWF96-Cys variant as the substrate and ADAMTS13 (11nM), in the presence and absence of the mAb 3E4, mAb 17G2 and mAb 15D1, as described in earlier sections of Chapter 5. The results are shown in Figure 5-4.

In the absence of antibodies, the VWF96-Cys variant was cleaved by ADAMTS13 with a k_{cat}/K_m of $3.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$); the ~ 42 -fold lower rate of proteolysis of this variant compared to the VWF96 ($k_{cat}/K_m=127 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (section 5.2) was due to the ablation of the Cys-rich domain exosite interaction. The presence of the mAb 15D1 had no influence on the rate of proteolysis ($k_{cat}/K_m=3.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). As with the VWF87(Δ Spacer) variant, the ~ 1.9 -fold activation of ADAMTS13-mediated proteolysis of the VWF96-Cys variant by the mAb 3E4 ($k_{cat}/K_m=5.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$) and mAb 17G2 ($k_{cat}/K_m=5.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$) indicated that the Cys-rich domain exosite interaction was not implicated in the activation mechanism. Using the mAb 3E4/17G2 cocktail enhanced the proteolysis of the VWF96-Cys variant by ~ 3.0 -fold ($k_{cat}/K_m=8.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). The results are summarized in Table 5-1 including standard error.

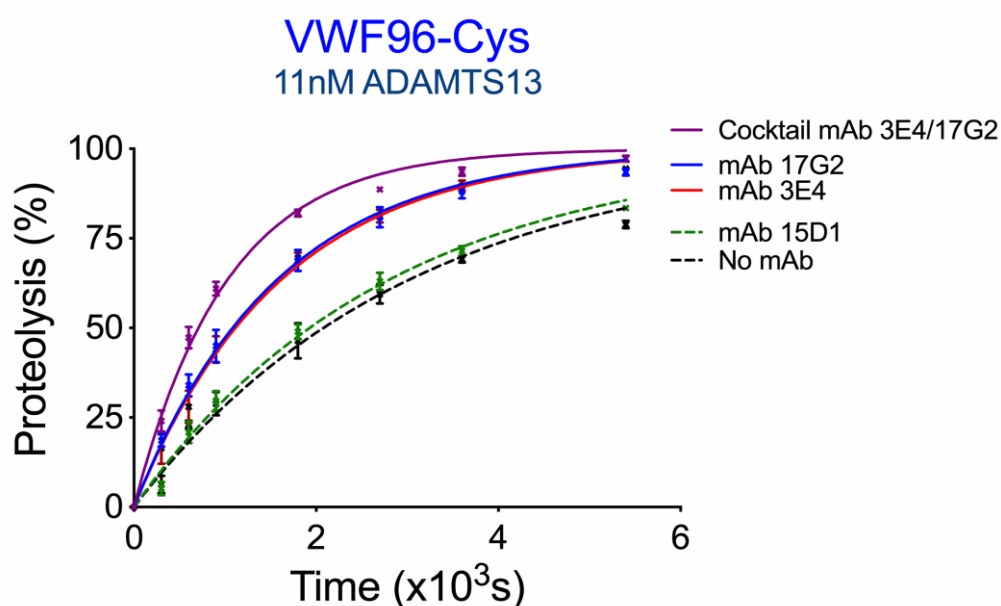


Figure 5-4 The effect of mAb 3E4 and mAb 17G2 on the proteolysis of VWF96-Cys by ADAMTS13

VWF96-Cys ($0.5 \mu\text{M}$) was incubated with purified ADAMTS13 (11 nM), in the presence and absence of $10 \mu\text{g/ml}$ monoclonal antibodies. Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. In the absence of antibodies, VWF96 was proteolysed with k_{cat}/K_m of $3.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$). Both mAb 3E4 and mAb 17G2, separately, enhanced the catalytic efficiency of ADAMTS13 by ~ 1.9 -fold ($k_{cat}/K_m=5.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{cat}/K_m=5.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively, $n=3$). Together, the antibodies enhanced the activity of ADAMTS13 by ~ 3.0 -fold ($k_{cat}/K_m=8.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=3$). The results are summarised in Table 5-1.

5.5 The mAb 3E4 and mAb 17G2 activate ADAMTS13 by increasing the k_{cat}

The mAb 3E4 and mAb 17G2 enhance the activity of ADAMTS13 by unfolding the C-terminal tail of ADAMTS13, but not via exposing the Spacer and Cys-rich domain exosite interactions. I decided to further characterise the antibody-mediated activating effect on ADAMTS13, by analysing the Michaelis Menten kinetics of VWF96 proteolysis in the presence and absence of the mAb 3E4 and mAb 17G2. This would shed light on whether the antibody-induced enhanced activity of ADAMTS13 is manifest through higher substrate binding affinity, or another mechanism.

Multiple concentrations of VWF96 (0-25 μ M) were incubated with ADAMTS13 in the presence and absence of mAb 17G2 or mAb 3E4. Each reaction was stopped between 0-120 minutes and the fraction of proteolysis analysed by VWF96 ELISA. Initial rates of proteolysis (<15% proteolysis) were calculated and plotted as a function of VWF96 concentration (see section 2.5). Figure 5-5 shows the data fitted to the Michaelis Menten equation. In the absence of antibodies, VWF96 was proteolysed with a k_{cat} of 2.5s⁻¹ and a K_m of 2.6 μ M; using these values, the calculated k_{cat}/K_m was 96.7x10⁴ M⁻¹s⁻¹ (n=31). Both antibodies, separately, influenced the k_{cat} more than the K_m . In the presence of mAb 3E4 or the mAb 17G2, the k_{cat} was ~1.7-fold higher (k_{cat} =4.3s⁻¹ and k_{cat} =4.2s⁻¹, respectively). The K_m of proteolysis was unaffected by the presence of the mAb 17G2 antibody (K_m =2.6 μ M), and only ~1.2-fold increased (K_m =3.0 μ M) in the presence of the mAb 3E4. Consistent with our earlier results, the presence of the mAb 3E4 and mAb 17G2 increased the k_{cat}/K_m by ~1.5-fold (k_{cat}/K_m =142x10⁴ M⁻¹s⁻¹, n=26) and ~1.7-fold (k_{cat}/K_m =166x10⁴ M⁻¹s⁻¹, n=31), respectively. The results are summarised in Table 5-1 including standard error.

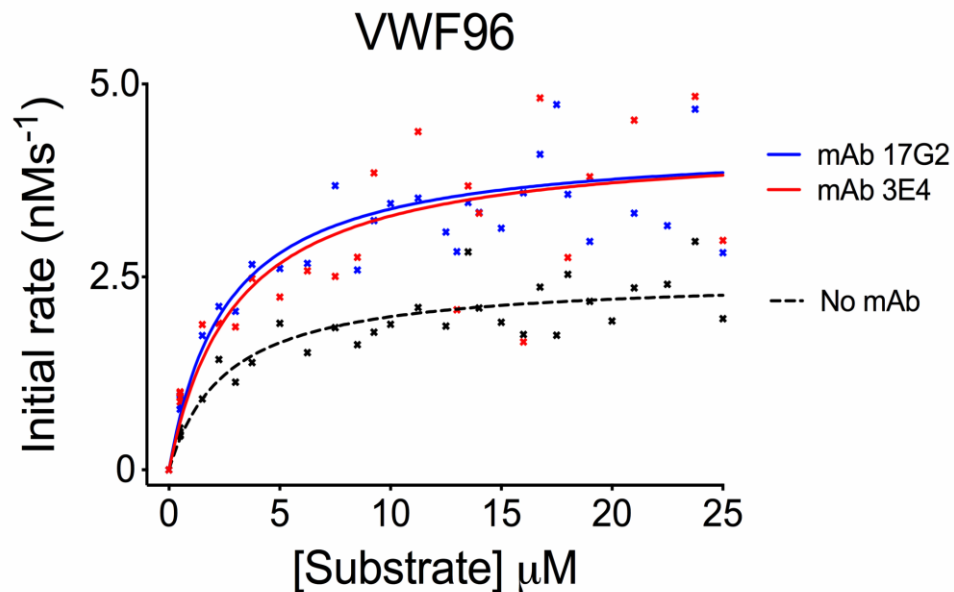


Figure 5-5 The effect of mAb 3E4 and mAb 17G2 on the Michaelis Menten kinetics of VWF96 proteolysis by ADAMTS13

Initial rates of VWF96 proteolysis by purified ADAMTS13 were analysed in the presence and absence of 10 μ g/ml of each monoclonal antibody. Initial rates (nMs⁻¹) were plotted as a function of substrate concentration (μ M) and the data were fitted to the Michaelis Menten equation in Graphpad Prism. The presence of mAb 3E4 and mAb 17G2, separately, primarily increased the k_{cat} of proteolysis by $\sim$ 1.7-fold, while showing a very modest (if any) influence on the K_m . The results are summarised in Table 5-1.

Table 5-1 Summary of the effects of monoclonal antibodies on the kinetics of VWF96 (variant) proteolysis by ADAMTS13 and MDTCS

REACTION CONDITIONS			TIME COURSE		MICHAELIS-MENTEN KINETICS					
Enzyme	Substrate	mAb	$k_{cat}/K_m \pm \text{SEM}$ ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)		$k_{cat} \pm \text{SEM}$ (s^{-1})		$K_m \pm \text{SEM}$ (μM)	$k_{cat}/K_m \pm \text{SEM}$ ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)		
ADAMTS13	VWF96	No mAb	127	± 5.2	2.5	± 0.1	2.6	± 0.6	96.7	± 24.0
		3E4	249	± 7.7	4.3	± 0.5	3.0	± 1.3	142	± 64.0
		17G2	229	± 9.9	4.2	± 0.4	2.6	± 1.0	166	± 66.4
		15D1	127	± 5.2						
		3E4/17G2	274	± 7.3						
	VWF87 (ΔSpacer)	No mAb	4.9	± 0.3						
		3E4	10.9	± 0.4						
		17G2	10.9	± 0.4						
		15D1	5.7	± 0.3						
	VWF96-Cys	No mAb	3.0	± 0.1						
		3E4	5.7	± 0.2						
		17G2	5.8	± 0.2						
		15D1	3.3	± 0.1						
		3E4/17G2	8.9	± 0.3						
MDTCS	VWF96	No mAb	91.9	± 4.1						
		3E4	78.2	± 2.3						
		17G2	89.4	± 2.2						
		15D1	78.3	± 3.2						
		3E4/17G2	84.5	± 2.5						

5.6 The conformational activation of ADAMTS13 is not induced by the recombinant D4-CK domains

Since the D4(-CK) domains of VWF had been proposed to physiologically enhance the activity of ADAMTS13 via a conformational activation mechanism,^(43, 44, 46) I decided to characterise the influence of recombinant D4-CK domains on the kinetics of VWF96 proteolysis, as performed with the mAb 3E4 and mAb 17G2. This would enable comparisons with the kinetics of ADAMTS13 activity enhancement induced by the mAb 3E4 and mAb 17G2, and further inform on whether the antibodies directly mimic the proposed physiological D4-CK-induced conformational activation of ADAMTS13.

I measured the catalytic efficiency of VWF96 proteolysis by ADAMTS13 (0.4nM), in the presence and absence of recombinant D4-CK domains (~2.5 μ M), which were previously generated in our lab. Surprisingly, there was no difference in the catalytic efficiency of VWF96 proteolysis in the presence ($k_{\text{cat}}/K_m=138 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=1$) and absence ($k_{\text{cat}}/K_m=156 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=1$) of D4-CK domains (Figure 5-6).

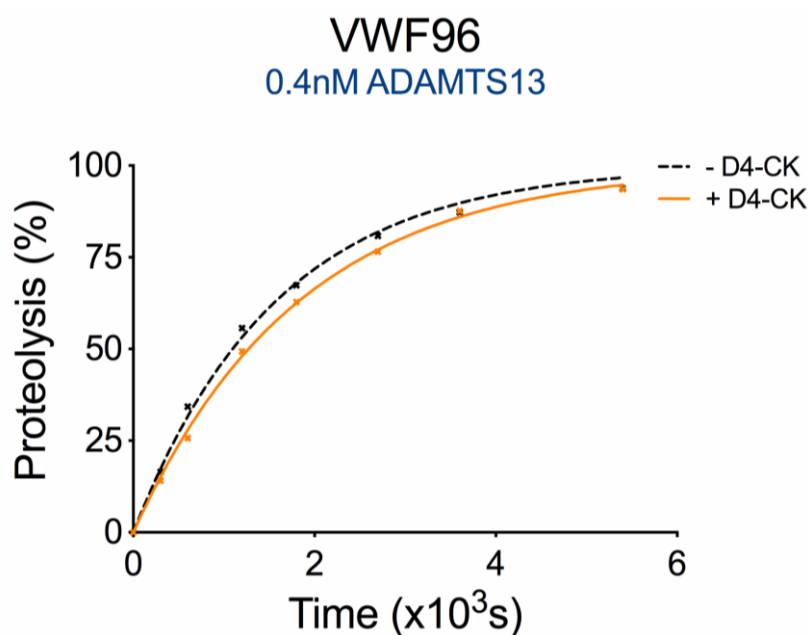


Figure 5-6 The effect of recombinant VWF D4-CK domains on the catalytic efficiency of VWF96 proteolysis by ADAMTS13

VWF96 (0.5 μ M) was incubated with purified ADAMTS13 (0.4nM) in the presence and absence of recombinant VWF D4-CK domains (~2.5 μ M). Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA, and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. No major difference in the k_{cat}/K_m of VWF96 proteolysis in the presence and absence of the D4-CK was observed ($k_{\text{cat}}/K_m=138 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{cat}}/K_m=156 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively, $n=1$).

Since the D4-CK used was already available in the lab, I needed to ensure that the inability of D4-CK to activate ADAMTS13 was not specific to the particular D4-CK preparation (eg. due to D4-CK aggregation, lower buffer pH or precipitation of calcium). I centrifuged a fresh D4-CK aliquot at high speed to remove any protein aggregates that had possibly formed. The concentration of D4-CK (as assessed by reading the absorbance at 280nm and using extinction coefficients) did not change after centrifugation, suggesting that protein aggregation had not taken place. To eliminate the possibility that factors associated with the buffer of the D4-CK preparation accounted for the inability to activate ADAMTS13, I exchanged the existing buffer with TBSC buffer (20mM Tris pH 7.5/50mM NaCl/5mM CaCl₂) using 7kDa MWC0 Zeba spin desalting columns (Thermo Fisher Scientific). I repeated the assay using 0.5nM ADAMTS13 but, again, there was no difference in the catalytic efficiency of VWF96 proteolysis in the presence ($k_{cat}/K_m=113 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=1$) and absence ($k_{cat}/K_m=95.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $n=1$) of $\sim 2.5\mu\text{M}$ D4-CK (Figure 5-7).

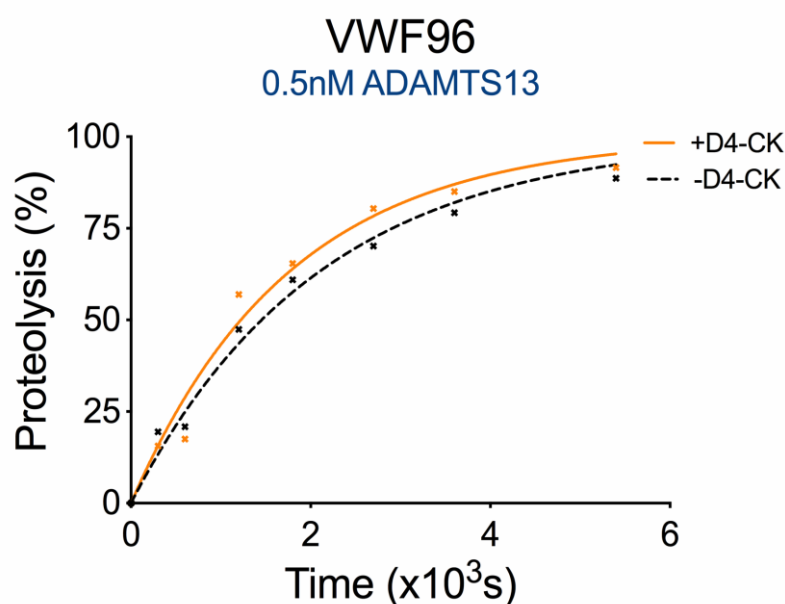


Figure 5-7 The effect of recombinant VWF D4-CK domains (buffer exchanged) on the catalytic efficiency of VWF96 proteolysis by ADAMTS13

VWF96 ($0.5\mu\text{M}$) was incubated with purified ADAMTS13 (0.4nM) in the presence and absence of recombinant VWF D4-CK domains ($\sim 2.5\mu\text{M}$) that were buffer exchanged and centrifuged to ensure that precipitation, aggregation or wrong buffer had not accounted for the inability to enhance the activity of ADAMTS13. Reaction sub-samples were stopped between 0-90 minutes, analysed by VWF96 ELISA, and the percentage of substrate proteolysed was plotted as a function of time (seconds). Data were fitted to calculate catalytic efficiencies. No major difference in the k_{cat}/K_m of VWF96 proteolysis in the presence and absence of the D4-CK was observed ($k_{cat}/K_m=113 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{cat}/K_m=95.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively, $n=1$).

5.7 DISCUSSION

In this chapter, I characterised the mechanism of ADAMTS13 activity enhancement induced by the anti-Spacer and anti-CUB monoclonal antibodies, mAb 3E4 and mAb 17G2, respectively. I originally hypothesized that binding of the antibodies induces a conformational activation in ADAMTS13 by disrupting an interaction between the Spacer and CUB domains that unfolds the C-terminal tail (TSP2-CUB2) and liberates the Spacer exosite, which, in turn, contributes to higher substrate binding affinity. Preliminary experiments using FRET-VWF73 (performed by An-Sofie Schelpe) showed that the mAb 3E4 and mAb 17G2 enhance the activity of ADAMTS13 by ~1.7-fold. However, studying the conformational activation of ADAMTS13 using FRET-VWF73 is not optimal, since the assay is performed at lower pH (pH 6), which was reported to, independently, also induce the conformational activation of ADAMTS13.⁽⁴³⁾ The VWF96 proteolysis assay developed as part of this PhD project is performed at physiological pH, which is preferable for studying the conformational activation of ADAMTS13. Furthermore, this assay allowed the VWF96 variants to be used as substrates to provide further information on the dependence of the conformational activation mechanism on exosite exposure.

I first used the VWF96 ELISA to measure the activating effect of the mAb 3E4 and mAb 17G2. Both antibodies induced the expected (~1.8-to-2-fold) rate enhancement in the proteolysis of VWF96 by ADAMTS13. The results showed excellent reproducibility, as seven replicate experiments generated the same result with essentially no inter-assay variability. The non-inhibitory, non-activating anti-Spacer antibody mAb 15D1 was used as a negative control in the experiments. This antibody had been generated and processed in exactly the same way as mAb 3E4 and mAb 17G2, verifying that the activating effects reported were specific to antibody binding and not associated with other factors related to the buffers of the antibodies, like pH or ionic strength, which had previously been shown to also influence the activity of ADAMTS13.^(43, 360)

I next assessed the ability of the antibodies to enhance the activity of MDTCS to cleave VWF96. The mAb 17G2 was not expected to interact with the MDTCS, since the CUB domains (ligand for mAb 17G2) were missing on this truncated variant. However, the experiment was still performed in the presence of the mAb 17G2 and acted as a negative control to, again, eliminate the possibility that the reported activating effect was induced by other factors associated with the buffer of this antibody. Neither antibody enhanced the activity of MDTCS significantly. This

confirmed that the activating effect was dependent on the presence of the C-terminal tail, which was perfectly consistent with the proposed mechanism supporting that the conformational activation is a result of the C-terminal tail of ADAMTS13 unfolding.^(43, 44, 46) It is worth noting that the k_{cat}/K_m of VWF96 proteolysis by MDTCS was not ~2-fold higher than the k_{cat}/K_m of VWF96 by ADAMTS13, as would be expected at physiological pH.⁽⁴³⁾ The MDTCS used had been concentrated in expression media (and not purified) and, as a result, no robust assay for MDTCS quantification was available. I assigned an active concentration to MDTCS by qualitatively comparing its rate of VWF96 proteolysis to wild-type ADAMTS13 (data not shown), which made direct comparison of their activities irrelevant.

It had been proposed that unfolding of the C-terminal tail activates ADAMTS13 by fully exposing the Spacer domain exosite that is implicated in a functional interaction with the VWF A2 domain. This mechanism, however, had not been confirmed experimentally; instead, it had been speculated based on the observations that isolated CUB domains could bind to the Spacer domain, and that this binding could be ablated by mutating the proposed ADAMTS13 Spacer exosite residues (R568K/F592Y/R660K/Y661F/Y665F).^(44, 46) I used the VWF87(Δ Spacer) and VWF96-Cys variants, which ablate the Spacer and Cys-rich domain exosite interactions, respectively, to investigate whether the activating effect of the antibodies is dependent on exposure of either exosite. This approach is somewhat preferable to mutating the exosite in the ADAMTS13 Spacer domain (performed previously) as it avoids interfering with the structure of the enzyme, which could potentially influence its activity in an exosite independent manner. Interestingly, the activating effects of mAb 3E4 and mAb 17G2 were still evident after ablation of both the Spacer and Cys-rich domain exosite interactions. These findings, therefore, contradict the proposed model of ADAMTS13 conformational activation and show for the first time that monoclonal antibodies, that are used to induce the unfolded, more active conformation of ADAMTS13 *in vitro*, do not function by facilitating Spacer or Cys-rich domain exosite exposure. In hindsight, the Spacer exosite contributes a ~15-fold to the rate of proteolysis, whereas the conformational activation only a ~2-fold; this might suggest distinct mechanisms.

The lack of involvement of the Spacer and Cys-rich domain exosites in the conformational activation of ADAMTS13 suggested that the activating effect was mediated through exposure of an exosite in another domain of ADAMTS13 that enhances the substrate binding affinity, or that the unfolding of the C-terminal tail allosterically influences the active site of ADAMTS13.

Using the VWF96 ELISA allowed me to more precisely characterise the activating effect of the mAb 3E4 and mAb 17G2 on ADAMTS13, by performing Michaelis Menten analysis on the proteolysis of VWF96 in the presence and absence of the antibodies. Both antibodies, separately, enhanced VWF96 proteolysis by primarily increasing the k_{cat} , while negligibly affecting the K_m . This approach allowed me to conclude that the activation of ADAMTS13 was primarily due to enhanced substrate turnover, suggesting that unfolding of the C-terminal tail mediated an allosteric enhancement in the capacity of the active site to cleave VWF96. In light of the allosteric activation mechanism proposed in the previous chapter, this result further corroborates the nature of ADAMTS13 as a conformationally flexible enzyme with the ability to allosterically transduce activating signals to the catalytic domain, influencing the active site.

The main limitation associated with the Michaelis Menten analysis is associated with some of the variability recorded in the initial rates of proteolysis. This variability originated from the ADAMTS13 preparations used. I observed differences in activities between different ADAMTS13 preparations, between different aliquots of the same preparation, and between different freeze-thawing cycles of the same aliquot. To control for variability and minimise potential error, I performed the experiments in the presence and absence of antibodies in parallel, using the same source ADAMTS13. Moreover, I performed reactions at multiple concentrations to strengthen the fit of the data.

An ELISA assay using antibodies that specifically recognise conformationally sensitive epitopes in the MP domain of ADAMTS13 had been published previously.⁽²⁶⁵⁾ The ELISA was performed by An-Sofie Schelpe and showed that incubation of ADAMTS13 with both mAb 3E4 and mAb 17G2, separately, exposed these cryptic epitopes in the MP domain. This is a separate line of evidence supporting that the antibody-induced conformational activation of ADAMTS13 is manifest through structural alterations of the catalytic domain.

My results support the contention that the antibody-mediated conformational activation of ADAMTS13 is manifest by C-terminal tail unfolding, which influences the structure and functionality of the active site, and does not augment Spacer/Cys-rich domain exosite exposure. The results of this project do not directly demonstrate that non-antibody-induced conformational activation of ADAMTS13 (pH, D4-CK) also relies on the same mechanism. One possibility would be that the structural manifestations proposed here are specific to antibody binding. Nevertheless, the fact that different antibodies, targeting different domains, enhance

the activity of ADAMTS13 in the same way gives confidence that the activation mechanism is a specific consequence of ADAMTS13 unfolding.

The D4-CK domains of VWF were proposed to physiologically induce the conformational activation of ADAMTS13. South *et al* reported that as little as 3nM D4-CK enhanced the k_{cat}/K_m of FRETs-VWF73 proteolysis by ADAMTS13 dose-dependently, but not when using the gain-of-function ADAMTS13 variant that contains mutations (R568K/F592Y/R660K/Y661F/Y665F) thought to induce the conformational activation. They also showed affinity between VWF D4-CK domains and the TSP8, CUB1 and CUB2 domains of ADAMTS13, but not when using the gain-of-function ADAMTS13, the MDTCS or a ADAMTS13 variant lacking the TSP8-CUB2 domains. The enhanced affinity of acquired TTP patient anti-ADAMTS13 antibodies to ADAMTS13 primed with D4-CK was also used to propose an explanation for the relative high frequency of anti-Spacer antibodies (as opposed to antibodies targeting other domains) in TTP; the Spacer domains expose cryptic epitopes during ADAMTS13 conformational activation that are, potentially, recognised as foreign, resulting in the generation of autoantibodies.^(44, 46)

Hence, I was interested in characterising the D4-CK-induced conformational activation in a similar way to the monoclonal antibodies. If, using recombinant D4-CK domains, I could reproduce the effects of the antibodies on the Michaelis Menten kinetics of VWF96 proteolysis, not only would this greatly contribute to understanding the mechanism by which D4-CK domains enhance the activity of ADAMTS13, but it would also validate the use the monoclonal antibodies as tools to mimic the physiological conformational activation of ADAMTS13. Surprisingly, my data contradict prior results, which associate the D4-CK domains with the conformational activation of ADAMTS13. In independent attempts, I have been unable to observe an enhancement in the activity of ADAMTS13 as a result of the presence of the D4-CK domains in proteolytic reactions. Different preparations of D4-CK and ADAMTS13 have been used in independent experiments to eliminate the possibility of preparation-specific factors that could have accounted for the inability of observing the activating effect of D4-CK. My collaborator on this project, An-Sofie Schelpe, also assessed the ability of recombinant D4-CK domains (independently generated in KU Leuven) and FL-VWF (Baxalta) to enhance the FRETs-VWF73 proteolysis by ADAMTS13. Again, no difference in the catalytic efficiency of ADAMTS13 was observed in the presence and absence of D4-CK domains (data not shown).

Muia *et al* reported that isolated VWF D4 domains and FL-VWF, but not VWF fragments that were cleaved within the D4 domain, could enhance the activity of ADAMTS13.⁽⁴³⁾ Based on this, they attributed physiological relevance to the D4-induced conformational activation of ADAMTS13. In their experiments they used 40 μ M D4 domains to activate ADAMTS13. This concentration is certainly supraphysiological, questioning the physiological relevance of these results. Further studies are required, but given the low concentration of ADAMTS13 (~5nM) and VWF (40nM) in circulation, higher affinity interactions would be required to confer a physiological role.

6 DISCUSSION

6.1 Summary of hypothesis

Unlike most haemostatic enzymes, the ability of ADAMTS13 to cleave its substrate does not depend upon specific on-demand activation/inhibition or regulation by a defined cofactor; yet, despite its generic metalloprotease active site, its protease function is incredibly specific. In this project, I aimed to explain the specificity of ADAMTS13 for nothing else than its physiological substrate, VWF. How does ADAMTS13, an apparently constitutively active plasma metalloprotease with long active half-life⁽³⁶⁷⁾, avoid off-target proteolysis? What gives ADAMTS13 its resistance to inhibition by broad-spectrum plasma protease inhibitors and family-specific inhibitors?^(365, 368)

The answer to ADAMTS13 unprecedented specificity is likely to be conferred by the exosite interactions that precede the proteolytic event. The functional interactions between exosites in the MP, Dis, Cys-rich and Spacer domains of ADAMTS13 and their complementary binding sites in the VWF A2 domain are an absolute minimum requirement for efficient proteolysis to occur.^(248, 250, 251, 255, 256, 263, 264, 266-268) This is inferred from the ability of ADAMTS13 to cleave VWF A2 domain peptide fragments that possess just the exosite binding sites and scissile bond with comparable efficiency to cleaving full-length VWF.⁽³⁶⁰⁾ Furthermore, in the absence of the Dis, Cys-rich and Spacer domain exosite interactions, proteolysis of VWF-derived substrates by the isolated MP domain has been reported to lack specificity as well as efficiency.^(249, 250)

I formed the hypothesis that the active site of ADAMTS13 is a dynamic structure; it can exist in both a latent/'closed' state that is not readily permissive to substrate/inhibitor access, and in an active/'open' state that can accommodate substrate and facilitate cleavage. Binding of one or more of its exosites causes a conformational change that favours the active/'open' state of the active site, allosterically activating ADAMTS13 to perform proteolysis.

6.2 Biochemical evidence of allosteric activation (summary of project results)

The aim of this project was to investigate the existence of an exosite-dependent allosteric mechanism that activates ADAMTS13, by studying the precise contribution of each exosite interaction to the binding and proteolysis biochemically. All exosite interactions were ablated (or severely disrupted), by generating variants of VWF96, which had the complementary binding sites for ADAMTS13 exosites mutated individually (Figure 4-8). The development of an

ELISA assay that specifically detected uncleaved VWF96 variants allowed precise quantitation of the relative concentration of these variants, and to quantitatively measure the rate of their proteolysis by ADAMTS13.

To compare the contribution of each exosite to the rate of proteolysis, it was important to generate VWF96 variants that (ideally) completely ablated the exosite interactions. For some exosite interactions, the precise exosite boundaries had not been defined prior to this project. The Spacer domain exosite had been proposed to involve the solvent exposed ADAMTS13 residues R659, R660, Y661 and Y665.^(235, 267, 268) The Spacer domain exosite binding site in the VWF A2 domain was shown to lie within the region E1660-R1668.^(251, 255, 360) However, no study had directly demonstrated binding of the putative Spacer exosite residues and individual residues in the binding site of the VWF A2 domain. Based on the fact that the charged residues R1659, E1660, D1663 and K1668, which are in the vicinity of the proposed Spacer domain exosite binding site E1660-R1668 in the VWF A2 domain, do not significantly influence ADAMTS13 binding and proteolysis,⁽³⁶¹⁾ I hypothesized that the hydrophobic residues L1664, V1665 and L1666 in the same region may facilitate the interaction with the Spacer domain exosite. My data identify the residues L1664, V1665 and L1666 as functionally important to the binding between the ADAMTS13 Spacer domain exosite and the VWF A2 domain and, consequently, proteolysis, supporting an interaction of hydrophobic nature (sections 4.9 and 4.10). This is not surprising, considering that the interaction facilitated by the adjacent Cys-rich domain exosite is also of hydrophobic nature.⁽²⁶⁶⁾ The hydrophobic residues V1665 and L1666 in the VWF A2 domain contribute to stabilising the structure of the VWF A2 domain in its globular form through intra-molecular interactions with the vicinal disulphide bond C1669-C1670.⁽⁵⁴⁾ However, when the VWF A2 domain is unfolded, these same residues are key to mediating inter-molecular interactions with the Spacer domain of ADAMTS13 that contribute to proteolysis. Direct interaction of these hydrophobic residues in the VWF A2 domain and the exosite in the ADAMTS13 Spacer domain is yet to be definitively demonstrated.

Evidence of direct binding between the R349 residue in the ADAMTS13 Dis domain and the D1614 residue in the VWF A2 domain had been provided by de Groot *et al.*⁽²⁵⁰⁾ Other proximal residues in the Dis domain (L350 and V352)⁽²⁵⁰⁾ and the VWF A2 domain (E1615/K1617)⁽²⁵⁶⁾ were reported to be functionally important, but no direct binding was demonstrated. Based on this, I originally hypothesized that the Dis domain exosite binding site in the VWF A2 domain covered the region A1612-K1617, hence generating the VWF96-Dis1 variant

ADEIK1612/1614-1617SNQQQ. The results of Kretz *et al*⁽³⁶³⁾(section 4.4) suggested that the Dis domain exosite interacts with a more extended area in the VWF A2 domain (D1614-D1622). Previously, Gao *et al* deleted a similar region (G1609-I1623) and proteolysis was abolished.⁽²⁵⁵⁾ However, this was an internal deletion that, apart from ablating the Dis domain exosite interaction, it also affected the relative positions of the other C-terminal exosite binding sites (for Cys-rich and Spacer domain exosites) in the VWF A2 domain, which could independently reduce the rate of proteolysis. Using the smaller fragments of the VWF A2 domain VWF47 (G1573-L1619) and VWF57 (G1573-G1629) in proteolytic reactions, I confirmed the functional importance of the VWF region P1620-G1629 (section 4.5). Most importantly, by performing reactions with MP-Dis (ADAMTS13 variant that is truncated after the Dis domain) I showed that the region P1620-G1629 specifically interacts with the Dis domain of ADAMTS13, and not with an adjacent exosite. I, therefore, substituted the residues DIKRD1614/1616-1618/1622AQEET in VWF96 to generate a new variant, VWF96-Dis2. Indeed, there was a clear functional deficit in the rate of VWF96-Dis2 proteolysis when compared with the original variant VWF96-Dis1, confirming the importance of the substituted residues for proteolysis.

The ADAMTS13 Dis domain exosite forms the most vital of all exosite interactions for the rate of proteolysis. The difference in the relative importance between the Dis domain exosite interaction and the rest of the exosite interactions was striking (sections 4.9 and 4.10). While the Spacer, Cys-rich and MP domain exosite interactions contribute by up to ~20-, ~20- and ~200-fold, respectively, to the k_{cat}/K_m of proteolysis, the Dis domain exosite contribution was measured to be up to ~780-fold. Ablating an interaction that allosterically activates ADAMTS13 was expected to (almost) abolish proteolysis. Therefore, these results would fit a model of ADAMTS13 allosteric activation that is highly dependent on the Dis domain exosite interaction.

The precise contribution of the exosites to the separate components of the proteolytic event, binding (K_D , K_m) and substrate turnover (k_{cat}), needed to be considered for conclusions to be made. Michaelis Menten analysis of the proteolysis of the VWF96 variants showed remarkable differences in the functionality of the different exosite interactions in proteolysis (section 4.10). Essentially all remote exosites (Spacer, Cys-rich and Dis domain) have a similar and significant (~15-fold) contribution to the binding between VWF96 and ADAMTS13. In contrast, the MP domain exosite interaction revealed, as expected, a low affinity interaction. That the MP

domain binds weakly is expected, as it needs to accommodate the scissile bond but not form a tight complex. As a separate line of evidence, ELISA-based assays measuring the equilibrium surface-phase binding affinity (K_D) between the VWF96 variants and ADAMTS13/MDTCS(E225Q) corroborated the magnitude of the contribution of the different exosite interactions to binding (section 4.11 and 4.12). These showed that approximation of ADAMTS13 and the VWF A2 domain is mediated by the high affinity interactions of the Spacer, Cys-rich and Dis domain exosites.

There is a key difference in the nature of contribution to the rate of proteolysis between the different remote exosites. While the influence of the Spacer and Cys-rich domain exosites to the rate of proteolysis is wholly explained by their influence on the binding affinity, the Dis domain appears to primarily affect the substrate turnover, as seen by the ~ 56 -fold influence on the k_{cat} . Such a big influence on the k_{cat} by a remote exosite in ADAMTS13 had not been reported previously. In light of these data, the functional importance of the Dis domain exosite interaction to proteolysis has been previously underestimated. Undoubtedly, the role of the Dis domain in proteolysis is distinct and superior from the role of any other of the domain exosites. The Dis domain exosite interaction not only influences the affinity of the two molecules for each other, but it also modulates the efficiency of the proteolytic event. How does a remote exosite, (ie. in a domain that is distinct from that of the active site) influence the catalysis to such a great extent? I propose that the Dis domain exosite interaction facilitates a conformational change within the catalytic domain of ADAMTS13 that shapes the active site in a way such that it can accommodate the scissile bond; this allosterically activates ADAMTS13.

6.3 Biophysical evidence of allosteric activation

Parallel to my project, biophysical data were generated in our lab by Dr Frank Xu (I also contributed to the provision of VWF fragments), which are consistent with a conformational change in ADAMTS13 as a result of VWF binding. Inactive MDTCS(E225Q) was expressed in S2 cells and purified by affinity chromatography and gel filtration. This was used in isothermal titration calorimetry (ITC) experiments to measure the solution-phase equilibrium binding affinity to tag-free VWF73 (D1596-R1668)(Figure 6-1). A K_D of $\sim 0.93\mu M$ was measured. The interaction was characterised by negative Gibbs free energy, indicating spontaneous binding. The exothermic enthalpy changes (negative ΔH value) indicate favourable hydrogen and Van der Waals bond formation. The data revealed unfavourable entropy changes (positive $-\Delta S$

value) indicating that the interaction is entropically driven, with alterations in the hydrophobic interactions and binding-induced conformational changes; these might reflect the hypothesized Dis domain exosite binding-induced conformational changes in the MP domain. Theoretically, one could use ITC to investigate the effect of ablating the exosite interactions on the energetic profile of binding. Using the VWF96 variant that has the exosite interaction responsible for conformational changes in ADAMTS13 ablated (ie. VWF96-Dis2 variant, as proposed here) should abolish the unfavourable entropic changes associated with binding. However, due to the low affinities associated with ablating the exosite interactions, high concentrations of VWF96 variants (>1mM) were required to perform the ITC experiments, which were not available.

MDTCS E225Q titrated by VWF73 wild type at molar ratio of 1:3.25 and 1:10 are combined (n=4).

K_D : $\sim 0.93\mu\text{M}$

N: ~ 0.4

ΔG : -8.5, ΔH : -26.1, $-\Delta S$: 17.6 (kcal/mol)

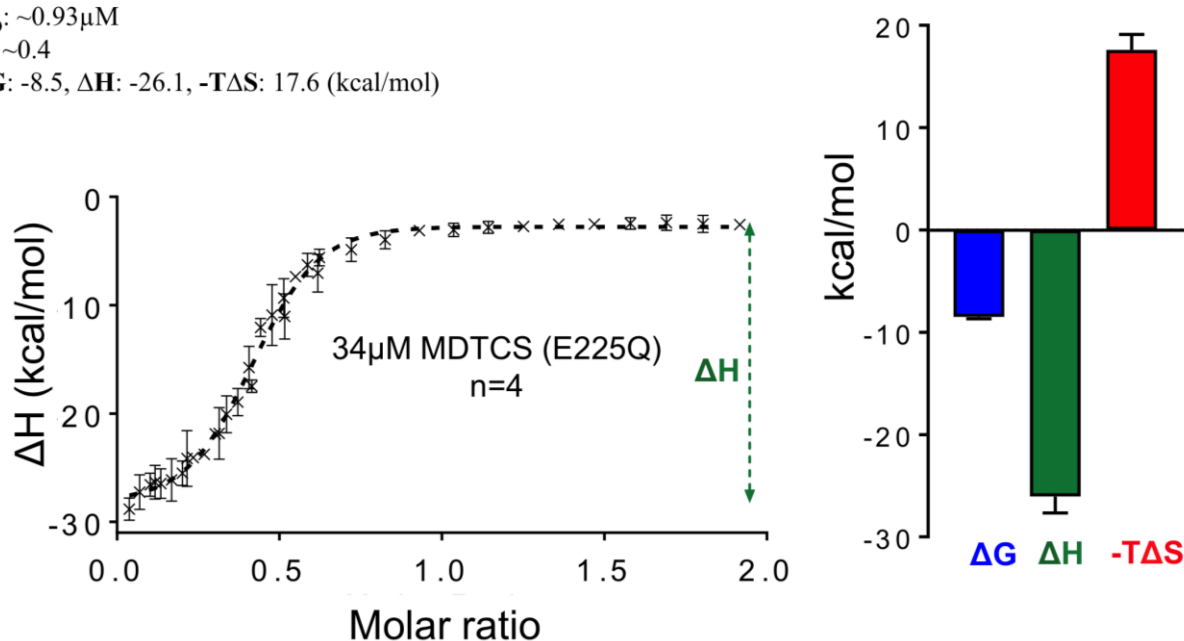


Figure 6-1 ITC experiments to investigate to binding affinity of tag-free VWF73 to MDTCS(E225Q)

Increasing concentrations of VWF73 were added to MDTCS(E225Q), and the enthalpy change (ΔH) associated with each addition was monitored. Based on these ITC data, the solution-phase, equilibrium K_D between MDTCS(E225Q) and VWF73 is $\sim 0.93\mu\text{M}$. The data suggest spontaneous binding (negative ΔG), involving hydrogen and Van der Waals bonding (favourable enthalpic changes, negative ΔH) and changes in the hydrophobic interactions and conformation (unfavourable entropic changes, positive $-\Delta S$).

6.4 Structural evidence of allosteric activation

Obtaining the crystal structure of ADAMTS13 in the presence and absence of VWF96 (or another VWF A2 domain fragment, like VWF73) could prove to be the gold standard in studying binding-induced conformational changes in the MP domain. Until recently, however, all attempts to solve the crystal structure of the ADAMTS13 MP domain have been unsuccessful. Interestingly, the crystal structure of the ADAMTS13 fragment spanning the region between the Dis and the Spacer domains (DTCS) was crystallized twice.^(235,253) What makes DTCS easier to crystallize than MDTCS? Potentially, the hypothesis that the MP domain is a flexible domain that can exist in equilibrium between ‘closed’ and ‘open’ active site states, could explain the inability to crystallize it, as flexible molecules are not conducive to crystallization.

The crystal structure of the ADAMTS4 MP domain has been solved in the presence and absence of an active site inhibitor.⁽²³⁷⁾ In the absence of the inhibitor (Figure 6-2A), the active site of ADAMTS4 adopts a ‘closed’ state, which is occluded by the Ca²⁺-binding loop that shapes the S2’ subsite. In this ‘closed’ state of the ADAMTS4 active site, the D328 residue chelates the Zn²⁺, which is, in turn, unavailable for proteolysis. In contrast, the presence of the inhibitor in the ADAMTS4 active site (Figure 6-2B) induces an ‘open’ state in the active site pocket, where the D328 residue no longer chelates the Zn²⁺. The crystal structures of the MP domains of ADAMTS1 and ADAMTS5 have also been solved in the presence of active site inhibitors;⁽²³⁶⁻²³⁸⁾ their active sites resemble the ‘open’ state seen in ADAMTS4. Whether the active sites of these enzymes also resemble a ‘closed’ state in the absence of a ligand, as seen in ADAMTS4, remains to be determined. Nevertheless, these observations might suggest flexibility in the active sites of other ADAMTS-family members, as hypothesized for ADAMTS13.

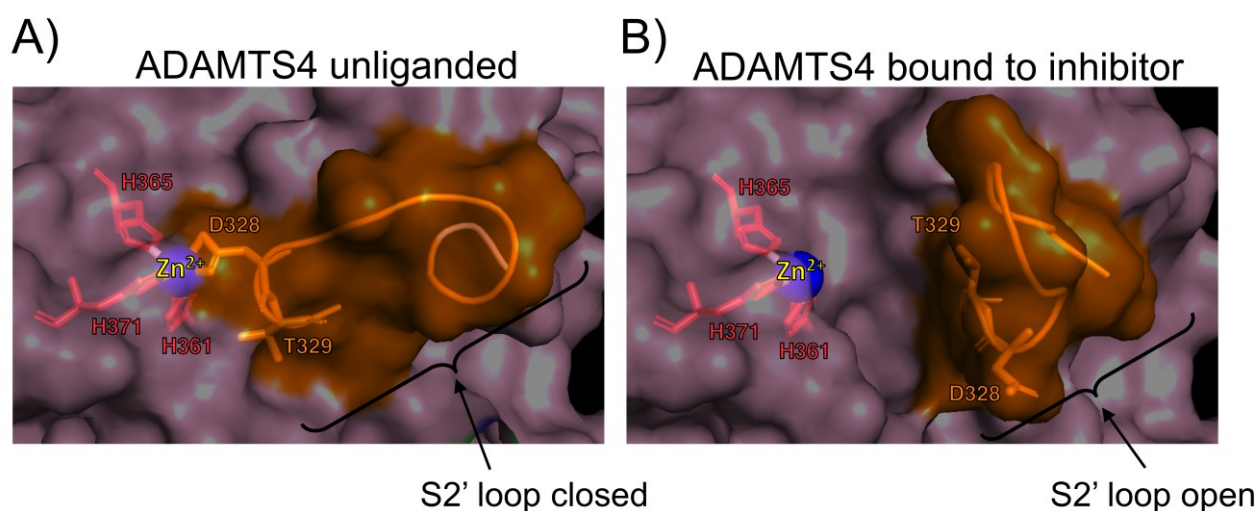


Figure 6-2 Crystal structures of the ADAMTS4 active site, with and without active site inhibitor bound

(A) In the absence of an active site inhibitor, the loop forming the S2' subsite folds towards the catalytic Zn^{2+} residue. The residue D328 of the S2' loop chelates the Zn^{2+} . (B) The presence of an active site inhibitor (inhibitor omitted in the figure for better visualisation of the active site) disrupts the chelation of the Zn^{2+} by the D328 residue and opens up the S2' loop, making room for accommodating the substrate. The ADAMTS4 structures were obtained from ⁽²³⁷⁾ and adapted to make the figures.

Recently, a stable complex between the MDTCS domains of ADAMTS13 and a monoclonal antibody Fab fragment raised against the MP domain of ADAMTS13, termed Fab 3H9 (developed by Prof Karen Vanhoorelbeke), was prepared in our lab and successfully crystallized by our collaborator Prof Jonas Emsley (manuscript in preparation). Even though the MDTCS domains are conserved in all members of the ADAMTS family of metalloproteases,⁽²³⁴⁾ ADAMTS13 is the first ADAMTS family member to have had all its MDTCS domains crystallized. It is possible that the Fab has stabilized an otherwise flexible MP domain (consistent with my hypothesis), enabling the formation of crystals. Figure 6-3A shows the solved crystal structure of the MDTCS(E225Q) with the Fab 3H9 removed. Intriguingly, the R193 in the Ca²⁺-binding loop appears to be involved in hydrogen bonding with the residues D217 and D252, stabilising a so-called 'gate-keeper' triad of residues (Figure 6-3B-D). Based on the relative positions (with respect to the active site) of the exosites that need to interact with the VWF A2 domain for proteolysis to take place, substrate scissile bond (and potentially inhibitor) access into the active site is predicted to be sterically hindered by this 'gate-keeper' triad. This crystal structure might, therefore, resemble the latent/'closed' active site state, which is also consistent with the fact that it was obtained in the absence of exosite binding. The proposed allosteric activation mechanism could, therefore, function by disrupting the interaction between the three residues, and repositioning the Ca²⁺-binding loop, which can now open and allow proteolysis. Interestingly, the ADAMTS4 'closed' and 'open' active site states are also shaped by the proximal Ca²⁺-binding loop. Considering the similarity between the 'closed' active site states of MDTCS(E225Q) (in the presence of the Fab 3H9) and the ADAMTS4 (in the absence of an inhibitor), the similarity between the 'open' active site states of the ADAMTS1/4/5, and the fact that the N-terminal domains (MDTCS), which contain functional exosites conferring specificity to proteolysis, are conserved in the ADAMTS family of proteases, the proposed MP domain flexibility and allosteric activation mechanism might represent a template for the function of other ADAMTS family members.

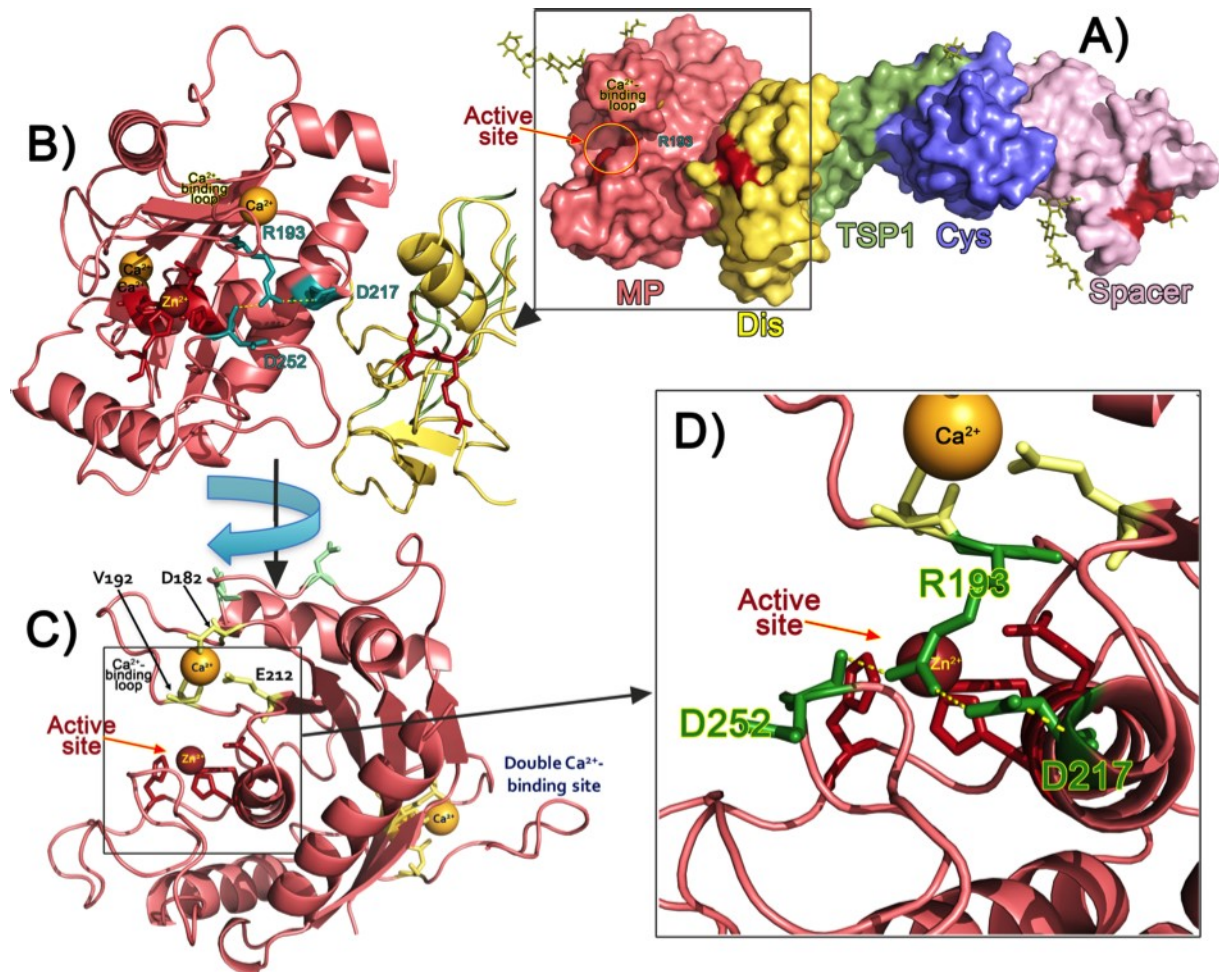


Figure 6-3 Crystal structure of the stable complex between the Fab 3H9 and MDTCS(E225Q)

(A) The crystal structure (surface view) of the stable complex between MDTCS(E225Q) and the Fab 3H9 (Fab 3H9 removed from structure). (B) The MP-Dis domains (cartoon view). The so-called 'gate-keeper' triad (R193, D217 and D252) is shown in teal. (C) The MP-Dis domains (cartoon view) rotated to bring the 'gate-keeper' triad to the front (triad omitted in this panel, see panel D). (D) Magnification of the 'gate-keeper' triad, stabilised by hydrogen bonding between the R193, D217 and D252.

An important consideration in drawing conclusions based on the crystal structure of MDTCS(E225Q) is the influence of the Fab 3H9 on the structure of the MP domain. It is possible that the 'closed' active site state observed could be a consequence of the binding of the Fab rather than being the natural latent conformation of the MP domain. To definitively conclude the existence of 'open' and 'closed' active site states, a crystal structure of the 'open' active site state is necessary. But how does one crystallize the open form of a conformationally flexible MP domain? The use of other Fab fragments that do not inhibit the active site to form a stable complex with MDTCS(E225Q) for co-crystallization has been trialled by my lab, but was unsuccessful. Theoretically, co-crystallizing MDTCS(E225Q) in complex with a VWF A2 domain fragment containing all the exosite binding sites (eg. VWF73 or VWF96) could be very informative, and this binding may also stabilise the fold of the MP domain. It would directly reveal the identity of the interacting residues involved in the exosite interactions. This would assist completely ablating the exosite interactions in future studies. Most importantly, crystallizing MDTCS(E225Q) during interaction with the Dis domain exosite would hopefully reveal the 'open' state of the active site and shed light to the structural changes that take place within the MP domain that make it permissive towards substrate/inhibitor access.

Complex formation and co-crystallization studies between the MDTCS(E225Q) and VWF73 are currently underway in our lab. However, we are currently facing unresolved problems that do not permit formation of a stable complex between the two binding partners. To first make a stable complex between the two proteins we need to fully understand the binding affinity between ADAMTS13 and the VWF A2 domain. As already explained, ELISA-based equilibrium binding assays have so far indicated relatively high binding affinities ($K_D \sim 7\text{-}40\text{ nM}$) between ADAMTS13 and VWF. However, since stable complex formation between MDTCS and VWF has proved to be more complicated, the true binding affinity between the two molecules could be closer to the K_m value, which is ~ 100 -fold higher ($K_m \sim 4\text{ }\mu\text{M}$).

The possibility of the VWF A2 domain fragments (eg. VWF73 and VWF96) adopting a tertiary structure in solution, which is less permissive to binding (due to compromised exosite binding site exposure) than when immobilised on a plate surface (potentially opening up the structure and more effectively exposing the exosite binding sites), has been introduced in Chapter 4 (section 4.13). Consistent with this, the K_D of solution-phase binding affinity between MDTCS(E225Q) and VWF73 obtained by ITC is $\sim 0.93\text{ }\mu\text{M}$, which is much closer to the K_m than the K_D from surface-phase binding assays.

Curiously, while uncleaved VWF73 and VWF96 bind to ADAMTS13/MDTCS, the affinity is lost after cleavage at the scissile bond, even though the C-terminal cleavage product still possesses the high affinity exosite binding sites that confer the affinity between the two molecules (unpublished data). How the cleavage product loses all affinity for ADAMTS13 despite the presence of all exosite binding sites remains an unresolved and intriguing question. Again, cleavage of the VWF A2 domain fragments could influence the tertiary structure of the cleavage products, and in so doing account for the reduction in the binding affinity.

6.5 How do ADAMTS13 domains communicate?

Future work on the allosteric activation of ADAMTS13 could address linking the effect of the Dis domain exosite interaction on substrate turnover to a conformational change in the MP domain, as well as the mode of inter-domain communication. How might the signal of Dis domain exosite interaction be transduced into a structural change within the active site of the MP domain? Interestingly, the crystal structure of the MDTCS(E225Q) in complex with the Fab 3H9 reveals a deep cleft between the MP and the Dis domains (Figure 6-3A). Potentially, this might be the interface of communication between the two domains. VWF binding is predicted to occur on either side of that cleft, and could be the link between the two domains; upon binding, the relative positions of the two domains change, such that the Dis domain can physically influence the conformation of the active site in the MP domain. This might also explain the critical importance of the low affinity MP domain exosite interaction (involving VWF A2 domain L1603); for VWF binding to impact on the relative positions of the MP and Dis domains, docking sites have to be available in both domains. The crystal structure of the complex between MDTCS(E225Q) and VWF73 will shed light on these questions.

It is becoming clearer that conformational changes and inter-domain communication, which impact upon the proteolytic function and specificity, could be a common theme in ADAMTS13. It is now established that the full length ADAMTS13 molecule can exist in folded and extended conformations, with its C-terminal domains involved in interactions with the N-terminal domains to maintain the folded conformation (Figure 1-16).^(43, 44, 46, 265, 376) Unfolding of ADAMTS13 enhances its specific activity by ~2-10-fold, which supports a functional link between the MP and non-catalytic domains of ADAMTS13 (see section 1.8.1).

Introducing the gain-of-function mutations at the Spacer domain residues thought to make up the Spacer domain exosite (R568K/F592Y/R660K/Y661F/Y665F) both unfolds ADAMTS13

and enhances its activity.⁽⁴⁶⁾ It is still unclear whether the mechanism of this conformational activation is, as previously proposed, dependent on full exposure of the Spacer exosite (otherwise sterically hindered by binding of the CUB domains) upon ADAMTS13 unfolding. Data from this project contribute to answering this question. By using monoclonal antibodies against the Spacer and CUB domains that unfold ADAMTS13, I show that unfolding can still cause activity enhancement even when the Spacer or Cys-rich domain exosite interactions are ablated (section 5.4); this argues against the proposed mechanism of liberation of the Spacer exosite upon ADAMTS13 unfolding.^(44, 46) Interestingly, Muia *et al* showed that the proteolysis of smaller VWF A2 domain-derived substrates that lack both Cys-rich and Spacer exosite binding sites (VWF25, G1598-D1622) is still enhanced by factors (pH and monoclonal antibodies) that unfold ADAMTS13.⁽⁴³⁾ Even though the authors did not comment further on the implications of this observation, in light of my data, this supports a link between the conformational activation of ADAMTS13 and the ADAMTS13 MP and Dis domains. Moreover, I show that antibody-induced activation of ADAMTS13 is primarily manifest by increasing the k_{cat} (substrate turnover), which supports structural implications upon ADAMTS13 unfolding that enhance the functionality of the active site (section 5.5). These data are in line with the ability of these activating antibodies to expose cryptic epitopes in the MP domain, as observed by my collaborator An-Sofie Schelpe, using an ELISA assay that specifically detects these cryptic epitopes.⁽²⁶⁵⁾

Another question that warrants further investigation concerns the physiological relevance of the conformational activation via ADAMTS13 unfolding. It was reported that binding of the VWF D4 domain to ADAMTS13 TSP8-CUB2 domains induces this conformational activation and was speculated that this could be a physiological way of enhancing ADAMTS13 activity.^(43, 44) It should be noted that for a 2-fold enhancing effect, 40 μ M D4 was required. To gain more insight into this mechanism, I tried to characterise, kinetically, the activity enhancement of ADAMTS13 using purified recombinant D4-CK domains. In multiple independent attempts using different preparations of ADAMTS13 and D4-CK domains (up to $\sim$ 2.5 μ M), I have not been able to reproduce the ability of D4-CK to enhance ADAMTS13 activity (section 5.6). However, a previous report suggested that 3nM of D4-CK could enhance the activity of ADAMTS13.⁽⁴⁶⁾ Since the VWF96 ELISA-based assay has been successful in accurately detecting and measuring the activating effect of antibody-induced ADAMTS13 unfolding, it is unlikely that the assay protocol itself could have accounted for the inability to see an activity

enhancement using the D4-CK domains. Nevertheless, given the sensitivity of this conformational activation mechanism to other factors that could easily confound the result (eg. buffer pH) more thorough investigation is required to draw conclusions on the ability of D4-CK to specifically conformationally activate ADAMTS13. Most importantly, the physiological relevance of this conformational activation of ADAMTS13 needs to be established; could a 2-fold activity enhancement be physiologically important, considering that low ADAMTS13 levels are not pathological unless <5%?

6.6 Conclusion – proposed model of allosteric activation

In conclusion, the data presented in this thesis are consistent with an allosteric link between the MP and the non-catalytic domains of ADAMTS13. I propose that the active site of ADAMTS13 is conformationally flexible, and can adopt latent/'closed' and active/'open' states, which exist in equilibrium. In unliganded ADAMTS13, the equilibrium favours the latent/'closed' state of the active site. This prevents off-target proteolysis and inhibitor access into the active site.

A shift of the active site state equilibrium towards the active/'open' state only occurs upon ADAMTS13 binding to its substrate, VWF, when VWF is unravelled by elevated shear. The Spacer, Cys-rich and Dis domain exosites interact with the unravelled VWF A2 domain at sites, which are found at the C-terminal side of the scissile bond. These interactions mediate approximation of the two molecules. Upon binding of the Dis domain exosite, a conformational change is also transduced to the active site favouring the active/'open' state. Docking of the MP domain (via lower affinity interactions) around the cleavage site directs the 'open' active site to precisely accommodate and cleave the scissile bond. I previously stated that ADAMTS13 is secreted as an active enzyme, with no activation step and no requirement for cofactors. Based on the proposed mechanism, this does not seem to be entirely true. I propose that VWF acts not only as the substrate, but also as a cofactor that specifically activates ADAMTS13. To definitively verify this mechanism, future studies need to link the critical influence of the Dis domain exosite interaction on substrate turnover (identified in this project), to a conformational change in the MP domain (suggested to exist by various lines of evidence). Additionally, solving the structure of the complex between ADAMTS13 and VWF73 will reveal the conformation of the active site upon exosite binding as well as the effect of exosite binding on the 'gate-keeper' triad proposed to stabilise a latent active site.

I propose that unfolding of ADAMTS13 via disruption of the inter-domain interactions that hold the molecule in a folded conformation also has implications on the ADAMTS13 MP and Dis domains and induces conformational changes within the MP domain that enhance the functionality of the active site. Whether this modest (~2-fold) activity enhancement is physiological and is manifest by the same mechanism of shifting the active site state equilibrium remains to be uncovered.

REFERENCES

1. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *The Journal of clinical investigation*. 1973;52(11):2757-64.
2. Nachman R, Levine R, Jaffe EA. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *The Journal of clinical investigation*. 1977;60(4):914-21.
3. Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *The Journal of clinical investigation*. 1985;76(3):1102-6.
4. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GA, Latt SA, et al. Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science*. 1985;228(4706):1401-6.
5. Sadler JE, Shelton-Inloes BB, Sorace JM, Harlan JM, Titani K, Davie EW. Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1985;82(19):6394-8.
6. Sadler JE, Shelton-Inloes BB, Sorace JM, Titani K. Cloning of cDNA and genomic DNA for human von Willebrand factor. *Cold Spring Harb Symp Quant Biol*. 1986;51 Pt 1:515-23.
7. Shelton-Inloes BB, Broze GJ, Jr., Miletich JP, Sadler JE. Evolution of human von Willebrand factor: cDNA sequence polymorphisms, repeated domains, and relationship to von Willebrand antigen II. *Biochemical and biophysical research communications*. 1987;144(2):657-65.
8. Shelton-Inloes BB, Titani K, Sadler JE. cDNA sequences for human von Willebrand factor reveal five types of repeated domains and five possible protein sequence polymorphisms. *Biochemistry*. 1986;25(11):3164-71.
9. Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, et al. Structure of the gene for human von Willebrand factor. *The Journal of biological chemistry*. 1989;264(33):19514-27.
10. Bonthron D, Orkin SH. The human von Willebrand factor gene. Structure of the 5' region. *European journal of biochemistry / FEBS*. 1988;171(1-2):51-7.
11. Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol*. 1994;14(2):999-1008.
12. Jahroudi N, Ardekani AM, Greenberger JS. An NF1-like protein functions as a repressor of the von Willebrand factor promoter. *The Journal of biological chemistry*. 1996;271(35):21413-21.
13. Rand JH, Badimon L, Gordon RE, Uson RR, Fuster V. Distribution of von Willebrand factor in porcine intima varies with blood vessel type and location. *Arteriosclerosis*. 1987;7(3):287-91.
14. Smith JM, Meinkoth JH, Hochstatter T, Meyers KM. Differential distribution of von Willebrand factor in canine vascular endothelium. *Am J Vet Res*. 1996;57(5):750-5.
15. Yamamoto K, de Waard V, Fearn C, Loskutoff DJ. Tissue distribution and regulation of murine von Willebrand factor gene expression in vivo. *Blood*. 1998;92(8):2791-801.
16. Gebrane-Younes J, Drouet L, Caen JP, Orcel L. Heterogeneous distribution of Weibel-Palade bodies and von Willebrand factor along the porcine vascular tree. *Am J Pathol*. 1991;139(6):1471-84.
17. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998;67:395-424.
18. Zhou YF, Eng ET, Zhu J, Lu C, Walz T, Springer TA. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120(2):449-58.
19. Fowler WE, Fretto LJ, Hamilton KK, Erickson HP, McKee PA. Substructure of human von Willebrand factor. *The Journal of clinical investigation*. 1985;76(4):1491-500.

20. Slayter H, Loscalzo J, Bockenstedt P, Handin RI. Native conformation of human von Willebrand protein. Analysis by electron microscopy and quasi-elastic light scattering. *The Journal of biological chemistry*. 1985;260(14):8559-63.
21. Schneider SW, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netz RR, et al. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(19):7899-903.
22. Siedlecki CA, Lestini BJ, Kottke-Marchant KK, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. 1996;88(8):2939-50.
23. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood*. 1994;83(12):3536-44.
24. Wise RJ, Barr PJ, Wong PA, Kiefer MC, Brake AJ, Kaufman RJ. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87(23):9378-82.
25. Rehemtulla A, Kaufman RJ. Preferred sequence requirements for cleavage of pro-von Willebrand factor by propeptide-processing enzymes. *Blood*. 1992;79(9):2349-55.
26. Haberichter SL. von Willebrand factor propeptide: biology and clinical utility. *Blood*. 2015;126(15):1753-61.
27. Verweij CL, Hart M, Pannekoek H. Expression of variant von Willebrand factor (vWF) cDNA in heterologous cells: requirement of the pro-polypeptide in vWF multimer formation. *EMBO J*. 1987;6(10):2885-90.
28. Wise RJ, Pittman DD, Handin RI, Kaufman RJ, Orkin SH. The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell*. 1988;52(2):229-36.
29. Haberichter SL, Fahs SA, Montgomery RR. von Willebrand factor storage and multimerization: 2 independent intracellular processes. *Blood*. 2000;96(5):1808-15.
30. Journet AM, Saffaripour S, Wagner DD. Requirement for both D domains of the propolypeptide in von Willebrand factor multimerization and storage. *Thrombosis and haemostasis*. 1993;70(6):1053-7.
31. Haberichter SL, Allmann AM, Jozwiak MA, Montgomery RR, Gill JC. Genetic alteration of the D2 domain abolishes von Willebrand factor multimerization and trafficking into storage. *Journal of thrombosis and haemostasis : JTH*. 2009;7(4):641-50.
32. Voorberg J, Fontijn R, van Mourik JA, Pannekoek H. Domains involved in multimer assembly of von willebrand factor (vWF): multimerization is independent of dimerization. *EMBO J*. 1990;9(3):797-803.
33. Purvis AR, Gross J, Dang LT, Huang RH, Kapadia M, Townsend RR, et al. Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(40):15647-52.
34. Dong Z, Thoma RS, Crimmins DL, McCourt DW, Tuley EA, Sadler JE. Disulfide bonds required to assemble functional von Willebrand factor multimers. *The Journal of biological chemistry*. 1994;269(9):6753-8.
35. Purvis AR, Sadler JE. A covalent oxidoreductase intermediate in propeptide-dependent von Willebrand factor multimerization. *The Journal of biological chemistry*. 2004;279(48):49982-8.
36. Huang RH, Wang Y, Roth R, Yu X, Purvis AR, Heuser JE, et al. Assembly of Weibel-Palade body-like tubules from N-terminal domains of von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(2):482-7.

37. Michaux G, Abbitt KB, Collinson LM, Haberichter SL, Norman KE, Cutler DF. The physiological function of von Willebrand's factor depends on its tubular storage in endothelial Weibel-Palade bodies. *Dev Cell*. 2006;10(2):223-32.
38. Fressinaud E, Mazurier C, Meyer D. Molecular genetics of type 2 von Willebrand disease. *International journal of hematology*. 2002;75(1):9-18.
39. Yee A, Gildersleeve RD, Gu S, Kretz CA, McGee BM, Carr KM, et al. A von Willebrand factor fragment containing the D'D3 domains is sufficient to stabilize coagulation factor VIII in mice. *Blood*. 2014;124(3):445-52.
40. Chiu PL, Bou-Assaf GM, Chhabra ES, Chambers MG, Peters RT, Kulman JD, et al. Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. *Blood*. 2015;126(8):935-8.
41. Feys HB, Anderson PJ, Vanhoorelbeke K, Majerus EM, Sadler JE. Multi-step binding of ADAMTS-13 to von Willebrand factor. *Journal of thrombosis and haemostasis : JTH*. 2009;7(12):2088-95.
42. Zanardelli S, Chion AC, Groot E, Lenting PJ, McKinnon TA, Laffan MA, et al. A novel binding site for ADAMTS13 constitutively exposed on the surface of globular VWF. *Blood*. 2009;114(13):2819-28.
43. Muia J, Zhu J, Gupta G, Haberichter SL, Friedman KD, Feys HB, et al. Allosteric activation of ADAMTS13 by von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111(52):18584-9.
44. South K, Freitas MO, Lane DA. A model for the conformational activation of the structurally quiescent metalloprotease ADAMTS13 by von Willebrand factor. *The Journal of biological chemistry*. 2017;292(14):5760-9.
45. South K, Lane DA. ADAMTS-13 and von Willebrand factor: a dynamic duo. *Journal of thrombosis and haemostasis : JTH*. 2017.
46. South K, Luken BM, Crawley JT, Phillips R, Thomas M, Collins RF, et al. Conformational activation of ADAMTS13. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111(52):18578-83.
47. Springer TA. Complement and the multifaceted functions of VWA and integrin I domains. *Structure*. 2006;14(11):1611-6.
48. Bienkowska J, Cruz M, Atiemo A, Handin R, Liddington R. The von willebrand factor A3 domain does not contain a metal ion-dependent adhesion site motif. *The Journal of biological chemistry*. 1997;272(40):25162-7.
49. Celikel R, Varughese KI, Madhusudan, Yoshioka A, Ware J, Ruggeri ZM. Crystal structure of the von Willebrand factor A1 domain in complex with the function blocking NMC-4 Fab. *Nat Struct Biol*. 1998;5(3):189-94.
50. Emsley J, Cruz M, Handin R, Liddington R. Crystal structure of the von Willebrand Factor A1 domain and implications for the binding of platelet glycoprotein Ib. *The Journal of biological chemistry*. 1998;273(17):10396-401.
51. Huizinga EG, Martijn van der Plas R, Kroon J, Sixma JJ, Gros P. Crystal structure of the A3 domain of human von Willebrand factor: implications for collagen binding. *Structure*. 1997;5(9):1147-56.
52. Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, de Groot PG, Sixma JJ, et al. Structures of glycoprotein Ibalph and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-9.
53. Jakobi AJ, Mashaghi A, Tans SJ, Huizinga EG. Calcium modulates force sensing by the von Willebrand factor A2 domain. *Nat Commun*. 2011;2:385.

54. Zhang Q, Zhou YF, Zhang CZ, Zhang X, Lu C, Springer TA. Structural specializations of A2, a force-sensing domain in the ultralarge vascular protein von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(23):9226-31.
55. Auton M, Cruz MA, Moake J. Conformational stability and domain unfolding of the Von Willebrand factor A domains. *Journal of molecular biology*. 2007;366(3):986-1000.
56. Zhang X, Halvorsen K, Zhang CZ, Wong WP, Springer TA. Mechanoenzymatic cleavage of the ultralarge vascular protein von Willebrand factor. *Science*. 2009;324(5932):1330-4.
57. Berndt MC, Ward CM, Booth WJ, Castaldi PA, Mazurov AV, Andrews RK. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry*. 1992;31(45):11144-51.
58. Cruz MA, Diacovo TG, Emsley J, Liddington R, Handin RI. Mapping the glycoprotein Ib-binding site in the von willebrand factor A1 domain. *The Journal of biological chemistry*. 2000;275(25):19098-105.
59. Matsushita T, Meyer D, Sadler JE. Localization of von willebrand factor-binding sites for platelet glycoprotein Ib and botrocetin by charged-to-alanine scanning mutagenesis. *The Journal of biological chemistry*. 2000;275(15):11044-9.
60. Shimizu A, Matsushita T, Kondo T, Inden Y, Kojima T, Saito H, et al. Identification of the amino acid residues of the platelet glycoprotein Ib (GPIb) essential for the von Willebrand factor binding by clustered charged-to-alanine scanning mutagenesis. *The Journal of biological chemistry*. 2004;279(16):16285-94.
61. Sugimoto M, Dent J, McClintock R, Ware J, Ruggeri ZM. Analysis of structure-function relationships in the platelet membrane glycoprotein Ib-binding domain of von Willebrand's factor by expression of deletion mutants. *The Journal of biological chemistry*. 1993;268(16):12185-92.
62. Lenting PJ, Pegon JN, Groot E, de Groot PG. Regulation of von Willebrand factor-platelet interactions. *Thrombosis and haemostasis*. 2010;104(3):449-55.
63. Auton M, Sowa KE, Behymer M, Cruz MA. N-terminal flanking region of A1 domain in von Willebrand factor stabilizes structure of A1A2A3 complex and modulates platelet activation under shear stress. *The Journal of biological chemistry*. 2012;287(18):14579-85.
64. Ulrichs H, Udvardy M, Lenting PJ, Pareyn I, Vandeputte N, Vanhoorelbeke K, et al. Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. *The Journal of biological chemistry*. 2006;281(8):4699-707.
65. Ulrichs H, Vanhoorelbeke K, Girma JP, Lenting PJ, Vauterin S, Deckmyn H. The von Willebrand factor self-association is modulated by a multiple domain interaction. *Journal of thrombosis and haemostasis : JTH*. 2005;3(3):552-61.
66. Martin C, Morales LD, Cruz MA. Purified A2 domain of von Willebrand factor binds to the active conformation of von Willebrand factor and blocks the interaction with platelet glycoprotein Ibalpha. *Journal of thrombosis and haemostasis : JTH*. 2007;5(7):1363-70.
67. Dumas JJ, Kumar R, McDonagh T, Sullivan F, Stahl ML, Somers WS, et al. Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibalpha complex reveals conformation differences with a complex bearing von Willebrand disease mutations. *The Journal of biological chemistry*. 2004;279(22):23327-34.
68. Nuytens BP, Thijs T, Deckmyn H, Broos K. Platelet adhesion to collagen. *Thrombosis research*. 2011;127 Suppl 2:S26-9.
69. Blenner MA, Dong X, Springer TA. Structural basis of regulation of von Willebrand factor binding to glycoprotein Ib. *The Journal of biological chemistry*. 2014;289(9):5565-79.

70. Kim J, Zhang CZ, Zhang X, Springer TA. A mechanically stabilized receptor-ligand flex-bond important in the vasculature. *Nature*. 2010;466(7309):992-5.
71. Flood VH, Schlauderaff AC, Haberichter SL, Slobodianuk TL, Jacobi PM, Bellissimo DB, et al. Crucial role for the VWF A1 domain in binding to type IV collagen. *Blood*. 2015;125(14):2297-304.
72. Saelman EU, Nieuwenhuis HK, Hese KM, de Groot PG, Heijnen HF, Sage EH, et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (alpha 2 beta 1-integrin). *Blood*. 1994;83(5):1244-50.
73. Kroner PA, Frey AB. Analysis of the structure and function of the von Willebrand factor A1 domain using targeted deletions and alanine-scanning mutagenesis. *Biochemistry*. 1996;35(41):13460-8.
74. Sobel M, Soler DF, Kermode JC, Harris RB. Localization and characterization of a heparin binding domain peptide of human von Willebrand factor. *The Journal of biological chemistry*. 1992;267(13):8857-62.
75. Sobel M, McNeill PM, Carlson PL, Kermode JC, Adelman B, Conroy R, et al. Heparin inhibition of von Willebrand factor-dependent platelet function in vitro and in vivo. *The Journal of clinical investigation*. 1991;87(5):1787-93.
76. Mohri H, Yoshioka A, Zimmerman TS, Ruggeri ZM. Isolation of the von Willebrand factor domain interacting with platelet glycoprotein Ib, heparin, and collagen and characterization of its three distinct functional sites. *The Journal of biological chemistry*. 1989;264(29):17361-7.
77. Sixma JJ, Schiphorst ME, Verweij CL, Pannekoek H. Effect of deletion of the A1 domain of von Willebrand factor on its binding to heparin, collagen and platelets in the presence of ristocetin. *European journal of biochemistry / FEBS*. 1991;196(2):369-75.
78. Adachi T, Matsushita T, Dong Z, Katsumi A, Nakayama T, Kojima T, et al. Identification of amino acid residues essential for heparin binding by the A1 domain of human von Willebrand factor. *Biochemical and biophysical research communications*. 2006;339(4):1178-83.
79. Rastegar-Lari G, Villoutreix BO, Ribba AS, Legendre P, Meyer D, Baruch D. Two clusters of charged residues located in the electropositive face of the von Willebrand factor A1 domain are essential for heparin binding. *Biochemistry*. 2002;41(21):6668-78.
80. Morales LD, Martin C, Cruz MA. The interaction of von Willebrand factor-A1 domain with collagen: mutation G1324S (type 2M von Willebrand disease) impairs the conformational change in A1 domain induced by collagen. *Journal of thrombosis and haemostasis : JTH*. 2006;4(2):417-25.
81. Bonnefoy A, Romijn RA, Vandervoort PA, I VANR, Vermynen J, Hoylaerts MF. von Willebrand factor A1 domain can adequately substitute for A3 domain in recruitment of flowing platelets to collagen. *Journal of thrombosis and haemostasis : JTH*. 2006;4(10):2151-61.
82. Hoylaerts MF, Yamamoto H, Nuyts K, Vreys I, Deckmyn H, Vermynen J. von Willebrand factor binds to native collagen VI primarily via its A1 domain. *Biochem J*. 1997;324 (Pt 1):185-91.
83. Mazzucato M, Spessotto P, Masotti A, De Appollonia L, Cozzi MR, Yoshioka A, et al. Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. *The Journal of biological chemistry*. 1999;274(5):3033-41.
84. Springer TA. Biology and physics of von Willebrand factor concatamers. *Journal of thrombosis and haemostasis : JTH*. 2011;9 Suppl 1:130-43.
85. Zhou M, Dong X, Baldauf C, Chen H, Zhou Y, Springer TA, et al. A novel calcium-binding site of von Willebrand factor A2 domain regulates its cleavage by ADAMTS13. *Blood*. 2011;117(17):4623-31.

86. Lynch CJ, Lane DA. N-linked glycan stabilization of the VWF A2 domain. *Blood*. 2016;127(13):1711-8.
87. McKinnon TA, Chion AC, Millington AJ, Lane DA, Laffan MA. N-linked glycosylation of VWF modulates its interaction with ADAMTS13. *Blood*. 2008;111(6):3042-9.
88. Chion A, O'Sullivan JM, Drakeford C, Bergsson G, Dalton N, Aguila S, et al. N-linked glycans within the A2 domain of von Willebrand factor modulate macrophage-mediated clearance. *Blood*. 2016;128(15):1959-68.
89. Luken BM, Winn LY, Emsley J, Lane DA, Crawley JT. The importance of vicinal cysteines, C1669 and C1670, for von Willebrand factor A2 domain function. *Blood*. 2010;115(23):4910-3.
90. Lynch CJ, Lane DA, Luken BM. Control of VWF A2 domain stability and ADAMTS13 access to the scissile bond of full-length VWF. *Blood*. 2014;123(16):2585-92.
91. Romijn RA, Bouma B, Wuyster W, Gros P, Kroon J, Sixma JJ, et al. Identification of the collagen-binding site of the von Willebrand factor A3-domain. *The Journal of biological chemistry*. 2001;276(13):9985-91.
92. Romijn RA, Westein E, Bouma B, Schiphorst ME, Sixma JJ, Lenting PJ, et al. Mapping the collagen-binding site in the von Willebrand factor-A3 domain. *The Journal of biological chemistry*. 2003;278(17):15035-9.
93. Lisman T, Raynal N, Groeneveld D, Maddox B, Peachey AR, Huizinga EG, et al. A single high-affinity binding site for von Willebrand factor in collagen III, identified using synthetic triple-helical peptides. *Blood*. 2006;108(12):3753-6.
94. Nishida N, Sumikawa H, Sakakura M, Shimba N, Takahashi H, Terasawa H, et al. Collagen-binding mode of vWF-A3 domain determined by a transferred cross-saturation experiment. *Nat Struct Biol*. 2003;10(1):53-8.
95. Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, Ijsseldijk MJ, et al. A3 domain is essential for interaction of von Willebrand factor with collagen type III. *Thrombosis and haemostasis*. 1996;75(6):950-8.
96. Vanhoorelbeke K, Depraetere H, Romijn RA, Huizinga EG, De Maeyer M, Deckmyn H. A consensus tetrapeptide selected by phage display adopts the conformation of a dominant discontinuous epitope of a monoclonal anti-VWF antibody that inhibits the von Willebrand factor-collagen interaction. *The Journal of biological chemistry*. 2003;278(39):37815-21.
97. Brondijk TH, Bihan D, Farndale RW, Huizinga EG. Implications for collagen I chain registry from the structure of the collagen von Willebrand factor A3 domain complex. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(14):5253-8.
98. Shapiro SE, Nowak AA, Wooding C, Birdsey G, Laffan MA, McKinnon TA. The von Willebrand factor predicted unpaired cysteines are essential for secretion. *Journal of thrombosis and haemostasis : JTH*. 2014;12(2):246-54.
99. O'Leary JM, Hamilton JM, Deane CM, Valeyev NV, Sandell LJ, Downing AK. Solution structure and dynamics of a prototypical chordin-like cysteine-rich repeat (von Willebrand Factor type C module) from collagen IIA. *The Journal of biological chemistry*. 2004;279(51):53857-66.
100. Zhou YF, Eng ET, Nishida N, Lu C, Walz T, Springer TA. A pH-regulated dimeric bouquet in the structure of von Willebrand factor. *EMBO J*. 2011;30(19):4098-111.
101. Lankhof H, Wu YP, Vink T, Schiphorst ME, Zerwes HG, de Groot PG, et al. Role of the glycoprotein Ib-binding A1 repeat and the RGD sequence in platelet adhesion to human recombinant von Willebrand factor. *Blood*. 1995;86(3):1035-42.
102. Beacham DA, Wise RJ, Turci SM, Handin RI. Selective inactivation of the Arg-Gly-Asp-Ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. *The Journal of biological chemistry*. 1992;267(5):3409-15.

103. Meitinger T, Meindl A, Bork P, Rost B, Sander C, Haasemann M, et al. Molecular modelling of the Norrie disease protein predicts a cystine knot growth factor tertiary structure. *Nat Genet.* 1993;5(4):376-80.
104. Vitt UA, Hsu SY, Hsueh AJ. Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Mol Endocrinol.* 2001;15(5):681-94.
105. Zhou YF, Springer TA. Highly reinforced structure of a C-terminal dimerization domain in von Willebrand factor. *Blood.* 2014;123(12):1785-93.
106. Katsumi A, Tuley EA, Bodo I, Sadler JE. Localization of disulfide bonds in the cystine knot domain of human von Willebrand factor. *The Journal of biological chemistry.* 2000;275(33):25585-94.
107. Canis K, McKinnon TA, Nowak A, Haslam SM, Panico M, Morris HR, et al. Mapping the N-glycome of human von Willebrand factor. *Biochem J.* 2012;447(2):217-28.
108. Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells. Identification of a large precursor polypeptide chain. *The Journal of biological chemistry.* 1983;258(4):2065-7.
109. Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. *J Cell Biol.* 1984;99(6):2123-30.
110. Mayadas TN, Wagner DD. Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proceedings of the National Academy of Sciences of the United States of America.* 1992;89(8):3531-5.
111. Dang LT, Purvis AR, Huang RH, Westfield LA, Sadler JE. Phylogenetic and functional analysis of histidine residues essential for pH-dependent multimerization of von Willebrand factor. *The Journal of biological chemistry.* 2011;286(29):25763-9.
112. Lynch DC, Williams R, Zimmerman TS, Kirby EP, Livingston DM. Biosynthesis of the subunits of factor VIIIIR by bovine aortic endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America.* 1983;80(9):2738-42.
113. Verweij CL, Hart M, Pannekoek H. Proteolytic cleavage of the precursor of von Willebrand factor is not essential for multimer formation. *The Journal of biological chemistry.* 1988;263(17):7921-4.
114. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol.* 1982;95(1):355-60.
115. Weibel ER, Palade GE. New Cytoplasmic Components in Arterial Endothelia. *J Cell Biol.* 1964;23:101-12.
116. Wagner DD, Saffaripour S, Bonfanti R, Sadler JE, Cramer EM, Chapman B, et al. Induction of specific storage organelles by von Willebrand factor propeptide. *Cell.* 1991;64(2):403-13.
117. Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H. Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. *EMBO J.* 1993;12(2):749-58.
118. Valentijn KM, Valentijn JA, Jansen KA, Koster AJ. A new look at Weibel-Palade body structure in endothelial cells using electron tomography. *J Struct Biol.* 2008;161(3):447-58.
119. Valentijn KM, Eikenboom J. Weibel-Palade bodies: a window to von Willebrand disease. *Journal of thrombosis and haemostasis : JTH.* 2013;11(4):581-92.
120. Berriman JA, Li S, Hewlett LJ, Wasilewski S, Kiskin FN, Carter T, et al. Structural organization of Weibel-Palade bodies revealed by cryo-EM of vitrified endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(41):17407-12.
121. Borchellini A, Fijnvandraat K, ten Cate JW, Pajkrt D, van Deventer SJ, Pasterkamp G, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of

experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996;88(8):2951-8.

122. Meriane F, Zerhouni L, Djeha N, Goudemand M, Mazurier C. Biological effects of a S/D-treated, very high purity, von Willebrand factor concentrate in five patients with severe von Willebrand disease. *Blood Coagul Fibrinolysis*. 1993;4(6):1023-9.

123. Lopes da Silva M, Cutler DF. von Willebrand factor multimerization and the polarity of secretory pathways in endothelial cells. *Blood*. 2016;128(2):277-85.

124. Valentijn KM, van Driel LF, Mourik MJ, Hendriks GJ, Arends TJ, Koster AJ, et al. Multigranular exocytosis of Weibel-Palade bodies in vascular endothelial cells. *Blood*. 2010;116(10):1807-16.

125. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002;100(12):4033-9.

126. Schick PK, Walker J, Profeta B, Denisova L, Bennett V. Synthesis and secretion of von Willebrand factor and fibronectin in megakaryocytes at different phases of maturation. *Arterioscler Thromb Vasc Biol*. 1997;17(4):797-801.

127. Cramer EM, Meyer D, le Menn R, Breton-Gorius J. Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. *Blood*. 1985;66(3):710-3.

128. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell*. 1986;46(2):185-90.

129. Fernandez MF, Ginsberg MH, Ruggeri ZM, Batlle FJ, Zimmerman TS. Multimeric structure of platelet factor VIII/von Willebrand factor: the presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood*. 1982;60(5):1132-8.

130. Miura Y, Takahashi T, Jung SM, Moroi M. Analysis of the interaction of platelet collagen receptor glycoprotein VI (GPVI) with collagen. A dimeric form of GPVI, but not the monomeric form, shows affinity to fibrous collagen. *The Journal of biological chemistry*. 2002;277(48):46197-204.

131. Nieswandt B, Bergmeier W, Eckly A, Schulte V, Ohlmann P, Cazenave JP, et al. Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. *Blood*. 2001;97(12):3829-35.

132. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, et al. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J*. 2001;20(9):2120-30.

133. Sarratt KL, Chen H, Zutter MM, Santoro SA, Hammer DA, Kahn ML. GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow. *Blood*. 2005;106(4):1268-77.

134. Saelman EU, Kehrel B, Hese KM, de Groot PG, Sixma JJ, Nieuwenhuis HK. Platelet adhesion to collagen and endothelial cell matrix under flow conditions is not dependent on platelet glycoprotein IV. *Blood*. 1994;83(11):3240-4.

135. Staatz WD, Rajpara SM, Wayner EA, Carter WG, Santoro SA. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg⁺⁺-dependent adhesion of platelets to collagen. *J Cell Biol*. 1989;108(5):1917-24.

136. Pugh N, Simpson AM, Smethurst PA, de Groot PG, Raynal N, Farndale RW. Synergism between platelet collagen receptors defined using receptor-specific collagen-mimetic peptide substrata in flowing blood. *Blood*. 2010;115(24):5069-79.

137. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. *Circ Res*. 2007;100(12):1673-85.

138. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996;84(2):289-97.
139. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *The Journal of clinical investigation*. 1986;78(6):1456-61.
140. Ruggeri ZM, Mendolicchio GL. Interaction of von Willebrand factor with platelets and the vessel wall. *Hamostaseologie*. 2015;35(3):211-24.
141. De Marco L, Girolami A, Russell S, Ruggeri ZM. Interaction of asialo von Willebrand factor with glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and mediates platelet aggregation. *The Journal of clinical investigation*. 1985;75(4):1198-203.
142. De Marco L, Girolami A, Zimmerman TS, Ruggeri ZM. Interaction of purified type IIB von Willebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and initiates aggregation. *Proceedings of the National Academy of Sciences of the United States of America*. 1985;82(21):7424-8.
143. Ribba AS, Loisel I, Lavergne JM, Juhan-Vague I, Obert B, Cherel G, et al. Ser968Thr mutation within the A3 domain of von Willebrand factor (VWF) in two related patients leads to a defective binding of VWF to collagen. *Thrombosis and haemostasis*. 2001;86(3):848-54.
144. Riddell AF, Gomez K, Millar CM, Mellars G, Gill S, Brown SA, et al. Characterization of W1745C and S1783A: 2 novel mutations causing defective collagen binding in the A3 domain of von Willebrand factor. *Blood*. 2009;114(16):3489-96.
145. Pareti FI, Fujimura Y, Dent JA, Holland LZ, Zimmerman TS, Ruggeri ZM. Isolation and characterization of a collagen binding domain in human von Willebrand factor. *The Journal of biological chemistry*. 1986;261(32):15310-5.
146. Pareti FI, Niiya K, McPherson JM, Ruggeri ZM. Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. *The Journal of biological chemistry*. 1987;262(28):13835-41.
147. Rand JH, Patel ND, Schwartz E, Zhou SL, Potter BJ. 150-kD von Willebrand factor binding protein extracted from human vascular subendothelium is type VI collagen. *The Journal of clinical investigation*. 1991;88(1):253-9.
148. Flood VH, Gill JC, Christopherson PA, Bellissimo DB, Friedman KD, Haberichter SL, et al. Critical von Willebrand factor A1 domain residues influence type VI collagen binding. *Journal of thrombosis and haemostasis : JTH*. 2012;10(7):1417-24.
149. Yago T, Lou J, Wu T, Yang J, Miner JJ, Coburn L, et al. Platelet glycoprotein Ibalph forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *The Journal of clinical investigation*. 2008;118(9):3195-207.
150. Lou J, Zhu C. Flow induces loop-to-beta-hairpin transition on the beta-switch of platelet glycoprotein Ib alpha. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(37):13847-52.
151. Doggett TA, Girdhar G, Lawshe A, Schmidtke DW, Laurenzi IJ, Diamond SL, et al. Selectin-like kinetics and biomechanics promote rapid platelet adhesion in flow: the GPIb(alpha)-vWF tether bond. *Biophys J*. 2002;83(1):194-205.
152. Fressinaud E, Baruch D, Girma JP, Sakariassen KS, Baumgartner HR, Meyer D. von Willebrand factor-mediated platelet adhesion to collagen involves platelet membrane glycoprotein IIb-IIIa as well as glycoprotein Ib. *J Lab Clin Med*. 1988;112(1):58-67.
153. Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*. 2000;101(1):47-56.

154. Knight CG, Morton LF, Onley DJ, Peachey AR, Ichinohe T, Okuma M, et al. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc Res.* 1999;41(2):450-7.
155. Zhang W, Deng W, Zhou L, Xu Y, Yang W, Liang X, et al. Identification of a juxtamembrane mechanosensitive domain in the platelet mechanosensor glycoprotein Ib-IX complex. *Blood.* 2015;125(3):562-9.
156. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell.* 1998;94(5):657-66.
157. Ichinohe T, Takayama H, Ezumi Y, Arai M, Yamamoto N, Takahashi H, et al. Collagen-stimulated activation of Syk but not c-Src is severely compromised in human platelets lacking membrane glycoprotein VI. *The Journal of biological chemistry.* 1997;272(1):63-8.
158. Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. *The Journal of clinical investigation.* 1998;101(2):479-86.
159. Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood.* 1999;94(1):172-8.
160. Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *The Journal of biological chemistry.* 1979;254(12):5357-63.
161. Bennett JS, Vilaire G, Cines DB. Identification of the fibrinogen receptor on human platelets by photoaffinity labeling. *The Journal of biological chemistry.* 1982;257(14):8049-54.
162. Hantgan RR, Hindriks G, Taylor RG, Sixma JJ, de Groot PG. Glycoprotein Ib, von Willebrand factor, and glycoprotein IIb/IIIa are all involved in platelet adhesion to fibrin in flowing whole blood. *Blood.* 1990;76(2):345-53.
163. Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *The Journal of biological chemistry.* 1992;267(16):11300-6.
164. Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *The Journal of clinical investigation.* 1991;87(4):1234-40.
165. Berliner SA, Roberts JR, Thorn ML, Ruggeri ZM. Generation and characterization of specific antibodies directed to the binding domain of adhesive molecules. *Pept Res.* 1988;1(2):60-4.
166. De Marco L, Girolami A, Zimmerman TS, Ruggeri ZM. von Willebrand factor interaction with the glycoprotein IIb/IIIa complex. Its role in platelet function as demonstrated in patients with congenital afibrinogenemia. *The Journal of clinical investigation.* 1986;77(4):1272-7.
167. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. *The Journal of biological chemistry.* 1995;270(40):23352-61.
168. Berliner S, Niiya K, Roberts JR, Houghten RA, Ruggeri ZM. Generation and characterization of peptide-specific antibodies that inhibit von Willebrand factor binding to glycoprotein IIb-IIIa without interacting with other adhesive molecules. Selectivity is conferred by Pro1743 and other amino acid residues adjacent to the sequence Arg1744-Gly1745-Asp1746. *The Journal of biological chemistry.* 1988;263(16):7500-5.
169. Peterson DM, Stathopoulos NA, Giorgio TD, Hellums JD, Moake JL. Shear-induced platelet aggregation requires von Willebrand factor and platelet membrane glycoproteins Ib and IIb-IIIa. *Blood.* 1987;69(2):625-8.

170. Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. *The Journal of clinical investigation*. 1983;72(1):1-12.
171. Moake JL, Turner NA, Stathopoulos NA, Nolasco L, Hellums JD. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood*. 1988;71(5):1366-74.
172. Santoro SA. Preferential binding of high molecular weight forms of von Willebrand factor to fibrillar collagen. *Biochim Biophys Acta*. 1983;756(1):123-6.
173. Sporn LA, Marder VJ, Wagner DD. von Willebrand factor released from Weibel-Palade bodies binds more avidly to extracellular matrix than that secreted constitutively. *Blood*. 1987;69(5):1531-4.
174. Federici AB, Bader R, Pagani S, Colibretti ML, De Marco L, Mannucci PM. Binding of von Willebrand factor to glycoproteins Ib and IIb/IIIa complex: affinity is related to multimeric size. *British journal of haematology*. 1989;73(1):93-9.
175. Martin SE, Marder VJ, Francis CW, Barlow GH. Structural studies of the functional heterogeneity of von Willebrand protein polymers. *Blood*. 1981;57(2):313-23.
176. Arya M, Anvari B, Romo GM, Cruz MA, Dong JF, McIntire LV, et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. *Blood*. 2002;99(11):3971-7.
177. Ott HW, Griesmacher A, Schnapka-Koepf M, Golderer G, Sieberer A, Spannagl M, et al. Analysis of von Willebrand factor multimers by simultaneous high- and low-resolution vertical SDS-agarose gel electrophoresis and Cy5-labeled antibody high-sensitivity fluorescence detection. *Am J Clin Pathol*. 2010;133(2):322-30.
178. Ganz PR, Atkins JS, Palmer DS, Dudani AK, Hashemi S, Luison F. Definition of the affinity of binding between human von Willebrand factor and coagulation factor VIII. *Biochemical and biophysical research communications*. 1991;180(1):231-7.
179. Vlot AJ, Koppelman SJ, Meijers JC, Dama C, van den Berg HM, Bouma BN, et al. Kinetics of factor VIII-von Willebrand factor association. *Blood*. 1996;87(5):1809-16.
180. Vlot AJ, Koppelman SJ, van den Berg MH, Bouma BN, Sixma JJ. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood*. 1995;85(11):3150-7.
181. Hilbert L, Nurden P, Caron C, Nurden AT, Goudemand J, Meyer D, et al. Type 2N von Willebrand disease due to compound heterozygosity for R854Q and a novel R763G mutation at the cleavage site of von Willebrand factor propeptide. *Thrombosis and haemostasis*. 2006;96(3):290-4.
182. Wise RJ, Dorner AJ, Krane M, Pittman DD, Kaufman RJ. The role of von Willebrand factor multimers and propeptide cleavage in binding and stabilization of factor VIII. *The Journal of biological chemistry*. 1991;266(32):21948-55.
183. Casonato A, Sartorello F, Cattini MG, Pontara E, Soldera C, Bertomoro A, et al. An Arg760Cys mutation in the consensus sequence of the von Willebrand factor propeptide cleavage site is responsible for a new von Willebrand disease variant. *Blood*. 2003;101(1):151-6.
184. Dasgupta S, Repesse Y, Bayry J, Navarrete AM, Wootla B, Delignat S, et al. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*. 2007;109(2):610-2.
185. Nesheim M, Pittman DD, Giles AR, Fass DN, Wang JH, Slonosky D, et al. The effect of plasma von Willebrand factor on the binding of human factor VIII to thrombin-activated human platelets. *The Journal of biological chemistry*. 1991;266(27):17815-20.

186. Saenko EL, Scandella D. A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor. *The Journal of biological chemistry*. 1995;270(23):13826-33.
187. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. *The Journal of clinical investigation*. 1988;82(4):1236-43.
188. Fay PJ, Coumans JV, Walker FJ. von Willebrand factor mediates protection of factor VIII from activated protein C-catalyzed inactivation. *The Journal of biological chemistry*. 1991;266(4):2172-7.
189. Koppelman SJ, van Hoeij M, Vink T, Lankhof H, Schiphorst ME, Damas C, et al. Requirements of von Willebrand factor to protect factor VIII from inactivation by activated protein C. *Blood*. 1996;87(6):2292-300.
190. Lenting PJ, van de Loo JW, Donath MJ, van Mourik JA, Mertens K. The sequence Glu1811-Lys1818 of human blood coagulation factor VIII comprises a binding site for activated factor IX. *The Journal of biological chemistry*. 1996;271(4):1935-40.
191. Nogami K, Shima M, Nishiya K, Hosokawa K, Saenko EL, Sakurai Y, et al. A novel mechanism of factor VIII protection by von Willebrand factor from activated protein C-catalyzed inactivation. *Blood*. 2002;99(11):3993-8.
192. Koedam JA, Hamer RJ, Beeser-Visser NH, Bouma BN, Sixma JJ. The effect of von Willebrand factor on activation of factor VIII by factor Xa. *European journal of biochemistry / FEBS*. 1990;189(2):229-34.
193. Lenting PJ, Donath MJ, van Mourik JA, Mertens K. Identification of a binding site for blood coagulation factor IXa on the light chain of human factor VIII. *The Journal of biological chemistry*. 1994;269(10):7150-5.
194. Borel-Derlon A, Federici AB, Roussel-Robert V, Goudemand J, Lee CA, Scharrer I, et al. Treatment of severe von Willebrand disease with a high-purity von Willebrand factor concentrate (Wilfactin): a prospective study of 50 patients. *Journal of thrombosis and haemostasis : JTH*. 2007;5(6):1115-24.
195. Kaufman RJ, Dorner AJ, Fass DN. von Willebrand factor elevates plasma factor VIII without induction of factor VIII messenger RNA in the liver. *Blood*. 1999;93(1):193-7.
196. van Meegeren ME, Mancini TL, Schoormans SC, van Haren BJ, van Duren C, Diekstra A, et al. Clinical phenotype in genetically confirmed von Willebrand disease type 2N patients reflects a haemophilia A phenotype. *Haemophilia*. 2015;21(5):e375-83.
197. Cao W, Krishnaswamy S, Camire RM, Lenting PJ, Zheng XL. Factor VIII accelerates proteolytic cleavage of von Willebrand factor by ADAMTS13. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(21):7416-21.
198. Terraube V, O'Donnell JS, Jenkins PV. Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance. *Haemophilia*. 2010;16(1):3-13.
199. Loscalzo J. From clinical observation to mechanism--Heyde's syndrome. *The New England journal of medicine*. 2013;368(6):579-80.
200. Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117(25):6777-85.
201. Leebeek FW, Eikenboom JC. Von Willebrand's Disease. *The New England journal of medicine*. 2016;375(21):2067-80.
202. Lillicrap D. von Willebrand disease: advances in pathogenetic understanding, diagnosis, and therapy. *Hematology Am Soc Hematol Educ Program*. 2013;2013:254-60.
203. Bharati KP, Prashanth UR. Von Willebrand disease: an overview. *Indian J Pharm Sci*. 2011;73(1):7-16.

204. Studt JD, Budde U, Schneppenheim R, Eisert R, von Depka Prondzinski M, Ganser A, et al. Quantification and facilitated comparison of von Willebrand factor multimer patterns by densitometry. *Am J Clin Pathol*. 2001;116(4):567-74.
205. Perez-Rodriguez A, Garcia-Rivero A, Loures E, Lopez-Fernandez MF, Rodriguez-Trillo A, Batlle J. Autosomal dominant C1149R von Willebrand disease: phenotypic findings and their implications. *Haematologica*. 2009;94(5):679-86.
206. Mannucci PM, Lombardi R, Castaman G, Dent JA, Lattuada A, Rodeghiero F, et al. von Willebrand disease "Vicenza" with larger-than-normal (supranormal) von Willebrand factor multimers. *Blood*. 1988;71(1):65-70.
207. Tsai HM, Nagel RL, Hatcher VB, Seaton AC, Sussman, II. The high molecular weight form of endothelial cell von Willebrand factor is released by the regulated pathway. *British journal of haematology*. 1991;79(2):239-45.
208. Tsai HM, Nagel RL, Hatcher VB, Sussman, II. Multimeric composition of endothelial cell-derived von Willebrand factor. *Blood*. 1989;73(8):2074-6.
209. Tsai HM, Nagel RL, Hatcher VB, Sussman, II. Endothelial cell-derived high molecular weight von Willebrand factor is converted into the plasma multimer pattern by granulocyte proteases. *Biochemical and biophysical research communications*. 1989;158(3):980-5.
210. Dent JA, Galbusera M, Ruggeri ZM. Heterogeneity of plasma von Willebrand factor multimers resulting from proteolysis of the constituent subunit. *The Journal of clinical investigation*. 1991;88(3):774-82.
211. Tsai HM, Nagel RL, Sussman, II. Subunit composition of plasma von Willebrand factor multimers: evidence for a non-proteolytic mechanism resulting in apparent increase in proteolytic fragments. *Thrombosis research*. 1991;63(1):179-88.
212. Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH. Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (types IIC, IID, and IIE). *The Journal of clinical investigation*. 1986;77(3):947-51.
213. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *The New England journal of medicine*. 1982;307(23):1432-5.
214. Moake JL, McPherson PD. Abnormalities of von Willebrand factor multimers in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *The American journal of medicine*. 1989;87(3N):9N-15N.
215. Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87(16):6306-10.
216. Tsai HM, Sussman, II, Nagel RL. Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. *Blood*. 1994;83(8):2171-9.
217. Furlan M, Robles R, Lammle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*. 1996;87(10):4223-34.
218. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood*. 1996;87(10):4235-44.
219. Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lammle B. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood*. 1997;89(9):3097-103.

220. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *The New England journal of medicine*. 1998;339(22):1578-84.
221. Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *The New England journal of medicine*. 1998;339(22):1585-94.
222. Furlan M, Robles R, Solenthaler M, Lammle B. Acquired deficiency of von Willebrand factor-cleaving protease in a patient with thrombotic thrombocytopenic purpura. *Blood*. 1998;91(8):2839-46.
223. Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood*. 2001;98(6):1654-61.
224. Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*. 2001;98(6):1662-6.
225. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413(6855):488-94.
226. Porter S, Clark IM, Kevorkian L, Edwards DR. The ADAMTS metalloproteinases. *Biochem J*. 2005;386(Pt 1):15-27.
227. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *The Journal of biological chemistry*. 2001;276(44):41059-63.
228. Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, et al. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem*. 2001;130(4):475-80.
229. Zhou W, Inada M, Lee TP, Benten D, Lyubsky S, Bouhassira EE, et al. ADAMTS13 is expressed in hepatic stellate cells. *Lab Invest*. 2005;85(6):780-8.
230. Turner N, Nolasco L, Tao Z, Dong JF, Moake J. Human endothelial cells synthesize and release ADAMTS-13. *Journal of thrombosis and haemostasis : JTH*. 2006;4(6):1396-404.
231. Shang D, Zheng XW, Niiya M, Zheng XL. Apical sorting of ADAMTS13 in vascular endothelial cells and Madin-Darby canine kidney cells depends on the CUB domains and their association with lipid rafts. *Blood*. 2006;108(7):2207-15.
232. Suzuki M, Murata M, Matsubara Y, Uchida T, Ishihara H, Shibano T, et al. Detection of von Willebrand factor-cleaving protease (ADAMTS-13) in human platelets. *Biochemical and biophysical research communications*. 2004;313(1):212-6.
233. Manea M, Kristoffersson A, Schneppenheim R, Saleem MA, Mathieson PW, Morgelin M, et al. Podocytes express ADAMTS13 in normal renal cortex and in patients with thrombotic thrombocytopenic purpura. *British journal of haematology*. 2007;138(5):651-62.
234. Apte SS. A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs: the ADAMTS family. *Int J Biochem Cell Biol*. 2004;36(6):981-5.
235. Akiyama M, Takeda S, Kokame K, Takagi J, Miyata T. Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(46):19274-9.
236. Gerhardt S, Hassall G, Hawtin P, McCall E, Flavell L, Minshall C, et al. Crystal structures of human ADAMTS-1 reveal a conserved catalytic domain and a disintegrin-like domain with a fold homologous to cysteine-rich domains. *Journal of molecular biology*. 2007;373(4):891-902.

237. Mosyak L, Georgiadis K, Shane T, Svenson K, Hebert T, McDonagh T, et al. Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5. *Protein science : a publication of the Protein Society*. 2008;17(1):16-21.
238. Shieh HS, Mathis KJ, Williams JM, Hills RL, Wiese JF, Benson TE, et al. High resolution crystal structure of the catalytic domain of ADAMTS-5 (aggrecanase-2). *The Journal of biological chemistry*. 2008;283(3):1501-7.
239. Crawley JT, de Groot R, Xiang Y, Luken BM, Lane DA. Unraveling the scissile bond: how ADAMTS13 recognizes and cleaves von Willebrand factor. *Blood*. 2011;118(12):3212-21.
240. Gomis-Ruth FX. Catalytic domain architecture of metzincin metalloproteases. *The Journal of biological chemistry*. 2009;284(23):15353-7.
241. Gardner MD, Chion CK, de Groot R, Shah A, Crawley JT, Lane DA. A functional calcium-binding site in the metalloprotease domain of ADAMTS13. *Blood*. 2009;113(5):1149-57.
242. de Groot R, Lane DA, Crawley JT. The ADAMTS13 metalloprotease domain: roles of subsites in enzyme activity and specificity. *Blood*. 2010;116(16):3064-72.
243. Bode W, Gomis-Ruth FX, Stockler W. Astacins, serralyins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett*. 1993;331(1-2):134-40.
244. Tallant C, Garcia-Castellanos R, Baumann U, Gomis-Ruth FX. On the relevance of the Met-turn methionine in metzincins. *The Journal of biological chemistry*. 2010;285(18):13951-7.
245. Anderson PJ, Kokame K, Sadler JE. Zinc and calcium ions cooperatively modulate ADAMTS13 activity. *The Journal of biological chemistry*. 2006;281(2):850-7.
246. Lancellotti S, Peyvandi F, Pagliari MT, Cairo A, Abdel-Azeim S, Chermak E, et al. The D173G mutation in ADAMTS-13 causes a severe form of congenital thrombotic thrombocytopenic purpura. A clinical, biochemical and in silico study. *Thrombosis and haemostasis*. 2016;115(1):51-62.
247. Ercig B, Wichapong K, Reutelingsperger CPM, Vanhoorelbeke K, Voorberg J, Nicolaes GAF. Insights into 3D Structure of ADAMTS13: A Stepping Stone towards Novel Therapeutic Treatment of Thrombotic Thrombocytopenic Purpura. *Thrombosis and haemostasis*. 2018;118(1):28-41.
248. Majerus EM, Anderson PJ, Sadler JE. Binding of ADAMTS13 to von Willebrand factor. *The Journal of biological chemistry*. 2005;280(23):21773-8.
249. Ai J, Smith P, Wang S, Zhang P, Zheng XL. The proximal carboxyl-terminal domains of ADAMTS13 determine substrate specificity and are all required for cleavage of von Willebrand factor. *The Journal of biological chemistry*. 2005;280(33):29428-34.
250. de Groot R, Bardhan A, Ramroop N, Lane DA, Crawley JT. Essential role of the disintegrin-like domain in ADAMTS13 function. *Blood*. 2009;113(22):5609-16.
251. Gao W, Anderson PJ, Majerus EM, Tuley EA, Sadler JE. Exosite interactions contribute to tension-induced cleavage of von Willebrand factor by the antithrombotic ADAMTS13 metalloprotease. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(50):19099-104.
252. Xiang Y, de Groot R, Crawley JT, Lane DA. Mechanism of von Willebrand factor scissile bond cleavage by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(28):11602-7.
253. Akiyama M, Nakayama D, Takeda S, Kokame K, Takagi J, Miyata T. Crystal structure and enzymatic activity of an ADAMTS-13 mutant with the East Asian-specific P475S polymorphism. *Journal of thrombosis and haemostasis : JTH*. 2013;11(7):1399-406.

254. Takeda S. Three-dimensional domain architecture of the ADAM family proteinases. *Semin Cell Dev Biol.* 2009;20(2):146-52.
255. Gao W, Anderson PJ, Sadler JE. Extensive contacts between ADAMTS13 exosites and von Willebrand factor domain A2 contribute to substrate specificity. *Blood.* 2008;112(5):1713-9.
256. Zanardelli S, Crawley JT, Chion CK, Lam JK, Preston RJ, Lane DA. ADAMTS13 substrate recognition of von Willebrand factor A2 domain. *The Journal of biological chemistry.* 2006;281(3):1555-63.
257. Assink K, Schiphorst R, Allford S, Karpman D, Etzioni A, Brichard B, et al. Mutation analysis and clinical implications of von Willebrand factor-cleaving protease deficiency. *Kidney international.* 2003;63(6):1995-9.
258. Fujimura Y, Matsumoto M, Kokame K, Isonishi A, Soejima K, Akiyama N, et al. Pregnancy-induced thrombocytopenia and TTP, and the risk of fetal death, in Upshaw-Schulman syndrome: a series of 15 pregnancies in 9 genotyped patients. *British journal of haematology.* 2009;144(5):742-54.
259. Schneppenheim R, Budde U, Oyen F, Angerhaus D, Aumann V, Drewke E, et al. von Willebrand factor cleaving protease and ADAMTS13 mutations in childhood TTP. *Blood.* 2003;101(5):1845-50.
260. Tan K, Duquette M, Liu JH, Dong Y, Zhang R, Joachimiak A, et al. Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. *J Cell Biol.* 2002;159(2):373-82.
261. Kuno K, Matsushima K. ADAMTS-1 protein anchors at the extracellular matrix through the thrombospondin type I motifs and its spacing region. *The Journal of biological chemistry.* 1998;273(22):13912-7.
262. Tortorella M, Pratta M, Liu RQ, Abbaszade I, Ross H, Burn T, et al. The thrombospondin motif of aggrecanase-1 (ADAMTS-4) is critical for aggrecan substrate recognition and cleavage. *The Journal of biological chemistry.* 2000;275(33):25791-7.
263. Soejima K, Matsumoto M, Kokame K, Yagi H, Ishizashi H, Maeda H, et al. ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. *Blood.* 2003;102(9):3232-7.
264. Zheng X, Nishio K, Majerus EM, Sadler JE. Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. *The Journal of biological chemistry.* 2003;278(32):30136-41.
265. Deforche L, Roose E, Vandenbulcke A, Vandeputte N, Feys HB, Springer TA, et al. Linker regions and flexibility around the metalloprotease domain account for conformational activation of ADAMTS13. *Journal of thrombosis and haemostasis : JTH.* 2015.
266. de Groot R, Lane DA, Crawley JT. The role of the ADAMTS13 cysteine-rich domain in VWF binding and proteolysis. *Blood.* 2015.
267. Jin SY, Skipwith CG, Zheng XL. Amino acid residues Arg(659), Arg(660), and Tyr(661) in the spacer domain of ADAMTS13 are critical for cleavage of von Willebrand factor. *Blood.* 2010;115(11):2300-10.
268. Pos W, Crawley JT, Fijnheer R, Voorberg J, Lane DA, Luken BM. An autoantibody epitope comprising residues R660, Y661, and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. *Blood.* 2010;115(8):1640-9.
269. Tao Z, Wang Y, Choi H, Bernardo A, Nishio K, Sadler JE, et al. Cleavage of ultralarge multimers of von Willebrand factor by C-terminal-truncated mutants of ADAMTS-13 under flow. *Blood.* 2005;106(1):141-3.
270. Luken BM, Turenhout EA, Hulstein JJ, Van Mourik JA, Fijnheer R, Voorberg J. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. *Thrombosis and haemostasis.* 2005;93(2):267-74.

271. Klaus C, Plaimauer B, Studt JD, Dorner F, Lammle B, Mannucci PM, et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. *Blood*. 2004;103(12):4514-9.
272. Zhou W, Dong L, Ginsburg D, Bouhassira EE, Tsai HM. Enzymatically active ADAMTS13 variants are not inhibited by anti-ADAMTS13 autoantibodies: a novel therapeutic strategy? *The Journal of biological chemistry*. 2005;280(48):39934-41.
273. Thomas MR, de Groot R, Scully MA, Crawley JT. Pathogenicity of Anti-ADAMTS13 Autoantibodies in Acquired Thrombotic Thrombocytopenic Purpura. *EBioMedicine*. 2015;2(8):942-52.
274. Zheng XL, Wu HM, Shang D, Falls E, Skipwith CG, Cataland SR, et al. Multiple domains of ADAMTS13 are targeted by autoantibodies against ADAMTS13 in patients with acquired idiopathic thrombotic thrombocytopenic purpura. *Haematologica*. 2010;95(9):1555-62.
275. Bork P, Beckmann G. The CUB domain. A widespread module in developmentally regulated proteins. *Journal of molecular biology*. 1993;231(2):539-45.
276. Bond JS, Beynon RJ. The astacin family of metalloendopeptidases. *Protein science : a publication of the Protein Society*. 1995;4(7):1247-61.
277. Gregory LA, Thielens NM, Arlaud GJ, Fontecilla-Camps JC, Gaboriaud C. X-ray structure of the Ca²⁺-binding interaction domain of C1s. Insights into the assembly of the C1 complex of complement. *The Journal of biological chemistry*. 2003;278(34):32157-64.
278. Romao MJ, Kolln I, Dias JM, Carvalho AL, Romero A, Varela PF, et al. Crystal structure of acidic seminal fluid protein (aSFP) at 1.9 Å resolution: a bovine polypeptide of the spermadhesin family. *Journal of molecular biology*. 1997;274(4):650-60.
279. Pimanda JE, Maekawa A, Wind T, Paxton J, Chesterman CN, Hogg PJ. Congenital thrombotic thrombocytopenic purpura in association with a mutation in the second CUB domain of ADAMTS13. *Blood*. 2004;103(2):627-9.
280. Sorvillo N, Kaijen PH, Matsumoto M, Fujimura Y, van der Zwaan C, Verbij FC, et al. Identification of N-linked glycosylation and putative O-fucosylation, C-mannosylation sites in plasma derived ADAMTS13. *Journal of thrombosis and haemostasis : JTH*. 2014;12(5):670-9.
281. Verbij FC, Stokhuijzen E, Kaijen PH, van Alphen F, Meijer AB, Voorberg J. Identification of glycans on plasma-derived ADAMTS13. *Blood*. 2016;128(21):e51-e8.
282. Ricketts LM, Dlugosz M, Luther KB, Haltiwanger RS, Majerus EM. O-fucosylation is required for ADAMTS13 secretion. *The Journal of biological chemistry*. 2007;282(23):17014-23.
283. Ling J, Su J, Ma Z, Ruan C. The WXXW motif in the TSR1 of ADAMTS13 is important for its secretion and proteolytic activity. *Thrombosis research*. 2013;131(6):529-34.
284. Feys HB, Liu F, Dong N, Pareyn I, Vauterin S, Vandeputte N, et al. ADAMTS-13 plasma level determination uncovers antigen absence in acquired thrombotic thrombocytopenic purpura and ethnic differences. *Journal of thrombosis and haemostasis : JTH*. 2006;4(5):955-62.
285. Soejima K, Nakamura H, Hirashima M, Morikawa W, Nozaki C, Nakagaki T. Analysis on the molecular species and concentration of circulating ADAMTS13 in Blood. *J Biochem*. 2006;139(1):147-54.
286. Rieger M, Ferrari S, Kremer Hovinga JA, Konetschny C, Herzog A, Koller L, et al. Relation between ADAMTS13 activity and ADAMTS13 antigen levels in healthy donors and patients with thrombotic microangiopathies (TMA). *Thrombosis and haemostasis*. 2006;95(2):212-20.
287. Nishio K, Anderson PJ, Zheng XL, Sadler JE. Binding of platelet glycoprotein Ib α to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(29):10578-83.

288. Shim K, Anderson PJ, Tuley EA, Wiswall E, Sadler JE. Platelet-VWF complexes are preferred substrates of ADAMTS13 under fluid shear stress. *Blood*. 2008;111(2):651-7.
289. Skipwith CG, Cao W, Zheng XL. Factor VIII and platelets synergistically accelerate cleavage of von Willebrand factor by ADAMTS13 under fluid shear stress. *The Journal of biological chemistry*. 2010;285(37):28596-603.
290. Dong JF. Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. *Journal of thrombosis and haemostasis : JTH*. 2005;3(8):1710-6.
291. Dong JF, Moake JL, Bernardo A, Fujikawa K, Ball C, Nolasco L, et al. ADAMTS-13 metalloprotease interacts with the endothelial cell-derived ultra-large von Willebrand factor. *The Journal of biological chemistry*. 2003;278(32):29633-9.
292. Chauhan AK, Motto DG, Lamb CB, Bergmeier W, Dockal M, Plaimauer B, et al. Systemic antithrombotic effects of ADAMTS13. *J Exp Med*. 2006;203(3):767-76.
293. Jin SY, Skipwith CG, Shang D, Zheng XL. von Willebrand factor cleaved from endothelial cells by ADAMTS13 remains ultralarge in size. *Journal of thrombosis and haemostasis : JTH*. 2009;7(10):1749-52.
294. Turner N, Nolasco L, Dong JF, Moake J. ADAMTS-13 cleaves long von Willebrand factor multimeric strings anchored to endothelial cells in the absence of flow, platelets or conformation-altering chemicals. *Journal of thrombosis and haemostasis : JTH*. 2009;7(1):229-32.
295. Chauhan AK, Goerge T, Schneider SW, Wagner DD. Formation of platelet strings and microthrombi in the presence of ADAMTS-13 inhibitor does not require P-selectin or beta3 integrin. *Journal of thrombosis and haemostasis : JTH*. 2007;5(3):583-9.
296. Joly BS, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. *Blood*. 2017;129(21):2836-46.
297. Shida Y, Nishio K, Sugimoto M, Mizuno T, Hamada M, Kato S, et al. Functional imaging of shear-dependent activity of ADAMTS13 in regulating mural thrombus growth under whole blood flow conditions. *Blood*. 2008;111(3):1295-8.
298. Donadelli R, Orje JN, Capoferri C, Remuzzi G, Ruggeri ZM. Size regulation of von Willebrand factor-mediated platelet thrombi by ADAMTS13 in flowing blood. *Blood*. 2006;107(5):1943-50.
299. Moschcowitz E. Hyaline thrombosis of the terminal arterioles and capillaries : A hitherto undescribed disease. *Proc N Y Patholl Soc*. 1924;24:21-4.
300. Moschcowitz E. An acute febrile pleiochromic anemia with hyaline thrombosis of the terminal arterioles and capillaries; an undescribed disease. *The American journal of medicine*. 1952;13(5):567-9.
301. AMOROSI EL, ULTMANN JE. THROMBOTIC THROMBOCYTOPENIC PURPURA: REPORT OF 16 CASES AND REVIEW OF THE LITERATURE. *Medicine*. 1966;45(2):139-60.
302. Veyradier A, Obert B, Houllier A, Meyer D, Girma JP. Specific von Willebrand factor-cleaving protease in thrombotic microangiopathies: a study of 111 cases. *Blood*. 2001;98(6):1765-72.
303. Zheng XL, Kaufman RM, Goodnough LT, Sadler JE. Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and nonidiopathic thrombotic thrombocytopenic purpura. *Blood*. 2004;103(11):4043-9.
304. Bianchi V, Robles R, Alberio L, Furlan M, Lammle B. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. *Blood*. 2002;100(2):710-3.

305. Scully M, Yarranton H, Liesner R, Cavenagh J, Hunt B, Benjamin S, et al. Regional UK TTP registry: correlation with laboratory ADAMTS 13 analysis and clinical features. *British journal of haematology*. 2008;142(5):819-26.
306. Rock GA, Shumak KH, Buskard NA, Blanchette VS, Kelton JG, Nair RC, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. *The New England journal of medicine*. 1991;325(6):393-7.
307. Scully M, Hunt BJ, Benjamin S, Liesner R, Rose P, Peyvandi F, et al. Guidelines on the diagnosis and management of thrombotic thrombocytopenic purpura and other thrombotic microangiopathies. *British journal of haematology*. 2012;158(3):323-35.
308. Schulman I, Pierce M, Lukens A, Currimbhoy Z. Studies on thrombopoiesis. I. A factor in normal human plasma required for platelet production; chronic thrombocytopenia due to its deficiency. *Blood*. 1960;16:943-57.
309. Upshaw JD, Jr. Congenital deficiency of a factor in normal plasma that reverses microangiopathic hemolysis and thrombocytopenia. *The New England journal of medicine*. 1978;298(24):1350-2.
310. Mariotte E, Azoulay E, Galicier L, Rondeau E, Zouiti F, Boisseau P, et al. Epidemiology and pathophysiology of adulthood-onset thrombotic microangiopathy with severe ADAMTS13 deficiency (thrombotic thrombocytopenic purpura): a cross-sectional analysis of the French national registry for thrombotic microangiopathy. *Lancet Haematol*. 2016;3(5):e237-45.
311. Donadelli R, Banterla F, Galbusera M, Capoferri C, Bucchioni S, Gastoldi S, et al. In-vitro and in-vivo consequences of mutations in the von Willebrand factor cleaving protease ADAMTS13 in thrombotic thrombocytopenic purpura. *Thrombosis and haemostasis*. 2006;96(4):454-64.
312. Hommais A, Rayes J, Houllier A, Obert B, Legendre P, Veyradier A, et al. Molecular characterization of four ADAMTS13 mutations responsible for congenital thrombotic thrombocytopenic purpura (Upshaw-Schulman syndrome). *Thrombosis and haemostasis*. 2007;98(3):593-9.
313. Lotta LA, Garagiola I, Palla R, Cairo A, Peyvandi F. ADAMTS13 mutations and polymorphisms in congenital thrombotic thrombocytopenic purpura. *Hum Mutat*. 2010;31(1):11-9.
314. Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, et al. Molecular characterization of ADAMTS13 gene mutations in Japanese patients with Upshaw-Schulman syndrome. *Blood*. 2004;103(4):1305-10.
315. Peyvandi F, Lavoretano S, Palla R, Valsecchi C, Merati G, De Cristofaro R, et al. Mechanisms of the interaction between two ADAMTS13 gene mutations leading to severe deficiency of enzymatic activity. *Hum Mutat*. 2006;27(4):330-6.
316. Hing ZA, Schiller T, Wu A, Hamasaki-Katagiri N, Struble EB, Russek-Cohen E, et al. Multiple in silico tools predict phenotypic manifestations in congenital thrombotic thrombocytopenic purpura. *British journal of haematology*. 2013;160(6):825-37.
317. Scully M, Knobl P, Kentouche K, Rice L, Windyga J, Schneppenheim R, et al. Recombinant ADAMTS-13: first-in-human pharmacokinetics and safety in congenital thrombotic thrombocytopenic purpura. *Blood*. 2017;130(19):2055-63.
318. Pos W, Sorvillo N, Fijnheer R, Feys HB, Kaijen PH, Vidarsson G, et al. Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain. *Haematologica*. 2011;96(11):1670-7.
319. Fujimura Y, Matsumoto M. Registry of 919 patients with thrombotic microangiopathies across Japan: database of Nara Medical University during 1998-2008. *Intern Med*. 2010;49(1):7-15.

320. Reese JA, Muthurajah DS, Kremer Hovinga JA, Vesely SK, Terrell DR, George JN. Children and adults with thrombotic thrombocytopenic purpura associated with severe, acquired Adamts13 deficiency: comparison of incidence, demographic and clinical features. *Pediatr Blood Cancer*. 2013;60(10):1676-82.
321. Jansson JH, Nilsson TK, Johnson O. von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Heart J*. 1991;66(5):351-5.
322. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *The New England journal of medicine*. 1995;332(10):635-41.
323. Whincup PH, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, et al. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Heart J*. 2002;23(22):1764-70.
324. Yoo G, Blomback M, Schenck-Gustafsson K, He S. Decreased levels of von Willebrand factor-cleaving protease in coronary heart disease and thrombotic thrombocytopenic purpura: study of a simplified method for assaying the enzyme activity based on ristocetin-induced platelet aggregation. *British journal of haematology*. 2003;121(1):123-9.
325. Fuchigami S, Kaikita K, Soejima K, Matsukawa M, Honda T, Tsujita K, et al. Changes in plasma von Willebrand factor-cleaving protease (ADAMTS13) levels in patients with unstable angina. *Thrombosis research*. 2008;122(5):618-23.
326. Kaikita K, Soejima K, Matsukawa M, Nakagaki T, Ogawa H. Reduced von Willebrand factor-cleaving protease (ADAMTS13) activity in acute myocardial infarction. *Journal of thrombosis and haemostasis : JTH*. 2006;4(11):2490-3.
327. Crawley JT, Lane DA, Woodward M, Rumley A, Lowe GD. Evidence that high von Willebrand factor and low ADAMTS-13 levels independently increase the risk of a non-fatal heart attack. *Journal of thrombosis and haemostasis : JTH*. 2008;6(4):583-8.
328. Matsukawa M, Kaikita K, Soejima K, Fuchigami S, Nakamura Y, Honda T, et al. Serial changes in von Willebrand factor-cleaving protease (ADAMTS13) and prognosis after acute myocardial infarction. *Am J Cardiol*. 2007;100(5):758-63.
329. Bongers TN, de Bruijne EL, Dippel DW, de Jong AJ, Deckers JW, Poldermans D, et al. Lower levels of ADAMTS13 are associated with cardiovascular disease in young patients. *Atherosclerosis*. 2009;207(1):250-4.
330. Chion CK, Doggen CJ, Crawley JT, Lane DA, Rosendaal FR. ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men. *Blood*. 2007;109(5):1998-2000.
331. Peyvandi F, Hollestelle MJ, Palla R, Merlini PA, Feys HB, Vanhoorelbeke K, et al. Active platelet-binding conformation of plasma von Willebrand factor in young women with acute myocardial infarction. *Journal of thrombosis and haemostasis : JTH*. 2010;8(7):1653-6.
332. Tanis B, Algra A, van der Graaf Y, Helmerhorst F, Rosendaal F. Procoagulant factors and the risk of myocardial infarction in young women. *European journal of haematology*. 2006;77(1):67-73.
333. Andersson HM, Siegerink B, Luken BM, Crawley JT, Algra A, Lane DA, et al. High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. *Blood*. 2012;119(6):1555-60.
334. Bongers TN, de Maat MP, van Goor ML, Bhagwanbali V, van Vliet HH, Gomez Garcia EB, et al. High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. *Stroke*. 2006;37(11):2672-7.
335. Wieberdink RG, van Schie MC, Koudstaal PJ, Hofman A, Witteman JC, de Maat MP, et al. High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke*. 2010;41(10):2151-6.

336. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, et al. Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Circulation*. 1999;100(7):736-42.
337. Hanson E, Jood K, Karlsson S, Nilsson S, Blomstrand C, Jern C. Plasma levels of von Willebrand factor in the etiologic subtypes of ischemic stroke. *Journal of thrombosis and haemostasis : JTH*. 2011;9(2):275-81.
338. van Schie MC, MP DEM, Dippel DW, de Groot PG, Lenting PJ, Leebeek FW, et al. von Willebrand factor propeptide and the occurrence of a first ischemic stroke. *Journal of thrombosis and haemostasis : JTH*. 2010;8(6):1424-6.
339. Denorme F, Kraft P, Pareyn I, Drechsler C, Deckmyn H, Vanhoorelbeke K, et al. Reduced ADAMTS13 levels in patients with acute and chronic cerebrovascular disease. *PloS one*. 2017;12(6):e0179258.
340. Sonneveld MA, Franco OH, Ikram MA, Hofman A, Kavousi M, de Maat MP, et al. Von Willebrand Factor, ADAMTS13, and the Risk of Mortality: The Rotterdam Study. *Arterioscler Thromb Vasc Biol*. 2016;36(12):2446-51.
341. Maino A, Siegerink B, Lotta LA, Crawley JT, le Cessie S, Leebeek FW, et al. Plasma ADAMTS-13 levels and the risk of myocardial infarction: an individual patient data meta-analysis. *Journal of thrombosis and haemostasis : JTH*. 2015;13(8):1396-404.
342. Sonneveld MA, Kavousi M, Ikram MA, Hofman A, Rueda Ochoa OL, Turecek PL, et al. Low ADAMTS-13 activity and the risk of coronary heart disease - a prospective cohort study: the Rotterdam Study. *Journal of thrombosis and haemostasis : JTH*. 2016;14(11):2114-20.
343. Green D, Tian L, Greenland P, Liu K, Kibbe M, Tracy R, et al. Association of the von Willebrand Factor-ADAMTS13 Ratio With Incident Cardiovascular Events in Patients With Peripheral Arterial Disease. *Clin Appl Thromb Hemost*. 2017;23(7):807-13.
344. Denorme F, Langhauser F, Desender L, Vandenbulcke A, Rottensteiner H, Plaimauer B, et al. ADAMTS13-mediated thrombolysis of t-PA-resistant occlusions in ischemic stroke in mice. *Blood*. 2016;127(19):2337-45.
345. Zhao BQ, Chauhan AK, Canault M, Patten IS, Yang JJ, Dockal M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood*. 2009;114(15):3329-34.
346. Gandhi C, Motto DG, Jensen M, Lentz SR, Chauhan AK. ADAMTS13 deficiency exacerbates VWF-dependent acute myocardial ischemia/reperfusion injury in mice. *Blood*. 2012;120(26):5224-30.
347. De Meyer SF, Savchenko AS, Haas MS, Schatzberg D, Carroll MC, Schiviz A, et al. Protective anti-inflammatory effect of ADAMTS13 on myocardial ischemia/reperfusion injury in mice. *Blood*. 2012;120(26):5217-23.
348. Nakano T, Irie K, Hayakawa K, Sano K, Nakamura Y, Tanaka M, et al. Delayed treatment with ADAMTS13 ameliorates cerebral ischemic injury without hemorrhagic complication. *Brain Res*. 2015;1624:330-5.
349. Sonneveld MA, van Dijk AC, van den Herik EG, van Loon JE, de Lau LM, van der Lugt A, et al. Relationship of Von Willebrand Factor with carotid artery and aortic arch calcification in ischemic stroke patients. *Atherosclerosis*. 2013;230(2):210-5.
350. Gandhi C, Ahmad A, Wilson KM, Chauhan AK. ADAMTS13 modulates atherosclerotic plaque progression in mice via a VWF-dependent mechanism. *Journal of thrombosis and haemostasis : JTH*. 2014;12(2):255-60.
351. Gandhi C, Khan MM, Lentz SR, Chauhan AK. ADAMTS13 reduces vascular inflammation and the development of early atherosclerosis in mice. *Blood*. 2012;119(10):2385-91.

352. Jin SY, Tohyama J, Bauer RC, Cao NN, Rader DJ, Zheng XL. Genetic ablation of Adamts13 gene dramatically accelerates the formation of early atherosclerosis in a murine model. *Arterioscler Thromb Vasc Biol.* 2012;32(8):1817-23.
353. Hassenpflug WA, Budde U, Obser T, Angerhaus D, Drewke E, Schneppenheim S, et al. Impact of mutations in the von Willebrand factor A2 domain on ADAMTS13-dependent proteolysis. *Blood.* 2006;107(6):2339-45.
354. Sutherland JJ, O'Brien LA, Lillicrap D, Weaver DF. Molecular modeling of the von Willebrand factor A2 Domain and the effects of associated type 2A von Willebrand disease mutations. *J Mol Model.* 2004;10(4):259-70.
355. Interlandi G, Ling M, Tu AY, Chung DW, Thomas WE. Structural basis of type 2A von Willebrand disease investigated by molecular dynamics simulations and experiments. *PloS one.* 2012;7(10):e45207.
356. Aponte-Santamaria C, Lippok S, Mittag JJ, Obser T, Schneppenheim R, Baldauf C, et al. Mutation G1629E Increases von Willebrand Factor Cleavage via a Cooperative Destabilization Mechanism. *Biophys J.* 2017;112(1):57-65.
357. Xu AJ, Springer TA. Mechanisms by which von Willebrand disease mutations destabilize the A2 domain. *The Journal of biological chemistry.* 2013;288(9):6317-24.
358. Lynch CJ, Cawte AD, Millar CM, Rueda D, Lane DA. A common mechanism by which type 2A von Willebrand disease mutations enhance ADAMTS13 proteolysis revealed with a von Willebrand factor A2 domain FRET construct. *PloS one.* 2017;12(11):e0188405.
359. Luken BM, Turenhout EA, Kaijen PH, Greuter MJ, Pos W, van Mourik JA, et al. Amino acid regions 572-579 and 657-666 of the spacer domain of ADAMTS13 provide a common antigenic core required for binding of antibodies in patients with acquired TTP. *Thrombosis and haemostasis.* 2006;96(3):295-301.
360. Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. *Blood.* 2004;103(2):607-12.
361. Wu JJ, Fujikawa K, McMullen BA, Chung DW. Characterization of a core binding site for ADAMTS-13 in the A2 domain of von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103(49):18470-4.
362. Pruss CM, Notley CR, Hegadorn CA, O'Brien LA, Lillicrap D. ADAMTS13 cleavage efficiency is altered by mutagenic and, to a lesser extent, polymorphic sequence changes in the A1 and A2 domains of von Willebrand factor. *British journal of haematology.* 2008;143(4):552-8.
363. Kretz CA, Dai M, Soylemez O, Yee A, Desch KC, Siemieniak D, et al. Massively parallel enzyme kinetics reveals the substrate recognition landscape of the metalloprotease ADAMTS13. *Proceedings of the National Academy of Sciences of the United States of America.* 2015;112(30):9328-33.
364. Desch KC, Kretz C, Yee A, Gildersleeve R, Metzger K, Agrawal N, et al. Probing ADAMTS13 Substrate Specificity using Phage Display. *PloS one.* 2015;10(4):e0122931.
365. Kelwick R, Desanlis I, Wheeler GN, Edwards DR. The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) family. *Genome biology.* 2015;16:113.
366. Majerus EM, Zheng X, Tuley EA, Sadler JE. Cleavage of the ADAMTS13 propeptide is not required for protease activity. *The Journal of biological chemistry.* 2003;278(47):46643-8.
367. Furlan M, Robles R, Morselli B, Sandoz P, Lammle B. Recovery and half-life of von Willebrand factor-cleaving protease after plasma therapy in patients with thrombotic thrombocytopenic purpura. *Thrombosis and haemostasis.* 1999;81(1):8-13.
368. Guo C, Tsigkou A, Lee MH. ADAMTS13 and 15 are not regulated by the full length and N-terminal domain forms of TIMP-1, -2, -3 and -4. *Biomed Rep.* 2016;4(1):73-8.

369. Iwaki M, Iwane AH, Ikebe M, Yanagida T. Biased Brownian motion mechanism for processivity and directionality of single-headed myosin-VI. *Biosystems*. 2008;93(1-2):39-47.
370. Whitelock JL, Nolasco L, Bernardo A, Moake J, Dong JF, Cruz MA. ADAMTS-13 activity in plasma is rapidly measured by a new ELISA method that uses recombinant VWF-A2 domain as substrate. *Journal of thrombosis and haemostasis : JTH*. 2004;2(3):485-91.
371. Sadler JE, Moake JL, Miyata T, George JN. Recent advances in thrombotic thrombocytopenic purpura. *Hematology Am Soc Hematol Educ Program*. 2004:407-23.
372. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETTS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *British journal of haematology*. 2005;129(1):93-100.
373. Di Stasio E, Lancellotti S, Peyvandi F, Palla R, Mannucci PM, De Cristofaro R. Mechanistic studies on ADAMTS13 catalysis. *Biophys J*. 2008;95(5):2450-61.
374. Kremer Hovinga JA, Coppo P, Lämmle B, Moake JL, Miyata T, Vanhoorelbeke K. Thrombotic thrombocytopenic purpura. *Nature Reviews Disease Primers*. 2017;3:17020.
375. Jian C, Xiao J, Gong L, Skipwith CG, Jin SY, Kwaan HC, et al. Gain-of-function ADAMTS13 variants that are resistant to autoantibodies against ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura. *Blood*. 2012;119(16):3836-43.
376. South K, Lane DA. ADAMTS-13 and von Willebrand factor: a dynamic duo. *Journal of thrombosis and haemostasis : JTH*. 2018;16(1):6-18.

APPENDIX I

Table A PCR primers

Purpose	Forward primer	Reverse primer
VWF96 PCR amp	GGCAACAGGACCAACACTGGG	TCACCTCTGCAGCACCAGGTCAGG
VWF96-XhoI	TAATACGACTCACTATAGGG	TATACTCGAGCCTCTGCAGCACCAGGTCAG
VWF87-XhoI	TAATACGACTCACTATAGGG	TATACTCGAGTCGGGGAGCCTCTCAAA
VWF57-XhoI	TAATACGACTCACTATAGGG	TATACTCGAGTCCAATGGGCACCACCTG
VWF47-XhoI	TAATACGACTCACTATAGGG	TATACTCGAGCAGCCTCTTGATCTCATCAGAGG
PCR screening	TAATACGACTCACTATAGGG	TAGTTATTGCTCAGCGGTGG
Inverse PCR		
VWF96-MP	AATGTCTACATGGTCACCGGAAATCC	GTTGGGCGCCTGCTCCCGG
VWF96-Dis1	CAGCAGCAGAGGCTGCCTGGAGACATCCAG	ATTAGAGGAAGGATTTCCGGTGAC
VWF96-Dis2	GAGCTGCCTGGAACCATCCAGGTGGTGCCCATTGGAG	CTCCTGCTCAGCAGAGGCAGGATTTCCGGTGACC
VWF96-Cys	TCCCCTCAGCAGCAGCAGGACTTTGAGACGCTCCCC	ATTGGGATAGCCCTGCCTCTCCAGCTC
VWF96-Spacer	AATCAGCAGAGGCTCGAGATCAAACG	CGTGTCAAGAGCCTCTCGGGG
Sequencing	TAATACGACTCACTATAGGG	TAGTTATTGCTCAGCGGTGG

APPENDIX II

Table B Permissions

Page No.	Type of work:	Name of work	Source of work	Copyright holder and contact	Permission requested on	I have permission yes /no	Permission note
33	Figure	Image 1 Densitogram of normal von Willebrand factor (vWF) bands in low resolution (1%) gel. Small, 1-5; intermediate, 6-13 _{SEP} and large, >13. The maximum is 25 multimers (top).	Analysis of von Willebrand Factor Multimers by Simultaneous High- and Low-Resolution Vertical SDS-Agarose Gel Electrophoresis and Cy5-Labeled Antibody High-Sensitivity Fluorescence Detection Ott <i>et al</i> , <i>Am J Clin Pathol</i> 2010;133:322-330	Copyright © 2010, Oxford University Press https://academic.oup.com/journals/pages/access_purchase/rights_and_permissions	22.10.2018	Yes	Thesis/Disser tation
120	Figure	Figure 3C	Massively parallel enzyme kinetics reveals the substrate recognition landscape of the metalloprotease ADAMTS13 Kretz <i>et al</i> , <i>Proc Natl Acad Sci U S A</i> . 2015 Jul 28;112(30):9328-33	Copyright © 2015 National Academy of Sciences http://www.pnas.org/page/about/rights-permissions	22.10.2018	Yes	No permission required by publisher for non-commercial purposes. Written permission also given by first author (via email).

**OXFORD UNIVERSITY PRESS LICENSE
TERMS AND CONDITIONS**

Oct 22, 2018

This Agreement between Anastasis Petri ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4454340135395
License date	Oct 22, 2018
Licensed content publisher	Oxford University Press
Licensed content publication	American Journal of Clinical Pathology
Licensed content title	Analysis of von Willebrand Factor Multimers by Simultaneous High- and Low-Resolution Vertical SDS-Agarose Gel Electrophoresis and Cy5-Labeled Antibody High-Sensitivity Fluorescence Detection
Licensed content author	Ott, Helmut W.; Griesmacher, Andrea
Licensed content date	Jan 2, 2010
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	The allosteric activation of ADAMTS13
Publisher of your work	Imperial College London
Expected publication date	Mar 2019
Permissions cost	0.00 GBP
Value added tax	0.00 GBP
Total	0.00 GBP
Title	The allosteric activation of ADAMTS13
Institution name	Imperial College London
Expected presentation date	Mar 2019
Portions	Image 1
Requestor Location	Anastasis Petri 5.S2 Commonwealth Building, Hammersmith Hospital Du Cane Road London, other United Kingdom Attn:
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	Anastasis Petri 5.S2 Commonwealth Building, Hammersmith Hospital Du Cane Road London, United Kingdom Attn: Anastasis Petri
Total	0.00 GBP
Terms and Conditions	

**STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL
FROM AN OXFORD UNIVERSITY PRESS JOURNAL**

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.

6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org. Should there be a problem clearing these rights, please contact journals.permissions@oup.com

7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.

8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.

9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.

10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

12. Other Terms and Conditions:

v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Requesting Permission

Anyone may, without requesting permission, use original figures or tables published in PNAS for noncommercial and educational use (i.e., in a review article, in a book that is not for sale), provided that the full journal reference is cited and, for articles published in volumes 90–105 (1993–2008), "Copyright (copyright year) National Academy of Sciences." Commercial reuse of figures and tables (i.e., in promotional materials, in a textbook for sale) requires permission from PNAS.

Text and data mining are permitted for noncommercial institutions with an active institutional site license to PNAS for internal noncommercial research purposes. Other requests should be sent to PNASpermissions@nas.edu.

PNAS authors need not obtain permission for the following cases:

1. to use their original figures or tables in their future works;
2. to make copies of their articles for their own personal use, including classroom use, or for the personal use of colleagues, provided those copies are not for sale and are not distributed in a systematic way;
3. to include their articles as part of their dissertations; or
4. to use all or part of their articles in printed compilations of their own works. The full journal reference must be cited and, for articles published in volumes 90–105 (1993–2008), "Copyright (copyright year) National Academy of Sciences."

For permission to reuse material in volumes 1–89 (1915–1992), requests should be sent to the original authors, who hold the copyright. The full journal reference must be cited.

For permission to reuse material in volumes 90–114 (1993–2017), requests should be sent to PNASpermissions@nas.edu and must include the following information:

1. Your full name, affiliation, and title
 2. Your complete mailing address, phone number, and email
 3. PNAS volume number, issue number, and issue date
 4. PNAS article title
 5. PNAS authors' names
-