Supplemental data

Supplemental Methods

Solid-phase binding assay: Human $\alpha_{IIb}\beta_3$ (Enzyme Research Laboratories) was coated directly onto a 96-well plate (Thermo Fischer Scientific) at 10 µg/ml in carbonate buffer, pH 9.6, and incubated overnight at 4 °C. Blocking and activation was achieved by incubation in 50 mM Tris pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.0035 % Triton X100, 3 % BSA for 1 h at 37 °C. C4 variants were biotinylated by mixing 100 µl of 100 µM C4 in PBS with 20 µl of biotin reagent (Thermo Scientific, 2.7 mg of NHS-biotin dissolved in 500 µl DMSO), incubated on ice for 2 h, before dialyzing against 20 mM HEPES pH 7.4, NaCl 100 mM for 24 h. Concentration series of 0.078 to 5 µM was obtained by diluting biotinylated C4 variants in Reaction Buffer (50 mM Tris pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.0035 % Triton X100, 1 % BSA), and incubated for 2 h at 37 °C in $\alpha_{IIb}\beta_3$-coated wells. The wells were subsequently washed with Reaction Buffer for 3 times 5 min. Binding was probed by incubation with streptavidin horseradish peroxidase conjugate (R&D Systems, 1:2000 dilution in Reaction Buffer) for 20 min at room temperature, before washing with Reaction Buffer for 3 times 5 min. Tetramethylbenzidine substrate kit (Thermo Fisher Scientific) was used to develop the assay, and when the color start to appear after a few minutes, the reaction was quenched by addition of 2 M H_2SO_4. Absorbance at 450 and 570 nm were measured by the Power Wave x340 (Bio-Tek Instruments). Experiments were performed in quadruplets.

Thermal stability assay: Nano differential scanning fluorimetry (nanoDSF) technology was employed to measure the unfolding temperatures of the C4 domains using a Prometheus NT.48 machine (NanoTemper Technologies). C4 samples of wt and Y2561 variant at both concentrations

of 1 mg/ml and 0.5 mg/ml in 20 mM Tris pH7.5, 150 mM NaCl were measured, and the ratio of tryptophan emission at 330 and 350 nm yields the protein unfolding curve. The unfolding temperature (T_m) can be calculated from the inflection point of the unfolding curve.

Docking of C4 domain with $\alpha_{IIb}\beta_3$ Integrin: An ensemble of 400 C4 conformations was assembled by taking snapshots every 5 ns from the 20 replicates. This was docked into the crystal structure of $\alpha_{IIb}\beta_3$ Integrin 2VDQ[1] using the software HADDOCK2.2[2] with default parameters, except as noted in the Supplementary Table 2 containing summary statistics and ambiguous interaction restraint definitions.

Supplementary Table 1. C_β chemical shifts of Cysteines involved in disulfde bridges

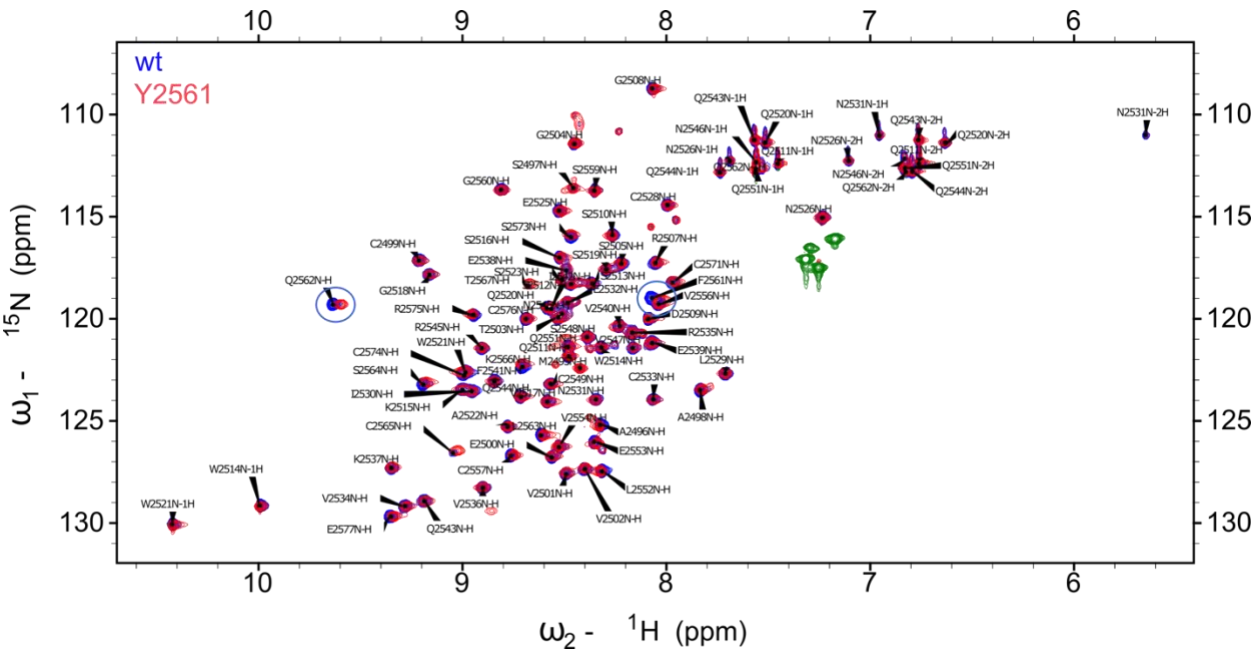
S-S bridge	Cysteine residue	C_β chemical shift (ppm)*
1	C2499	41.76
	C2533	39.31
2	C2528	37.55
	C2570	39.22
C4	C2565	47.20
	C2574	48.58
4	C2549	40.11
	C2571	40.36
V	C2557	36.29
	C2576	40.21

*Mean chemical shift values for C_β of disulphide bonded cysteines are 40.7 ± 3.8 ppm and 28.3 ±2.2 ppm for reduced cysteines[26]. Thus, all disulfide bonds are formed in the NMR sample environment.

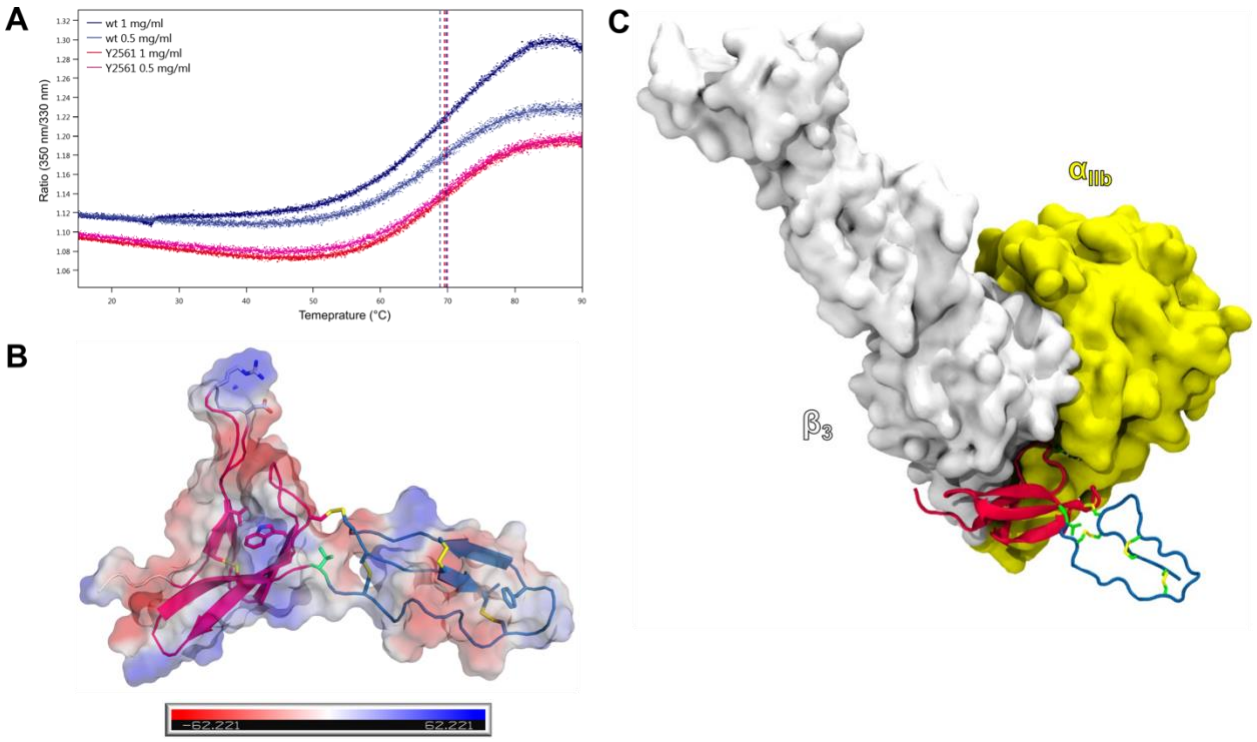
Supplementary Table 2. Summary of modified HADDOCK parameters and results.

	Parameters
Number of integrin conformations	2
Number of C4 conformations	400
Number of rigid-body complexes	6000

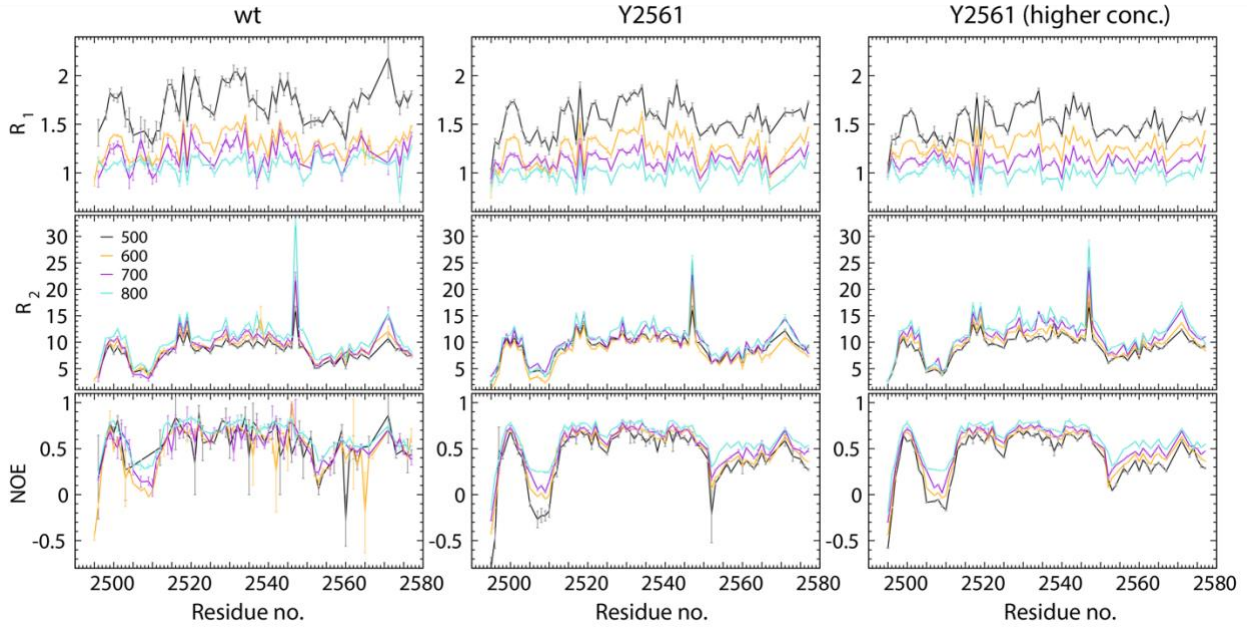
Number chosen for refinement and analysis	200
Initial seed	1231
Ambiguous Interaction Restraints	
$\alpha_{IIb}\beta_3$ Integrin	Active α_{IIb}: 160,189-190,192,224-226,231-232 Active β_3: 121-124,213-218,220 Passive α_{IIb}: 156,159,161-162,191,223,227-230,262 Passive β_3: 125-127,179-180,182,212,219,251-253
vWF C4	Active (RGD-motif): 2507-2509 Passive: 2505-2506, 2510-2511
Summary Results	
Cluster size distribution with >3 members according to fraction of common contacts at 5 Å	(Threshold 0.5): 142,10, 6, 5 (Threshold 0.7): 77,8,7,6,6,5,4,4,4 (Threshold 0.9): 16,11
HADDOCK ranking of the lowest energy member in the largest cluster	(Threshold 0.5): 3 (Threshold 0.7): 4 (depicted) (Threshold 0.9): 9



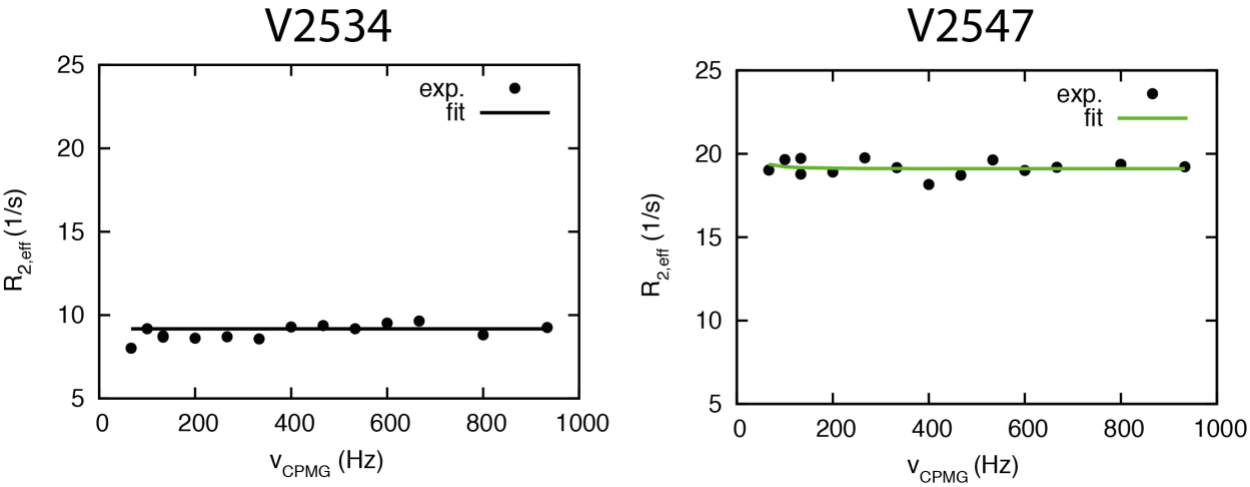
Supplementary Figure 1. Backbone resonance assignment of the VWF C4 domain. ^{1}H-^{15}N-HSQC of the VWF C4 domain (blue) with peak labels corresponding to their positions in the full length VWF sequence (UniProt P04275). ^{1}H-^{15}N-HSQC spectrum overlay from variant Y2561 (red), with blue circles highlighting chemical shift perturbations of residues Y2561 and Q2562, compared with residues F2561 and Q2562 in wt.



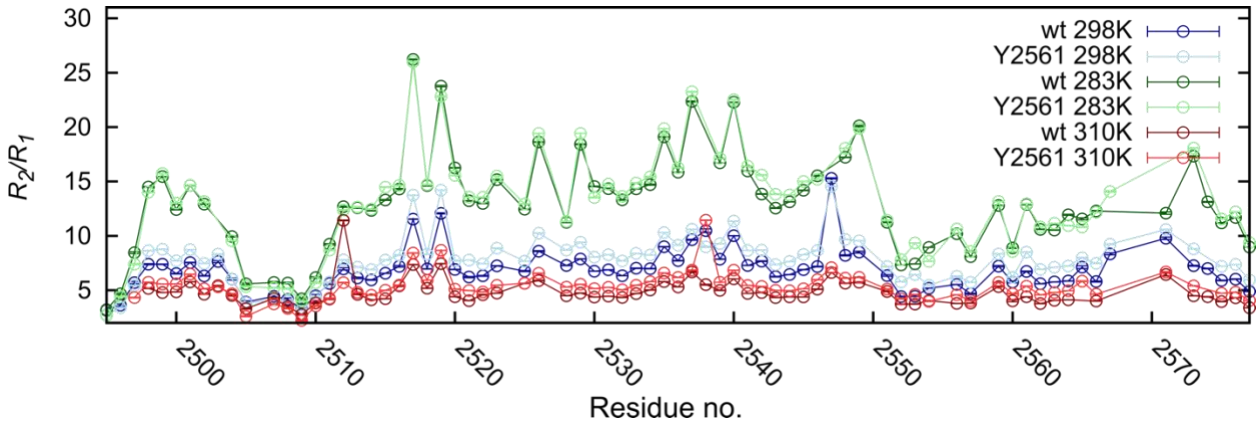
Supplementary Figure 2. Thermal stability, surface charge of C4 structure, and platelet integrin binding model. (A) Thermal stability of wt and Y2561 measured by nanoDSF. The unfolding temperatures are indicated by dashed lines at the inflection points (69.9 °C for wt 1 mg/ml; 69.1 °C for wt 0.5 mg/ml; 69.6 °C for Y2561 1 mg/ml; 70 °C for Y2561 0.5 mg/ml). (B) Surface representation of the C4 structure, colored according to the electrostatic potentials indicated by the color-code bar at the bottom. (C) Model of C4 binding to platelet integrin $\alpha_{IIb}\beta_3$ by docking the C4 structure onto the fibrinogen bound $\alpha_{IIb}\beta_3$ (PDB 2VDQ). $\alpha_{IIb}\beta_3$ is shown as surface representation and C4 as ribbon diagram.



Supplementary Figure 3. ^{15}N relaxation data of the wt and Y2561 variant VWF C4 domains at four different frequencies: black, 500 MHz; magenta, 600 MHz; yellow, 700 MHz; cyan, 800 MHz.



Supplementary Figure 4. CPMG relaxation dispersion of wt VWF C4 domain. Residues with no exchange contributions to R_2 (left, e.g. V2534) exhibit flat dispersion profiles in CPMG experiments. V2547 with high exchange contributions exhibit flat profiles but retaining a high $R_{2,eff}$, demonstrating that dynamics for this residue are not in the CPMG (ms) time scale but faster.



Supplementary Figure 5. ^{15}N relaxation of wt (strong colors) and Y2561 (pale colors) VWF C4 domain at three different temperatures: green, 283 K; blue, 298 K; red, 310 K. The data were obtained at 600 MHz.

References

1. Springer TA, Zhu J, Xiao T. Structural basis for distinctive recognition of fibrinogen γC peptide by the platelet integrin αIIbβ3. *J. Cell Biol.* 2008;182(4):791–800.

2. Dominguez C, Boelens R, Bonvin AMJJ. HADDOCK: A Protein−Protein Docking Approach Based on Biochemical or Biophysical Information. *J Am Chem Soc.* 2003;125(7):1731–1737.