Supplemental Material

Supplemental Materials and Methods

Fibrinogen, Fibrinogen Fragments, and mAbs

Human fibrinogen was obtained from Enzyme Research Labs (South Bend, IN) and fibrinogen fragments D100 and 'D98' were obtained from Haematologic Technologies Inc. (Essex Junction, VT). The former was made by treating fibrinogen in 10 mM sodium citrate, 10 mM $NaPO_4$ with a combination of glu-plasminogen at 1/1000th the concentration of fibrinogen and streptokinase (2 U/mg) at 37°C for 1 hour. After the digest was passed through a benzamidine Sepharose column, the plasmin was inhibited with EGR-chloromethyl ketone and fragment D separated from fragment E on a cation exchange column. SDS-PAGE analysis under non-reducing conditions identified a major band at 128 kD and a minor band at 113 kD. After reduction, the dominant γ-chain band had an Mr of of 42.6 kD and the dominant β-chain band had an Mr of 40.6 kD. Analysis by nanoflow liquid chromatography coupled to mass spectrometry (LC-MS/MS; see below) indicated that this fragment had the same γ-chain N- and C-termini as the γ_{1A} γ-chain of the D100 fragment reported by Podolnikova et al.[1,2] (γ-63 and γ-411, respectively), and thus we refer to this fragment as D100.

Fragment 'D98' was prepared by a modification of the procedure of Podolnikova et al.,[1] which referenced Ugarova and Budzynski[3] and Lishko et al.[2] They prepared D98 from fibrinogen by two sequential plasmin digestions: the first to make D100 (fibrinogen in 0.1 M NaCl, 0.05 M Tris/HCl, 1 mM NaN_3, 5 mM $CaCl_2$, 7 μM merthiolate, pH 7,4; 13 U human plasmin added per mg fibrinogen; fibrinogen digested at 37°C for 24 hours),[3] and the second to make D98 [D100 digested with plasmin at a 100:1 ratio (w/w) for 20 hours at 22°C].[2] In our preparation, fibrinogen was digested as indicated for the first digestion noted above[3] and the digestion was monitored by

reaction with mAb 7E9, which reacts with the C-terminus of mouse[4] and human fibrinogen. mAb 7E9 reacted with fibrinogen and the D100 fragment prepared as indicated above, but did not react with the fibrinogen D fragment prepared as per the first digestion reported by Ugarova and Budzynski[3] to produce D100. This fragment was further purified by Sephacryl S-200 and CM-50 Sephadex chromatography. SDS-PAGE analysis of the fragment under non-reducing conditions revealed major bands at Mr 123 and 115 kD. After reduction, the β-chain was found to have the same Mr as in fragment D100 (40.6 kD) and the γ-chain was represented by a dominant band that migrated at an Mr of 39 kD. LC-MS/MS demonstrated that the most N-terminal peptides identified by propionic anhydride labelling followed by trypsin digestion began at γ-88 or γ-89, and the labeling pattern indicated that the γ-89 residue had a free N-terminus. In addition, the γ-76-85 peptide observed in D100 was markedly reduced. Lishko et al. reported that the N-terminus of D98 was γ-86.[2] Since our digestion strategy was not designed to identify peptides beginning at γ-86, it is possible that some peptides had N-termini between γ-86 and γ-88, and we cannot exclude the presence of γ-chain peptides with N-termini between γ-76 and γ-86. As judged by two different digestion strategies, the predominant C-terminus was at γ-405 (Table S2). Lishko et al. reported C-termini for D98 at γ-396 and γ-404/γ-405, but they didn't specify the percentages.[2] Since for the purposes of our study loss of an intact C-terminal γ-12 peptide is the hallmark feature of D98,[2] while still recognizing that our fragment appears to differ modestly from that described by Podolnikova et al. and Lishko,[1,2] we have designated this fragment 'D98.'

The mAbs 10E5 (anti-αIIbβ3),[5] 7E3 (anti-αIIbβ3 + αVβ3),[6] and 7E9 (anti-γ-12)[4] were produced at the National Cell Culture Center (Minneapolis, MN). The anti-Ligand Induced Binding Site (LIBS) mAb AP5[7] was generously provided by Dr. Peter Newman (Blood Center of Wisconsin), and the anti-αVβ3 mAb LM609[8,9] was generously provided by Dr. David Cheresh (University of California at San Diego). Alexa488 labeling of 10E5, 7E3, and AP5 was carried out according to the manufacturer's instruction. Alexa488-fibrinogen was obtained from Invitrogen.

Molecular modeling of the αIIbβ3 headpiece-fibrinogen γ-module complex

We used the crystal structures of the αIIbβ3 γ-12 complex (PDB ID:2VDO) and the fibrinogen γ-module (PDB ID: 1FIC) to build a three-dimensional molecular model of the complex formed by the C-terminal γ-module of fibrinogen and αIIbβ3 head domain. Specifically, atomic coordinates of chain A αIIb residues 1-450 and chain B β3 residues 109-352 were taken from the crystal structure of aIIbβ3 bound to the fibrinogen γ-12 peptide (PDB ID: 2VDO). Only the last 7 residues of the bound fibrinogen dodecapeptide (chain C and residues 405-411) were retained from this crystal structure to ensure that the γ-chain peptide domain would bind as expected at the RGD-binding site. The rest of the ligand coordinates were taken from chain A residues 144-392 of the crystal structure of the carboxyl terminal fragment of the human fibrinogen γ-chain (PDB ID: 1FIC). Since the region made up of residues 393-403 is flexible as judged by its high B factor in the crystal structure, and since we employed rigid protein-protein docking algorithms, we removed this region from the structure so that it would not interfere with the docking.

Molecular docking between the αIIbβ3 head domain in the presence of the last 7 residues of the bound dodecapeptide (γ-405-411) and the fibrinogen C-terminal γ-module (γ-144-392) was carried out using three different protein-protein docking web servers: ClusPro,[10] Haddock,[11] and SwarmDock.[12] Three different sets of restraints were used to guide the docking by each of the programs, yielding a total of 9 separate docking experiments (See Supplemental Table 1 and Supplemental Figure 1). For all three, we used γ-316-322 and γ-370-381 since these residues were suggested by Remijn et al. and Podolnikova et al. to be involved in the interaction between the fibrinogen γ-module and αIIbβ3.[13,14] The αIIb residues used as restraints in the first series of dockings were based on those suggested by the studies of Kamata et al. and Podolnikova et al.[15,16] No β3 restraints were used in this first series. In the second series, to broaden the search,

we incorporated all of the αIIb and β3 head piece residues that lie on the surface of the receptor where the γ-12 exits from the RGD pocket. In the third series, we combined the experimental evidence that mAb 10E5 inhibits fibrinogen binding to αIIbβ3[5,17] with its solved crystal structure in complex with the αIIbβ3 headpiece,[18] and included as restraints the αIIbβ surface residues (i.e., those with solvent exposed surface area equal or larger than 4 Å^2) within 12 Å from 10E5 in the PDB 2VDO crystal structure. Similarly, we retained only those αIIbβ3 head piece-fibrinogen γ-module docked structures that showed at least partial overlap between the fibrinogen and the location of 10E5. Specifically, for each fibrinogen residue within segments 316-322 and 370-381, heavy atom distances between these residues and αIIbβ3 residues near the 10E5 binding site were computed. A contact was considered formed when the heavy atom distance was below 4 Å. Docking experiments yielded 350 predicted αIIbβ3 head piece-fibrinogen γ-module complexes with ClusPro[10] (117,119 and 114 for series 1, 2, and 3, respectively), 92 with Haddock[11] (40, 40, and 12 for series 1, 2, and 3, respectively), and 897 with SwarmDock[12] (324, 386, and 187 for series 1, 2, and 3, respectively). Among them, 100 exhibited at least six ligand-receptor contacts between residues 316-322 and 370-381 on the fibrinogen and 10E5 binding site residues on αIIb, and were therefore selected for further studies. These 100 structures were made complete by modeling the missing loop connecting residues 144-392 of the γ-module with its C-terminus (residue 405-411) using the Rosetta protein modeling suite version 2014.[19] Complexes for which Rosetta failed to build the linker, mostly due to the long distance from docked protein to the fibrinogen C-terminus were discarded, leaving 55 suitable configurations of the complex formed by the C-terminal γ-module of fibrinogen and αIIbβ3. Out of the 55 models, 10 formed contacts with γ-316-322 and 45 formed contacts with γ-370-381, while no models made contacts with both γ-316-322 and γ-370-381.

Following optimal superposition of the αIIbβ3 head domain Cα atoms, the 55 conformations of the fibrinogen γ-module (Cα atoms of residues 144-389) were clustered based on root mean

square deviation (RMSD) using the clustering plugin of VMD.[20] Using a maximum number of 15 clusters and a 7.0 Å RMSD cutoff, a total of 13 clusters were identified, with the three most populated clusters formed by 7, 5, and 4 predicted complexes, respectively. For each cluster, RMSD calculations (using Cα atoms of residues 144-389 as a reference) were performed on all structure pairs, and the structure with the lowest RMSD was selected as the cluster representative structure. The representative structures of the most populated clusters were relaxed in an explicit solvent environment with molecular dynamics (MD) simulations. Specifically, structures were immersed in a dodecahedron water box with a minimum distance of 10 Å from the protein surface to the box edges. Calcium and magnesium ions in the αIIbβ3 crystal structure were kept, and sodium chloride ions were added at physiological concentration to neutralize the system, resulting in a system size of ~125,000 atoms. MD simulations were performed using GROMACS version 4.6.5. Simulations used a 2 fs time step, Particle Mesh Ewald (PME) electrostatics, and a constant-temperature, constant-pressure (NPT) ensemble at 300 K and 1 atm, respectively. After a 6 ns equilibration with decreasing positional restraints, a production run was carried out keeping a 250 $kJ \cdot mol^{-1} \cdot nm^{-2}$ positional restraint on the Cα atoms of both the last 7 residues of fibrinogen C-terminal γ-12 and the αIIbβ3 residues while the rest of the fibrinogen γ-module was free to move during simulation.

Generation of mutants and stable cell lines

Human αIIb and β3 cDNAs were generously provided by Dr. Peter Newman (Blood Center of Southeastern Wisconsin). αIIbF992A/F993A (FF), β3N339S, β3Δ717, β3D119A, and αIIb(FF)β3D119A mutants were generated using the QuikChange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutant cDNAs were sequenced to confirm that the mutations were introduced as predicted. Human embryonic kidney (HEK) 293 cells (ATCC) were transfected with either normal or mutant cDNAs using

Lipofectamine 2000 (Invitrogen). Cells were selected in 80 μg/ml G418 (Life Technologies) followed by sorting based on their binding of Alexa488-conjugated mAb 10E5, which binds to the cap region of the αIIb β-propeller domain.[18] Surface expression of the normal and mutant receptors was analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) using mAbs 10E5, 7E3, and/or 7H2. Although HEK293 cells make variable amounts of αV, which can combine with transfected β3 to form αVβ3, we found little or no αV on cells expressing αIIbβ3 as judged by the binding of mAb LM609. Moreover, mAb LM609, which blocks αVβ3 did not affect the adhesion assay results.

The αIIb(αV) swap mutant was made by restriction enzyme digestion of αIIb and αV cDNA followed by gel purification of the desired fragments and ligation of the purified cDNA fragments. The swap was confirmed by sequencing the cDNA. The cell line was made by transfection as described above followed by sorting based on binding of Alexa488-conjugated mAb PT25-2, which binds to the αIIb β-propeller domain (mAb 10E5 does not bind to the mutant). Surface expression was analyzed by flow cytometry using mAb PT25-2.

Tissue Culture

Cells were grown in Dullbecco's Modified Eagle Medium (DMEM) (Life Technologies, 11965) supplemented with 10% fetal bovine serum (Life Technologies, 10082147), MEM non-essential amino acids, and antibiotics. Cells were grown at 37°C in the presence of 5% CO_2 and harvested at 70-80% confluence using trypsin-EDTA solution containing 0.02 mM trypsin and 0.48 mM EDTA ("0.05%"; Life Technologies, 25300) for 5 min at 22˚C.

Platelet and Cell Adhesion Assay

96 well polystyrene plates (Greiner Bio-one, 655096) were coated with 10 μg/mL human fibrinogen or fibrinogen fragment 'D98' in Tris/HCl for 1 hour at room temperature (RT). Wells were washed with HEPES-modified Tyrode's buffer (HBMT; 10 mM HEPES, 12 mM $NaHCO_3$,

138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.35% bovine serum albumin, 0.1% glucose, pH 7.4) and blocked with HBMT at 4°C overnight. Cells expressing normal and mutant αIIbβ3 were harvested and washed with HBMT. Blood from healthy donors was obtained after obtaining informed consent in accord with a protocol approved by the Rockefeller University IRB and washed platelets were prepared as described previously.[21] Cells or platelets were then labelled with calcein AM (Life Technologies) (10 μM for cells and 7 μM for platelets), for 30 min at RT, washed in HBMT, and resuspended at 2 X 10^6/ml cells or 2 X 10^8/ml platelets in the same buffer. EDTA and/or mAbs were added to cell or platelet suspensions for 20 min at RT before adding the cells to the wells. Platelets were activated by adding 10 μM TRAP to the wells before adding the platelets with or without EDTA, mAbs, or tirofiban. In some experiments, either the immobilized fibrinogen or 'D98' was pre-treated with EDTA (10 mM) for 20 minutes at 22°C, or the αIIbβ3-HEK cells was pre-treated with EDTA (10 mM) in HBMT buffer for 20 minutes at 22°C. In the former case, the EDTA solution was removed and then the αIIbβ3-HEK cells in HBMT with 2 mM Ca^{2+}, 1 mM Mg^{2+} was added. In the latter case, the cells were added to the wells after the pre-incubation. Adhesion was performed for 1 hour at RT in the presence of 1 mM Mg^{2+} and 2 mM Ca^{2+}. Non-adherent cells or platelets were removed by three washes with HBMT and the fluorescence intensity was measured by reading the plates at 480 nm in a fluorescent plate reader (BioTek Synergy Neo). For cells, adhesion was expressed as arbitrary fluorescence intensity units (AFU) after subtracting the background value in the absence of cells (net value) or as a percentage of the adhesion of control cells tested in the same experiment. In some studies comparing different cell lines, the net value was adjusted for differences in surface expression of αIIbβ3 by dividing the net value by surface expression as derived from the binding of mAb 10E5 or PT25-2. For platelets, adhesion was expressed as % of the adhesion of unactivated platelets to fibrinogen in the same experiment.

Mass spectrometry (MS) analysis of fibrinogen fragments D100 and 'D98'

Data acquisition: Proteins were separated by SDS-PAGE under reducing conditions and excised and digested with trypsin as described previously.[22] In some experiments, peptides were labeled with propionic anhydride prior to digesting so as to label primary amines (lysines and N-termini). Extracted peptides were analyzed by LC-MS/MS (Ultimate 3000 nano-HPLC system coupled to a Q-Exactive Plus mass spectrometer, Thermo Scientific). Peptides were separated on a C_{18} column (12 cm / 75 µm, 3 µm beads, Nikkyo Tecnologies) at 300 nl/min with a gradient increasing from 2% Buffer B/98% buffer A to 45% buffer B/55% Buffer A in either 37 min or 54 min (buffer A: 0.1% formic acid, buffer B: 0.1% formic acid in 80% acetonitrile) and analyzed by data dependent acquisition (DDA) or by parallel reaction monitoring (PRM). The fibrinogen α, β, and γ-chains were confidently matched with multiple peptides at a 1% peptide false discovery rate and with sequence coverage of >60%.

Data analysis: MS/MS spectra were extracted and queried against UniProt's Human data base concatenated (December 2015) with common contaminants[23] or a custom database containing the fibrinogen γ-chain using Proteome Discoverer 1.4 (Thermo Scientific) and MASCOT 2.5.1 (Matrix Science). Acetylation (protein N-termini) and oxidation (M) were allowed as variable modifications while carbamidomethylation (C) was set as fixed. For experiments that used propionic anhydride blocking of primary amines,[24] propionyl was allowed as a variable modification of peptide N-termini and lysines.10 ppm was used as mass accuracy for precursors ions while 20 mDa was used for fragment ions. Semi-tryptic search constraints were used. Matched peptides were filtered using a Percolator[25] calculated 1% false discovery rate and requiring that a peptide was matched as rank 1 with a precursor mass accuracy better than 5 ppm.

N-terminal and C-terminal analysis: Three different digestion strategies (trypsin, endopeptidase Lys-C, and propionic anhydride blocking of primary amines followed by trypsin digestion) were

applied to the ‘D98’ and D100 gel bands. Both endopeptidase Lys-C and blocking followed by trypsin allowed for detailed analysis of the N- and C-termini of the fibrinogen γ-chain. The expected peptide fragments, in addition to control peptides used for normalization in-between samples, were targeted in Parallel Reaction Monitoring (PRM) experiments.[26] The targeted peptides and respective signals are listed in Supplementary Table S2. Multiple charge states for each peptide were used for quantitation. Peptide MS signals were extracted using Skyline.[27]

Figure and Table Legends

Table S1. Docking restraints. The three sets of residues designated as being at the fibrinogen-interface set as restraints in protein-protein docking. The fibrinogen γ-chain residues 316-322 and 370-381, which come from previous experimental studies,[14,28] were used in all three sets. For αIIb and β3, the residues defined were based on previous experimental data (set 1),[29,30] the assumption that fibrinogen binds on the side of the receptor where the γ-12 peptide exits the RGD pocket (set 2), and the assumption that fibrinogen binds near the 10E5 binding site (set 3), defined as integrin surface exposed residues within 12 Å of 10E5 in the 2VDO crystal structure.

Table S2. Abundance of C-terminal peptides of γ-chain in fibrinogen fragment D100 and 'D98' based on LC-MS/MS analysis. MS extracted signals (<5ppm) from endopeptidase Lys-C-generated the γ-chain peptides are displayed in (A) and γ-chain peptides generated by propionic anhydride treatment followed by trypsinization are listed in (B). Signals for peptides at the N- and C-termini of γ-chain in fibrinogen as well as peptides far from both termini are shown in the 'Measured signal' column for D100 and 'D98.' The majority of the signals were generated in PRM experiments. Peptides far from both termini, indicated at the bottom of each table with an asterisk, were used as loading controls. Adjusted signals are shown in the 'Normalized signal' column. For the endopeptidase Lys-C experiment (A), signals from the 'D98' gel band were multiplied by a factor of 1.2. For the propionic anhydride/trypsin experiments the 'D98' signals were multiplied by 3.25. The first and last residues of the listed peptides are noted in the 'N-term' and 'C-term' columns.

Figure S1. Three sets of restraints on αIIbβ3 used during protein-protein docking. (A) Restraint set 1 shown as orange sticks. (B) Restraint set 2 shown as cyan sticks. (C) Restraint set 3 shown as pink sticks.

Figure S2. Comparison of the effects of TRAP and ADP + epinephrine on platelet adhesion to fibrinogen and 'D98.' Calcein-labeled washed platelets (2 X 10^5/μl; 50 μl) were tested either before (unactivated) or after activation with 10 μM TRAP or the combination of ADP + epinephrine (10 μM each) for adhesion to fibrinogen or 'D98' immobilized in microtiter wells by coating at 10 μg/mL. Platelets were incubated for 1 hour at 22°C. Unactivated platelets adhered well to fibrinogen and both activators produced modest increases in adhesion. In contrast, unactivated platelets adhered poorly to 'D98' whereas TRAP-activated platelets adhered much better (*P* = 0.009) and ADP + epinephrine produced a modest increase in adhesion that was not statistically significant (*P* = 0.06).

Figure S3. Comparison of the effect of EDTA treatment of αIIbβ3 expressing cells and EDTA treatment of ligands on adhesion. (A, B) Calcein-labeled αIIbβ3-HEK cells (2 X 10^3/μl; 50 μl) were allowed to adhere to microtiter wells pre-coated with fibrinogen or 'D98' (each at 10 μg/mL coating concentration) for 1 hour at 22°C after pre-treating either the αIIbβ3-HEK cells or the ligands (fibrinogen or 'D98') with EDTA (10 mM). The fluorescence intensity was measured after washing away unbound cells. (A) Pre-incubating fibrinogen with EDTA did not inhibit adhesion of the αIIbβ3-HEK cells to fibrinogen, whereas EDTA treatment of αIIbβ3-HEK cells eliminated their adhesion to fibrinogen. (B) Pre-treating 'D98' with EDTA did not enhance adhesion of the αIIbβ3-HEK cells to 'D98', whereas EDTA treatment of αIIbβ3-HEK cells led to their enhanced adhesion to 'D98'.

Figure S4. Swapping the αIIb 148-166 loop with the αV 144-154 loop eliminates adhesion to fibrinogen and EDTA-induced adhesion to fibrinogen fragment 'D98'. (A, B) Calcein-labeled HEK293 cells (2 X 10^3/μl; 50 μl) expressing normal αIIbβ3, normal αVβ3, or αIIbβ3 in which the αV 144-154 loop has been swapped for the αIIb 148-166 loop [αIIb(αV)β3] were allowed to adhere to microtiter wells pre-coated with fibrinogen or 'D98' (each at 10 μg/mL coating concentration) for 1 hour at 22°C. The fluorescence intensity was measured after washing away unbound cells. (A) Both normal αIIbβ3-HEK and normal αVβ3-HEK cells adhered to fibrinogen whereas the αIIb(αV)β3-HEK cells did not adhere (n = 5; $P \leq 0.01$ for each compared to either normal receptor). Adding EDTA nearly eliminated adhesion of αIIbβ3-HEK and αVβ3-HEK to fibrinogen (n = 5; $P \leq 0.01$ for both). Adding DTT (5 mM) did not affect the adhesion of either αIIbβ3-HEK or αVβ3-HEK, but dramatically increased the adhesion of αIIb(αV)β3-HEK cells (n = 5; $P < 0.001$). (B) EDTA (10 mM) enhanced the adhesion of αIIbβ3-HEK cells to 'D98' (n = 3; $P = 0.02$) while it had no effect on the adhesion of normal αVβ3-HEK or αIIb(αV)β3-HEK cells (n = 3; $P = 0.02$ for both compared to normal αIIbβ3). Surface expression of receptors for each cell line was measured based on mAb 7H2 binding and is expressed as GMFI: normal αIIbβ3 33 ± 5 AFU; normal αVβ3 195 ± 50 AFU; αIIb(αV)β3 Swap 77 ± 37 AFU. (C) αVb3 crystal structure 1L5G aligned with αIIbβ3 crystal 2VDO showing the overlap of the αIIb cap 3 loop (147-167) shown in blue and the αV 143-154 loop shown in cyan. The rest of αV and β3 in 1L5G and αIIb and β3 in 2VDO are omitted for clarity.

Figure S5. RMSD measurement of fibrinogen γ-chain C-terminal during MD simulation starting from the cluster 1 (upper panel) or cluster 2 (lower panel) representative

structures. Simulation structures were fitted using integrin heavy atoms first, and then heavy atom RMSD of the fibrinogen γ-chain C-terminal was measured.

Figure S6. αIIbβ3 crystal structure 2VDO with residues within 5Å distance to 10E5 highlighted as pink sticks.

Table S1

Set number	Fibrinogen residues	αIIb residues	β3 residues
Set 1	316-322, 370-381	69, 70, 71, 74, 100, 102, 157, 159, 162, 235, 243, 247, 252, 428, 429, 110-114, 117, 124, 125, 147, 155, 160, 163-166, 189-191, 193, 200, 203, 204, 207, 212-215, 222, 224, 226, 231, 232, 234, 260, 264, 284, 285	None
Set 2	316-322, 370-381	73-88, 100-228, 270-276	120-220, 309-314, 330-345
Set 3	316-322, 370-381	74-83, 85, 114, 116-122, 142, 144-162, 164-165, 197, 199, 201-202, 204-211, 213-221, 223-224	None

Table S2

A.

N-term	C-term	Peptide Sequence	Normalized signal		Measured signal	
			D100	'D98'	D100	'D98'
63	75	AIQLTYNPDESSK	6.7E+10	2.5E+07	6.74E+10	2.23E+07
63	85	AIQLTYNPDESSKPNMIDAATLK	5.8E+10	7.7E+08	5.79E+10	6.88E+08
76	85	PNMIDAATLK	2.6E+10	1.6E+08	2.56E+10	1.40E+08
386	393	IIPFNRLT	1.4E+07	0.0E+00	0.00E+00	0.00E+00
386	394	IIPFNRLTI	0.0E+00	0.0E+00	0.00E+00	0.00E+00
386	395	IIPFNRLTIG	0.0E+00	0.0E+00	0.00E+00	0.00E+00
386	396	IIPFNRLTIGE	0.0E+00	0.0E+00	0.00E+00	0.00E+00
386	397	IIPFNRLTIGEG	3.0E+08	2.1E+08	1.35E+06	0.00E+00
386	398	IIPFNRLTIGEGQ	4.6E+07	2.9E+08	4.61E+07	2.58E+08
386	399	IIPFNRLTIGEGQQ	2.7E+08	5.0E+08	2.71E+08	4.48E+08
386	400	IIPFNRLTIGEGQQH	3.4E+08	9.4E+08	3.44E+08	8.39E+08
386	401	IIPFNRLTIGEGQQHH	9.4E+07	1.9E+09	9.36E+07	1.68E+09
386	402	IIPFNRLTIGEGQQHHL	2.1E+08	9.2E+09	2.11E+08	8.21E+09
386	403	IIPFNRLTIGEGQQHHLG	0.0E+00	2.8E+09	0.00E+00	2.48E+09
386	404	IIPFNRLTIGEGQQHHLGG	2.1E+08	5.0E+08	2.13E+08	4.41E+08
386	405	IIPFNRLTIGEGQQHHLGGA	2.6E+10	1.0E+11	2.60E+10	9.10E+10
386	406	IIPFNRLTIGEGQQHHLGGAK	3.8E+10	1.2E+08	3.81E+10	1.03E+08
128	140	*VAQLEAQC[+57]QEPC[+57]K	-	-	1.13E+11	9.24E+10
141	151	*DTVQIHDITGK	-	-	5.89E+10	8.69E+10
163	170	*QSGLYFIK	-	-	6.96E+10	5.87E+10
197	205	*RLDGSVDFK	-	-	2.98E+10	2.12E+10

Control

B.

N-term	C-term	Peptide Sequence	Normalized signal		Measured signal	
			D100	'D98'	D100	'D98'
88	108	K[56]MLEEIMK[56]YEASILTHDSSIR	1.0E+10	4.7E+09	1.02E+10	1.45E+09
89	108	[56]MLEEIMK[56]YEASILTHDSSIR	4.9E+07	1.0E+10	2.47E+06	8.04E+08
392	400	LTIGEGQQH	2.2E+09	3.8E+09	2.22E+09	1.17E+09
392	401	LTIGEGQQHH	2.7E+08	2.0E+09	2.68E+08	6.11E+08
392	402	LTIGEGQQHHL	1.6E+08	6.4E+09	1.60E+08	1.99E+09
392	403	LTIGEGQQHHLG	9.4E+07	6.7E+08	9.37E+07	2.05E+08
392	404	LTIGEGQQHHLGG	1.0E+08	5.8E+07	1.04E+08	1.79E+07
392	405	LTIGEGQQHHLGGA	1.6E+10	5.8E+10	1.62E+10	1.79E+10
392	407	LTIGEGQQHHLGGAK[+56]Q	7.4E+08	1.9E+07	7.36E+08	5.82E+06
392	408	LTIGEGQQHHLGGAK[+56]QA	1.3E+09	0.0E+00	1.26E+09	0.00E+00
392	409	LTIGEGQQHHLGGAK[+56]QAG	1.6E+09	0.0E+00	1.57E+09	0.00E+00
392	410	LTIGEGQQHHLGGAK[+56]QAGD	0.0E+00	0.0E+00	0.00E+00	0.00E+00
392	411	LTIGEGQQHHLGGAK[+56]QAGDV	2.0E+11	0.0E+00	2.04E+11	0.00E+00
248	256	*VELEDWNGR	-	-	2.19E+11	6.37E+10
257	275	*TSTADYAMFKVGPEADKYR	-	-	9.85E+09	3.22E+09

Control

Figure S1

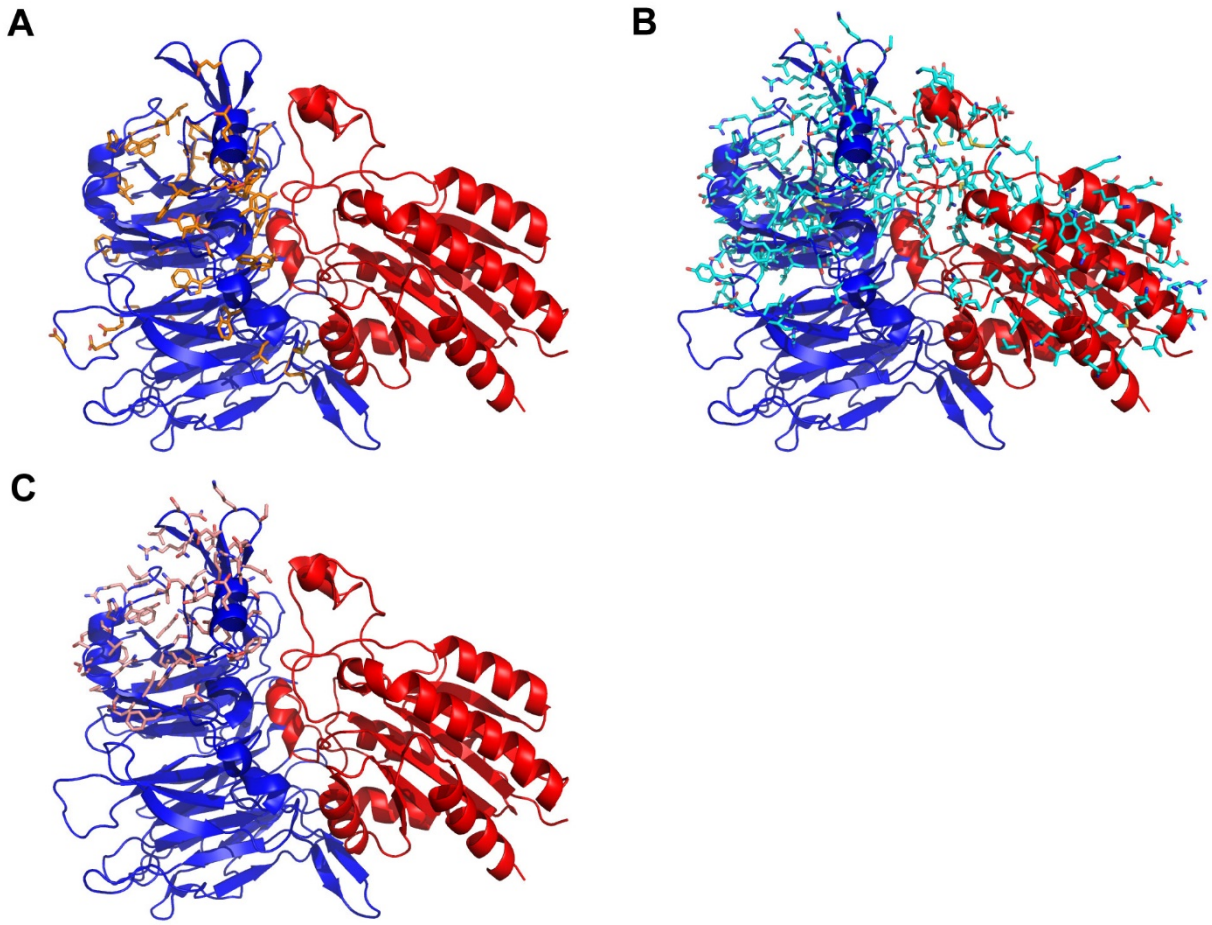


Figure S2

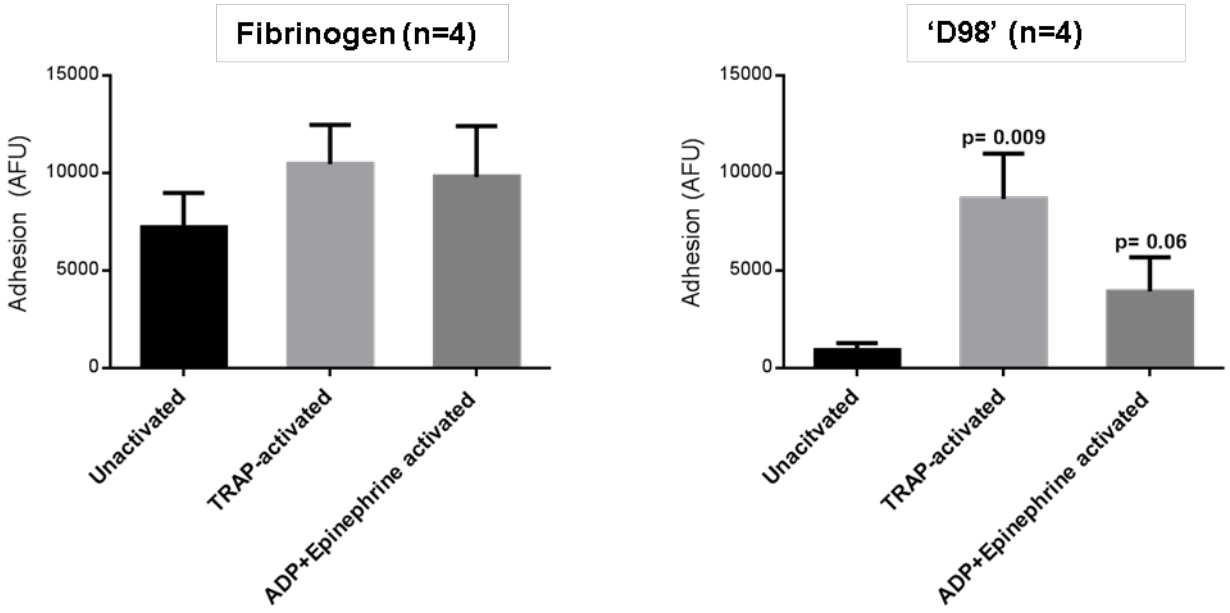

Fibrinogen (n=4)
Adhesion (AFU)
15000
10000
5000
0
Unactivated
TRAP-activated
ADP+Epinephrine activated
'D98' (n=4)
Adhesion (AFU)
15000
10000
5000
0
p= 0.009
p= 0.06
Unacitvated
TRAP-activated
ADP+Epinephrine activated

Figure S3

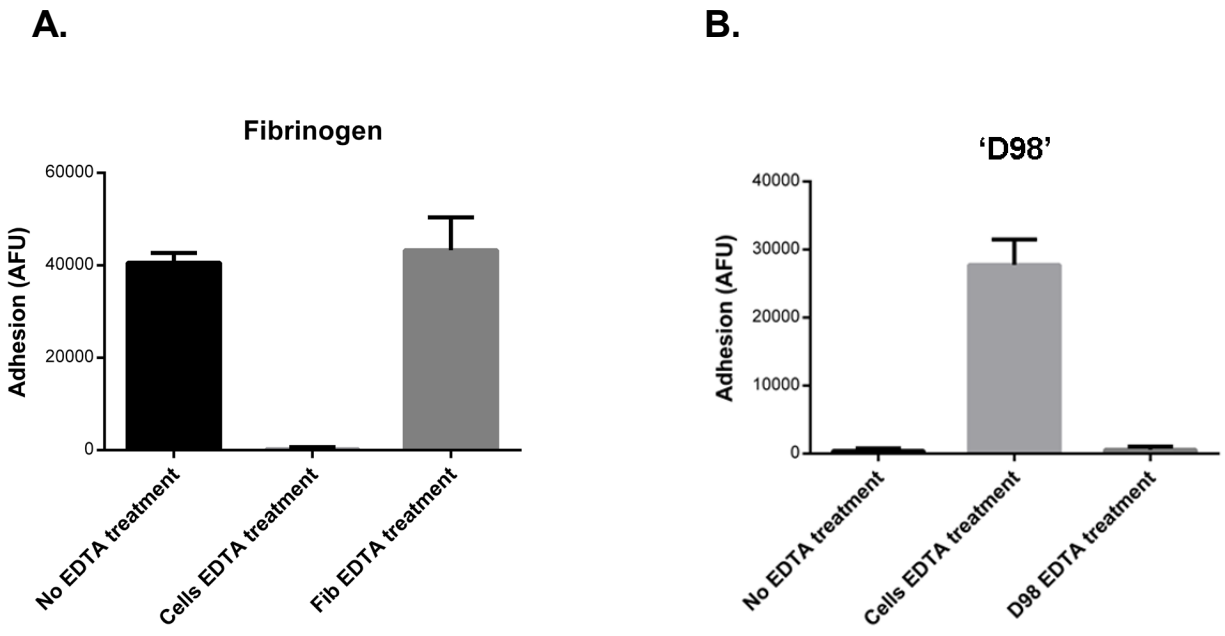
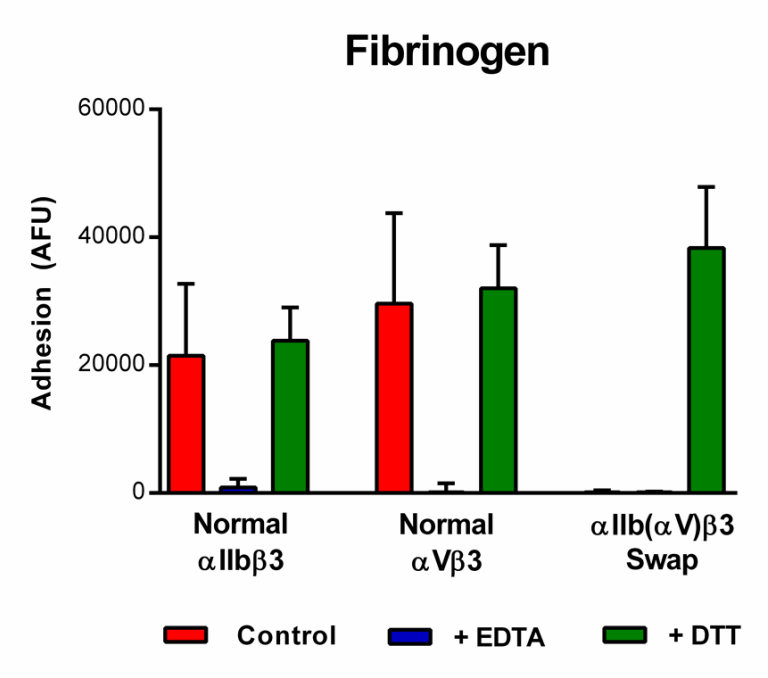

A.
Fibrinogen
Adhesion (AFU)
60000
40000
20000
0
No EDTA treatment
Cells EDTA treatment
Fib EDTA treatment
B.
'D98'
Adhesion (AFU)
40000
30000
20000
10000
0
No EDTA treatment
Cells EDTA treatment
D98 EDTA treatment

Figure S4

A.


Fibrinogen
Adhesion (AFU)
60000
40000
20000
0
Normal αIIbβ3
Normal αVβ3
αIIb(αV)β3 Swap
Control
+ EDTA
+ DTT

B.

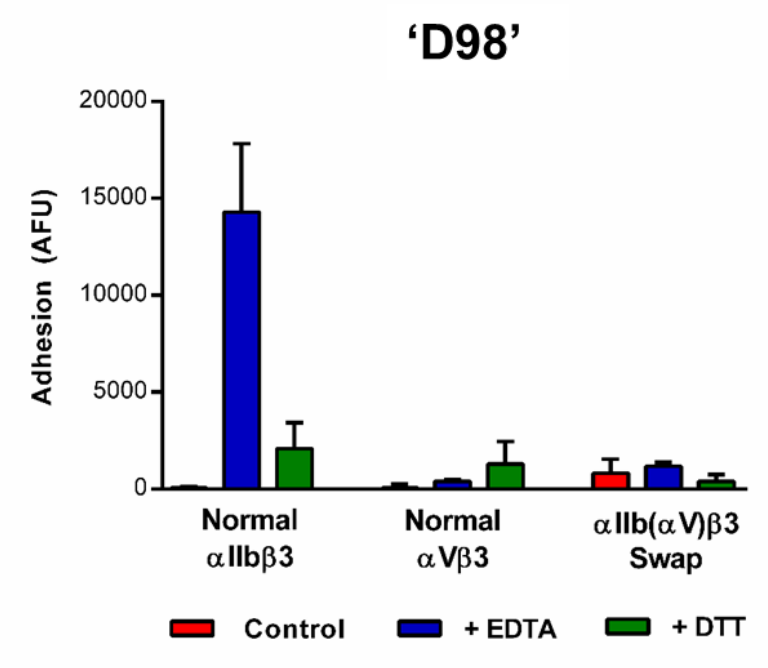

'D98'
Adhesion (AFU)
20000
15000
10000
5000
0
Normal αIIbβ3
Normal αVβ3
αIIb(αV)β3 Swap
Control
+ EDTA
+ DTT

Figure S4

C.

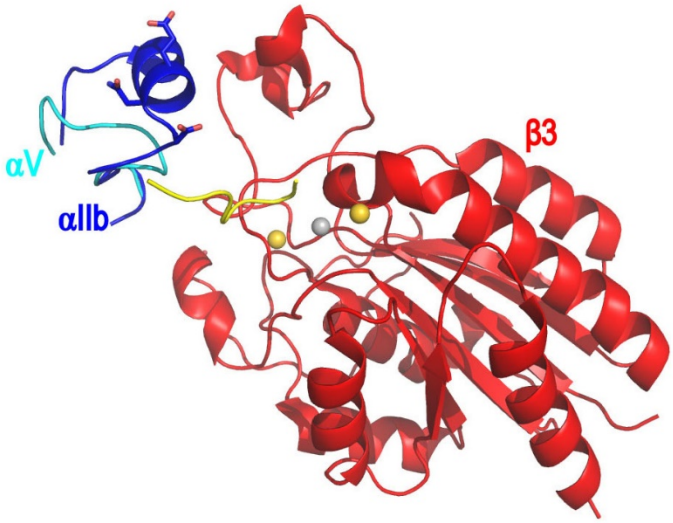


Figure S5

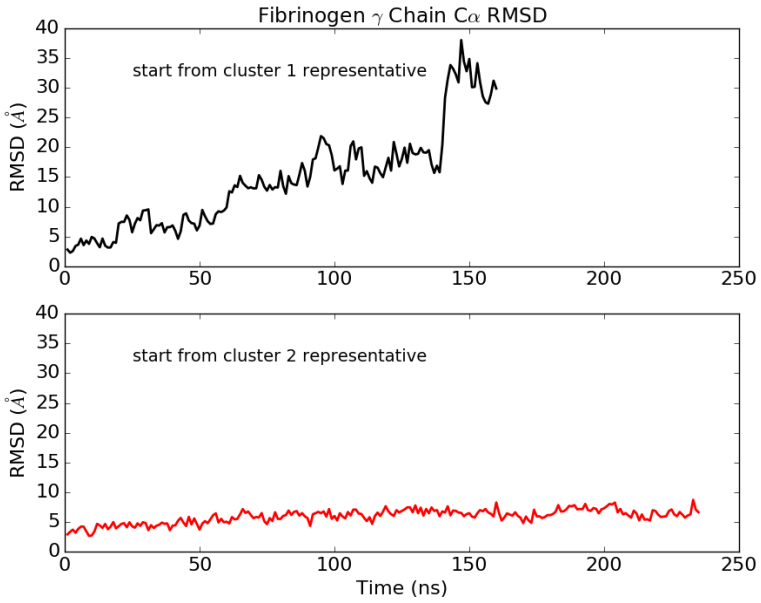
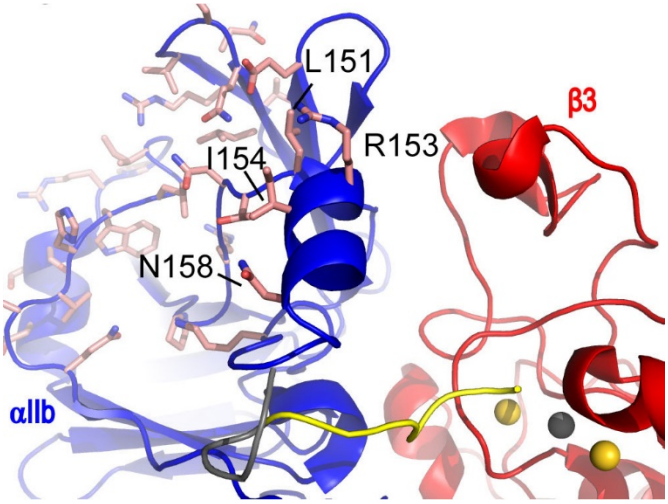

Fibrinogen γ Chain Cα RMSD
start from cluster 1 representative
start from cluster 2 representative
RMSD (Å)
Time (ns)

Figure S6



References

1. Podolnikova NP, Yakubenko VP, Volkov GL, Plow EF, Ugarova TP. Identification of a novel binding site for platelet integrins alpha IIb beta 3 (GPIIbIIIa) and alpha 5 beta 1 in the gamma C-domain of fibrinogen. *J Biol Chem*. 2003;278(34):32251-32258.
2. Lishko VK, Kudryk B, Yakubenko VP, Yee VC, Ugarova TP. Regulated unmasking of the cryptic binding site for integrin alpha M beta 2 in the gamma C-domain of fibrinogen. *Biochemistry*. 2002;41(43):12942-12951.
3. Ugarova TP, Budzynski AZ. Interaction between complementary polymerization sites in the structural D and E domains of human fibrin. *J Biol Chem*. 1992;267(19):13687-13693.
4. Jirouskova M, Smyth SS, Kudryk B, Coller BS. A hamster antibody to the mouse fibrinogen gamma chain inhibits platelet-fibrinogen interactions and FXIIIa-mediated fibrin cross-linking, and facilitates thrombolysis. *Thromb Haemost*. 2001;86(4):1047-1056.
5. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J Clin Invest*. 1983;72:325-338.
6. Coller BS. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet GPIIb/IIIa complex. *J Clin Invest*. 1985;76:101-108.
7. Honda S, Tomiyama Y, Pelletier AJ, et al. Topography of ligand-induced binding sites, including a novel cation-sensitive epitope (AP5) at the amino terminus, of the human integrin beta 3 subunit. *J Biol Chem*. 1995;270(20):11947-11954.
8. Cheresh DA. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc Natl Acad Sci USA*. 1987;84:6471-6475.
9. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin αVβ3 for angiogenesis. *Science*. 1994;264(5158):569-571.
10. Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: a fully automated algorithm for protein-protein docking. *Nucleic Acids Res*. 2004;32(Web Server issue):W96-W99.
11. de Vries SJ, van Dijk M, Bonvin AM. The HADDOCK web server for data-driven biomolecular docking. *Nat Protoc*. 2010;5(5):883-897.
12. Moal IH, Bates PA. SwarmDock and the use of normal modes in protein-protein docking. *Int J Mol Sci*. 2010;11(10):3623-3648.
13. Remijn JA, Ijsseldijk MJ, van Hemel BM, et al. Reduced platelet adhesion in flowing blood to fibrinogen by alterations in segment gamma316-322, part of the fibrin-specific region. *Br J Haematol*. 2002;117(3):650-657.
14. Podolnikova NP, Gorkun OV, Loreth RM, Yee VC, Lord ST, Ugarova TP. A cluster of basic amino acid residues in the gamma370-381 sequence of fibrinogen comprises a binding site for platelet integrin alpha(IIb)beta3 (glycoprotein IIb/IIIa). *Biochemistry*. 2005;44(51):16920-16930.
15. Kamata T, Tieu KK, Irie A, Springer TA, Takada Y. Amino acid residues in the alpha IIb subunit that are critical for ligand binding to integrin alpha IIbbeta 3 are clustered in the beta-propeller model. *J Biol Chem*. 2001;276(47):44275-44283.
16. Podolnikova NP, Yakovlev S, Yakubenko VP, Wang X, Gorkun OV, Ugarova TP. The interaction of integrin alphaIIbbeta3 with fibrin occurs through multiple binding sites in the alphaIIb beta-propeller domain. *J Biol Chem*. 2014;289(4):2371-2383.
17. Seligsohn U, Mibashan RS, Rodeck CH, Nicolaides KH, Millar DS, Coller BS. Prevention program of type I Glanzmann-thrombasthenia in Israel: Prenatal diagnosis. In: Kaplan C, ed. Curr Stud Hematol Blood Transf. Basel: Karger; 1988:174-179.

18. Xiao T, Takagi J, Coller BS, Wang J, Springer TA. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature*. 2004;432:59-67.
19. Kaufmann KW, Lemmon GH, Deluca SL, Sheehan JH, Meiler J. Practically useful: what the Rosetta protein modeling suite can do for you. *Biochemistry*. 2010;49(14):2987-2998.
20. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996;14(1):33-38.
21. Blue R, Murcia M, Karan C, Jirouskova M, Coller BS. Application of high throughput screening to identify a novel alphaIIb-specific small molecule inhibitor of alphaIIbbeta3-mediated platelet Interaction with fibrinogen. *Blood*. 2008;111:1248-1256.
22. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem*. 1996;68(5):850-858.
23. Bunkenborg JG, G.E.; Paz, M.I.; Andersen, J.S.; Molina, H. The minotaur proteome: avoiding cross-species identifications deriving from bovine serum in cell culture models. *Proteomics*. 2010;10(16):3040.
24. Garcia BA, Mollah S, Ueberheide BM, et al. Chemical derivatization of histones for facilitated analysis by mass spectrometry. *Nat Protoc*. 2007;2(4):933-938.
25. Spivak M, Weston J, Bottou L, Kall L, Noble WS. Improvements to the percolator algorithm for Peptide identification from shotgun proteomics data sets. *J Proteome Res*. 2009;8(7):3737-3745.
26. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics*. 2012;11(11):1475-1488.
27. MacLean B, Tomazela DM, Shulman N, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010;26(7):966-968.
28. Remijn JA, MJ IJ, van Hemel BM, et al. Reduced platelet adhesion in flowing blood to fibrinogen by alterations in segment gamma316-322, part of the fibrin-specific region. *Br J Haematol*. 2002;117(3):650-657.
29. Podolnikova NP, Yakovlev S, Yakubenko VP, Wang X, Gorkun OV, Ugarova TP. The interaction of integrin alphaIIbbeta3 with fibrin occurs through multiple binding sites in the alphaIIb beta-propeller domain. *J Biol Chem*. 2014;289(4):2371-2383.
30. Kamata T, Tieu KK, Irie A, Springer TA, Takada Y. Amino acid residues in the alpha IIb subunit that are critical for ligand binding to integrin alpha IIbbeta 3 are clustered in the beta-propeller model. *J Biol Chem*. 2001;276(47):44275-44283.