A directional switch of integrin signalling and a new anti-thrombotic strategy

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Integrins have a critical role in thrombosis and haemostasis1. Antagonists of the platelet integrin αIbβ3 are potent anti-thrombotic drugs, but also have the life-threatening adverse effect of causing bleeding2,3. It is therefore desirable to develop new antagonists that do not cause bleeding. Integrins transmit signals bidirectionally4,5. Integrin ligation mediates thrombus formation and clot retraction. An EXE-motif-containing G8α13–integrin interaction selectively abolishes outside-in signalling without affecting integrin ligation, and suppresses occlusive arterial thrombosis without affecting bleeding time. Thus, we have discovered a new mechanism for the directional switch of integrin signalling and, on the basis of this mechanism, designed a potent new anti-thrombotic drug that does not cause bleeding.

Figure 1 | Mutually exclusive binding of talin and Gα13 to β3. a. The sequence of the human β3 cytoplasmic domain and its alignment with other β subunits, showing conserved EXE motifs and binding sites for talin, kindlins and SRC. b. Co-immunoprecipitation of wild-type (WT) and truncated mutant β3 with Gα13 and talin using anti-β3 or control pre-immune (pre-im) rabbit serum. Immunoprecipitates (IP) and CHO cell lysates (10% of that used in immunoprecipitation) were immunoblotted (IB) with indicated antibodies. c. Binding of purified recombinant Gα13 to glutathione-S-transferase (GST)–β3 cytoplasmic domain fusion protein (GST–β3CD) (Fig. 1f, g and Extended Data Fig. 2e). d. Overexpression of the integrin-binding talin head domain (THD) in αIbβ3-expressing cells inhibited Gα13-co-immunoprecipitation with β3 (Fig. 1e). Purified recombinant THD and Gα13 competed directly for binding to purified glutathione S-transferase (GST)–β3 cytoplasmic domain fusion protein (GST–β3CD) (Fig. 1f, g and Extended Data Fig. 2e).
disruption of the relationship between G\textsubscript{\alpha 13} and G\textsubscript{\beta 3} implies that the interaction of these two proteins with G\textsubscript{\beta 3} selectively mediates inside-out and outside-in interactions, respectively. This hypothesis was tested using talin knock-out\textsuperscript{16} and shRNA-induced talin knockdown platelets, which are defective in adenosine diphosphate (ADP)/fibrinogen-induced, integrin-dependent aggregation (Fig. 2d, e and Extended Data Fig. 4a, c). Their defective aggregation was fully corrected with manganese or an integrin-activating antibody (LIBS6) (Fig. 2e and Extended Data Fig. 4c), which activates integrins independently of inside-out signalling. These data confirm a role for talin in inside-out signalling\textsuperscript{6,15,17,18}. It is established that inside-out signalling is not the only pathway of vinculin-mediated integrin activation. Integrin–fibrinogen interaction may occur independently of inside-out signalling when fibrinogen changes conformation, either by immobilization or conversion to fibrin\textsuperscript{19,19}. This is because the initial contact of the exposed ligand recognition sequence, RGDS, with resting integrins triggers ligand-induced integrin activation\textsuperscript{20}. Interestingly, adhesion of resting talin-knockout or -knockdown platelets to immobilized fibrinogen was defective (Fig. 2f and Extended Data Fig. 4b), indicating the importance of talin in platelet adhesion to immobilized fibrinogen in the absence of inside-out signalling. However, addition of manganese or integrin-activating antibody fully corrected talin-knockout and -knockdown platelet adhesion and spreading (and also the spreading of talin-binding-defective mutant G\textsubscript{\beta 3}-expressing Chinese hamster ovary (CHO) cells\textsuperscript{15}) on immobilized fibrinogen (Fig. 2f, g and Extended Data Fig. 4b, d, e). Thus, the role of talin in resting platelet adhesion to fibrinogen is solely due to its importance in ligand-induced integrin activation. Because cell spreading requires the early phase of outside-in signalling, these data further demonstrate that talin is not required for the early phase of outside-in signalling leading to cell spreading once its role in integrin activation is bypassed.

To assess whether G\textsubscript{\alpha 13} binding to the EXE motif selectively mediates outside-in signalling without perturbing talin-dependent integrin function, wild-type and AAA mutant G\textsubscript{\beta 3}-transfected ITGB3 (G\textsubscript{\beta 3})\textsuperscript{17} bone marrow stem cells (from G\textsubscript{\beta 3} mice) were transplanted into irradiated G\textsubscript{\beta 3} mice. The platelets from the recipient mice expressed similar levels of wild-type or AAA mutant G\textsubscript{\beta 3} (Fig. 3a and Extended Data Fig. 5a). The AAA mutation inhibited integrin interaction with G\textsubscript{\alpha 13}, but not talin (or SRC) (Fig. 3b and Extended Data Fig. 5b), during integrin signalling. The AAA mutation also had no effect on agonist-induced soluble fibrinogen binding (Fig. 3c). Thus, the EXE motif is not required for talin-dependent inside-out signalling. By contrast, the AAA mutant G\textsubscript{\beta 3}-expressing platelets were defective in spreading on immobilized fibrinogen (Fig. 3d and Extended Data Fig. 5c, d). Thus, G\textsubscript{\alpha 13}-binding deficiency in G\textsubscript{\beta 3} causes a selective defect in integrin outside-in signalling and platelet spreading. Similarly, AAA and more conserved DED or QSE G\textsubscript{\beta 3} mutants expressed in CHO cells, all defective in G\textsubscript{\alpha 13} binding (Extended Data Fig. 2e), and were also defective in spreading on fibrinogen (Fig. 3e, f and Extended Data Fig. 6a–c). However, AAA mutant G\textsubscript{\beta 3} expressed in CHO cells had no negative effect on THBD binding, in contrast to the Y747A mutant (Extended Data Fig. 6d, e). In addition, AAA-expressing cells showed defects in integrin-dependent activation of SRC (as shown by phosphorylation at Tyr-416) and transient inhibition of RHODA during cell spreading (Fig. 3g and Extended Data Fig. 6f), both of which are important elements of outside-in signalling. Together with previous studies that identified β3 sequences mediating talin binding (Fig. 1a), these data suggest that talin and G\textsubscript{\alpha 13} dynamically interact with distinct recognition sequences in the same region of β3 to serve as a molecular switch controlling the direction of integrin signalling.

The specific role of the EXE motif in outside-in signalling prompted us to design selective inhibitors of outside-in signalling. We synthesized several myristoylated (Myr) EXE-motif-containing G\textsubscript{\beta 3} peptides: mP5...
Figure 3 | The selective role of Gα13/EEX binding in integrin outside-in signalling. a, Flow cytometric analysis of β3 expression in platelets from β3-/- mice transplanted with wild-type or AAA mutant β3-transfected bone marrow stem cells. β3-/- platelets served as negative control. Picture shows relative fluorescence (FL1) on the x-axis and number of events (count) on the y-axis. b, Mouse platelets expressing wild-type (EEE) or AAA mutant β3 were stimulated with 0.025 U ml⁻¹ α-thrombin, solubilized at various time points, immunoprecipitated with anti-β3 or pre-immune rabbit serum and immunoblotted for Gα13, talin and β3. c, PAR4 agonist peptide (PAR4-AP)-induced binding of Oregon Green-labelled fibrinogen to wild-type or AAA mutant α5β3, expressing platelets with β3-/- platelets as a negative control. d, Confocal images of β3-/- platelets and β3+/+ platelets expressing wild-type or AAA-mutant β3 spreading on fibrinogen and surface area quantification (mean ± s.e.m.). Merged anti-β3 (green) and Alexa Fluor 546-conjugated phalloidin (red) fluorescence. e, Flow cytometric analysis of wild-type or β3 AAA mutant α5β3 expression in CHO-1b9 cells. f, Confocal images of phalloidin (red)/anti-β3 (green)-double stained wild-type and AAA-mutant α5β3-expressing CHO cell spreading on fibrinogen (45 min). g, RHOA activation and SRC Tyr 416 phosphorylation in wild-type or AAA mutant α5β3-expressing CHO-1b9 cells adherent to fibrinogen (mean ± s.d., n = 3).

Figure 4 | A new anti-thrombotic that does not cause bleeding. a, The effects of 500 µM mP33, mP or mP3 on co-immunoprecipitation of β3 with Gα13 or talin in thrombin-stimulated platelets in comparison with scrambled (Scr) controls (also see Extended Data Fig. 7). b, Confocal images of phalloidin (red)/anti-β3 (green)-double-stained human platelets treated with 100 µM mP or mP3 (red) spreading on immobilized fibrinogen (1 h). c, PAR4-AP-induced Oregon Green-labelled fibrinogen binding to human platelets pre-treated with DMSO or 100 µM mP or mP3 on clot retraction of human platelet-rich plasma, with or without 1 mM manganese (Mn) (mean ± s.d., n = 3). d, Effects of mP or mP3 (250 µM) on resting platelet adhesion to immobilized fibrinogen as compared with scrambled peptides (mean ± s.d., n = 4). e, Effect of mP or mP3 (250 µM) on clot retraction of human platelet-rich plasma, with or without 1 mM manganese (Mn) (mean ± s.d., n = 3). f, Effects of 10 µM mP or mP3 micelles on platelet aggregation induced by 0.03 U ml⁻¹ thrombin. g, Comparison of mP3 micelle (5 µmol kg⁻¹) with Integrilin (12 µmol kg⁻¹) and their respective controls in inhibiting laser-induced arteriolar thrombosis in mice. Representative images at 60 s after injury are shown. Platelet thrombi were indicated by Dylight 649-labelled nonblocking rat anti-mouse GPIbγ (red). h, Quantiﬁcation of g. The median time-integrated platelet ﬂuorescence (Fmean) during thrombosis at 30 injury sites in three mice. i, Comparison of mP3 (5 µmol kg⁻¹) with Integrilin (5 µmol kg⁻¹) and their respective controls in inhibiting FeCl3-induced carotid artery thrombosis in mice. j, Comparison of mP3 (5 µmol kg⁻¹) with Integrilin (5 µmol kg⁻¹) and controls in mouse tail bleeding time analysis.
arterial thrombosis were performed as described previously. Data were analysed using t-test or one-way analysis of variance.

Tail bleeding time. Analysis was performed as described previously. Time to stable cessation of the bleeding is defined as no re-bleeding for 60 s. Bleeding exceeding 15 min was immediately stopped. Data were analysed using the Mann–Whitney test.

Online Content Any additional Methods, Extended data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions B.S. performed most of the experiments and participated in experimental design, data analysis and manuscript writing. X.Z., K.A.O., A.S.-T. and M.K.D. each performed parts of the experiments and participated in aspects of data analyses and manuscript writing. K.K. and J.C. performed laser-induced thrombosis experiments and data analysis; S.C.-T.L. provided talin constructs and purified proteins, and participated in discussions and data analyses; X.D. designed and directed the research, analysed data and wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to X.D. (xd@uic.edu).
Co-immunoprecipitation. As described previously, platelets or CHO-1b9 cells expressing recombinant integrin α<sub>IIb</sub>β<sub>3</sub><sup>-/-</sup> were solubilized in NP40 lysis buffer (50 mM Tris, pH 7.4, 10 mM MgCl<sub>2</sub>, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF), with complete protease inhibitor cocktail tablets (1 tablet per 5 ml buffer, Roche). Lysis debris was cleared after centrifugation at 14,000 g for 10 min. Lysates were then immunoprecipitated with rabbit anti-Go213 IgG, anti-integrin β<sub>3</sub> rabbit serum or an equal amount of rabbit IgG or pre-immune serum for 2 h before Protein A/G sepharose beads were added. After incubation of Protein A/G sepharose beads for 45 min at 4 °C, beads were centrifuged down and washed for six times with NP40 lysis buffer. Immunoprecipitates were analysed by immunoblotting.

RHOA activity assay. Platelets or α<sub>IIb</sub>β<sub>3</sub>-expressing CHO cells modified Tyrode's buffer or adherent on immobilized fibronectin were solubilized in cold NP40 lysis buffer at 4 °C, and debris-cleared lysates were incubated for 1 h with purified GST–RBD beads, washed, and then immunoblotted with an anti-RhoA monoclonal antibody, as described previously.

Bone marrow transplantation. As described previously<sup>12</sup>, bone marrow stem cells were isolated from femur and tibia of 6–8-week-old integrin β<sub>3</sub><sup>-/-</sup> or C57/B6J mice using the MACS lineage cell depletion kit (Miltenyi Biotech). Stem cells were subsequently infected twice with concentrated lentivirus-containing shRNA or cDNA constructs, as described in Animals and Reagents section, using a Lentix-1 concentrator (Clontech). The cells were then retrovirally injected into irradiated recipient mice (5 Gy for integrin β<sub>3</sub><sup>-/-</sup> mice and 9.6 Gy for C57/B6J mice, one million cells per recipient mice) one day after irradiation.

Platelet adhesion assay. As described previously<sup>12</sup>, washed platelets were preincubated with vehicle or peptides, or with either 1 mM MnCl<sub>2</sub> or 0.18 μg/ml IL-8 before experimentation. Carotid arterial thrombosis was induced with a filter paper disc (diameter = 2 mm) soaked with 1.2 μg/ml FeCl<sub>3</sub> and the concentration of PDAM-conjugated peptide was estimated by measuring absorbance at 405 nm. Statistical significance was determined using t-test (n = 3).

Cell spreading, immunofluorescence and confocal microscopy. Washed platelets or α<sub>IIb</sub>β<sub>3</sub>-expressing CHO cells suspended in modified Tyrode’s buffer were added to 100 μg/ml fibronectin (Enzyme Research Laboratories)-coated cover slides and incubated at 37 °C for various lengths of time. Cells were fixed, permeabilized, blocked with 0.5% BSA in modified Tyrode’s buffer, stained with mAb15 (followed by Fluor 488-conjugated anti-mouse secondary antibody, and the corresponding control peptides mP13Scr (Myr-EEARERKDWAKFT), mP23Scr (Myr-EEARERKDWAKFT), mP23Scr (Myr-EEARE), and mP23Scr (Myr-EFAFEE)). The peptides were prepared in DMSO for use and conjugated with PDAM overnight in the dark in DMSO or adherent on immobilized fibrinogen were solubilized in cold NP40 lysis buffer or adherent on immobilized fibrinogen were solubilized in cold NP40 lysis buffer at 4 °C, and debris-cleared lysates were incubated for 1 h with purified GST–RBD beads, washed, and then immunoblotted with an anti-RhoA monoclonal antibody, as described previously. For the fibrinogen binding assay, washed platelets or α<sub>IIb</sub>β<sub>3</sub>-expressing CHO cells suspended in modified Tyrode’s buffer were added to 100 μg/ml fibronectin (Enzyme Research Laboratories)-coated cover slides and incubated at 37 °C for various lengths of time. Cells were fixed, permeabilized, blocked with 0.5% BSA in modified Tyrode’s buffer, stained with mAb15 (followed by Fluor 488-conjugated anti-mouse secondary antibody) and/or Alexa Fluor 546-conjugated phallolidin, and viewed with a Zeiss LSM510 META confocal microscope, as described previously<sup>12</sup>, or with Leica DM IRB fluorescence microscope, Photometrics CoolSNAP HQ camera and μManager software. Cell surface area was measured by NIH Image analysis of 5–10 random images. Statistical significance was determined using t-test.

Clot retraction assay. As previously described<sup>3</sup>, human PRP was incubated with vehicle or peptides for 5 min at room temperature (22 °C) before stimulation with thrombin. The two-dimensional size of retracted clots was quantified using Image J software, and statistical significance was determined using t-test (n = 3).

Peptide inhibitors. Myristoylated peptides were synthesized and purified at the Research Resource Center at the University of Illinois at Chicago. These peptides include: mP13 (Myr-KFEEERARKWDT), mP12 (Myr-EV33), mP23 (Myr-FEEER) and the corresponding control peptides mP13Scr (Myr-EEARERKDWAFTK), mP23Scr (Myr-EEARE), and mP23Scr (Myr-EFAFEE). The peptides were prepared in DMSO for use in vitro, and in micellar formulation for in vivo (and in vitro) use. For micellar formulation, PEG<sub>2000</sub>-DSPE, 1-α-phosphatidylcholine, and peptides were mixed at a molar ratio of 45:5:2. The micelles were suspended to form micelle colloid in HEPES-saline buffer (10 mM HEPES, 150 mM NaCl, pH 7.4), peptide concentration 1 mM as described previously<sup>12</sup>. mP13 is similar to mP13Scr in uptake by platelets (Extended Data Fig. 9a) and does not cause significant changes in hemorragin in vivo (Extended Data Fig. 9b).

Estimation of peptide concentration in platelets. mP13 and mP23Scr peptides were dissolved and conjugated with PDAM overnight in the dark in DMSO, or conjugated in methanol and incorporated into the micelle as described above. Platelets were incubated with the PDAM-conjugated peptides for 5 min at room temperature, pelleted via centrifugation, washed and lysed with NP40 lysis buffer, and the concentration of PDAM-conjugated peptide was estimated by measuring fluorescence intensity (absorbance 340 nm/emission 395 nm) as described previously<sup>12</sup>. Platelet lysates (without peptide incubation) were used as a blank control. Standard curve was obtained using known concentrations of peptides added to platelet lysates.

In vivo FeCl<sub>3</sub>-induced thrombosis and tail bleeding time. 7–8-week-old male mice were anesthetized by isoflurane inhalation. Retro-orbital injection of peptide micelle or integrin (5 μmol kg<sup>-1</sup> mouse weight) were performed 15 min before experimentation. Carotid arterial thrombosis was induced with a filter paper disc (diameter = 2 mm) soaked with 1.2 μl of 7.5% FeCl<sub>3</sub>. Blood flow was...
monitored with a TS420 flow meter using a MA-0.5SB doppler probe (Transonic Systems). Data were analysed using one-way ANOVA. Tail bleeding time analysis were performed as described previously29. Time to stable cessation of bleeding was defined as no evidence of rebleeding for 60 s. Bleeding exceeding 15 min was immediately stopped by applying pressure. Statistical significance was determined using the Mann–Whitney test. Similar results were also obtained with a nonparametric ANOVA. For bleeding assays measuring total blood loss, cut mouse tails were immersed in microcentrifuge tubes with 1.5 ml of 0.15 M NaCl at 37 °C for 15 min. The haemoglobin concentration in the tube was determined using a HemoCue photometer. Data were analysed using one-way ANOVA. The experiments were performed in double-blinded fashion.

Intravital microscopy and laser-induced thrombosis. Similar to the methods described previously27, wild-type male mice (6–8 weeks old) were anaesthetized via intraperitoneal injection of ketamine and xylazine and placed on a thermo-controlled blanket (37 °C). The cremaster muscle was exteriorized and superfused with thermo-controlled (37 °C) bicarbonate-buffered saline for the duration of experiments. Fluorescence and bright-field images were recorded using an Olympus BX61W microscope with a 60×/1.0 NA water immersion objective and a high-speed camera (Hamamatsu C9300) through an intensifier (Video Scope International). Fluorescence images were captured at 20 frames per second, and data were analysed using Slidebook v5.5 (Intelligent Imaging Innovations). Arteriolar wall injury was induced with a micropoint laser ablation system (Photonics Instruments). Platelet accumulation was visualized by infusion of Dylight 649-labelled anti-mouse CD42c (Emfret, 0.05 μg g⁻¹ body weight) into mice. Vehicle control, Integrilin, scrambled peptide or mP6, were infused 3 min before laser injury. Laser-induced thrombi were generated at different sites in the blood vessel, with new sites upstream of earlier thrombi. Data were collected for 5 min following laser injury. The kinetics of platelet accumulation was analysed by median fluorescence values of the antibodies as a function of time in approximately 30 thrombi in three mice per group. Statistical difference of fluorescence intensity (mean ± s.d.) at selected time points was also determined using Welch’s t-test. The experiments were performed in double-blinded fashion.

Statistics. For parametric data, statistical significance was analysed using Student’s t-test (or Welch t-test for samples with nonequal variances) or ANOVA following determination of normal distribution and equal variances. For nonparametric data (bleeding time analysis), Mann–Whitney test was applied. Analyses were performed with GraphPad Prism 4 software. Sample size estimation was performed with Fisher’s exact test using GraphPad InStat 3.

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Extended Data Figure 1 | A schematic showing how selective inhibitors of integrin outside-in signalling work as anti-thrombotics. Blue arrows indicate steps that are inhibited.
Extended Data Figure 2 | The importance of the conserved EXE motif in integrin–Gα13 interactions. a, b, Lysates from CHO cells expressing similar levels of wild-type (WT) αIIbβ3 (a), and β3, EXE motif mutants (E731A, E732A, and E733A to AAA) complexed with wild-type αIIb (b) were immunoprecipitated with anti-Gα13 antibody or equal amount of control rabbit IgG. Immunoprecipitates and lysates (equivalent of 10% used for immunoprecipitation) were immunoblotted with anti-Gα13 and anti-β3 antibodies.

c, d, GST–β3CD (WT) or GST–β3(AAA)CD (AAA mutant) proteins immobilized in glutathione-coated microtitre wells were incubated with increasing concentrations of Gα13 (c), or increasing concentrations of THD (d). After washing, bound Gα13 and THD were respectively detected using anti-Gα13 or anti-talin (mouse IgG was used as a specificity control) followed by secondary horseradish peroxidase-labelled anti-IgG antibody. e, In addition to the AAA mutation, conserved mutations of EEE to DED and EEE to QSE (as found in β5) were introduced to the β3 cytoplasmic domain. These mutants were co-transfected with wild-type αIIb, into CHO cells, which were sorted to achieve comparable expression levels with wild-type-αIIbβ3-expressing cells (as shown in Extended Data Fig. 4e). Lysates from these cells were immunoprecipitated with anti-β3 or equal amount of pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-Gα13 or anti-β1 antibodies. Gα13 is associated with β1, which is increased after thrombin stimulation.
Extended Data Figure 3 | Ligand occupancy induces switch of integrin αIIbβ3 from the talin-bound to the Go13-bound state. a, To determine the effect of integrin activation and ligand occupancy on Go13–β3 association, human platelets were incubated with or without 1 mM MnCl2 and 30 μg ml⁻¹ fibrinogen for 5 min at 22 °C. Platelet lysates were then immunoprecipitated with anti-β3 or pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-β3 or anti-Go13. b, c, Washed human platelets were stimulated with 0.025 U ml⁻¹ a-thrombin with or without adding 2 mM EDTA (an inhibitor of the ligand binding function of integrins), stirred (1,000 r.p.m.) at 37 °C, solubilized at various time points, and immunoprecipitated with anti-β3 or equal amounts of pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-Go13, anti-talin or anti-β3 antibodies. b, Western blot results. c, Turbidity changes in platelet suspension indicating integrin-dependent platelet aggregation. Note the inhibitory effect of EDTA on talin dissociation and Go13 binding to β3. d, As additional controls for Fig. 2a to exclude the possibility of loss of talin and β3 in platelet lysates to insoluble fraction during integrin signalling, washed human platelets were stimulated with 0.025 U ml⁻¹ a-thrombin in the absence or presence of 2 mM integrin inhibitor RGDS, stirred (1,000 r.p.m.) at 37 °C, and then solubilized at various time points as in Fig. 2a. Solubilized platelets were centrifuged at 14,000 g for 10 min to separate lysates from insoluble pellets. Pellets were dissolved in SDS sample buffer to the same volume as the lysates after diluting them 1:1 with 2× SDS sample buffer, and both were immunoblotted with anti-β3 and anti-talin antibodies. Note that the levels of talin and β3 in platelet lysates kept essentially constant during the course of platelet aggregation and, with low concentrations of thrombin used to stimulate platelets, very little insoluble β3 and talin were present in the pellet, which were detectable only after prolonged exposure (5-min exposure compared to 10 s of normal exposure time) and with no obvious variation during the course of platelet aggregation.
Extended Data Figure 4 | Effects of shRNA-induced talin knockdown and talin knockout on integrin signalling. a, Western blot comparison of talin 1 expression levels in mouse platelets derived from control shRNA- or talin-shRNA-transfected bone marrow stem cells. Western blots of Gα13, and integrin β1 and β3 are also shown. b, Adhesion of unstimulated mouse platelets to immobilized fibrinogen for 1 h. Adherent platelets were quantified as percentage of total platelets loaded (mean ± s.d., n = 4). c, Turbidity changes in mouse platelet suspension stimulated with 5 μM ADP in the presence of 20 μg ml⁻¹ fibrinogen, with or without 1 mM MnCl₂, as detected using an aggregometer. d, Fluorescence microscopy images of phalloidin-stained mouse platelet spreading on fibrinogen for 1 h, with or without 1 mM MnCl₂. e, Quantification of surface areas of individual adherent platelets as shown in Fig. 2g (mean ± s.e.m.).
Extended Data Figure 5 | Effects of AAA mutation on integrin outside-in signalling in platelets. **a**, Flow cytometric analysis of integrin αIIbβ3 expression levels in β3−/− mouse platelets transfected with wild-type or AAA mutant β3 using bone marrow stem cell transplantation technology in comparison with C57BL/6 mouse platelets. β3−/− platelets were used as a negative control. αIIbβ3 complex was detected using an anti-mouse αIIb antibody. **b**, Mouse platelets expressing recombinant wild-type or AAA mutant β3 as in a were lysed and immunoprecipitated with anti-β3 or equal amounts of pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-SRC or anti-β3 antibodies. **c, d**, Spreading of phalloidin-stained wild-type platelets (EEE), AAA mutant platelets and AAA mutant platelets incubated with mP6Scr or mP6 on immobilized fibrinogen for 1 h. **c**, Typical fluorescence microscopy images. **d**, Quantification of surface areas of individual platelets (mean ± s.e.m.).
Extended Data Figure 6 | Effects of mutational disruption of the EXE motif on integrin outside-in signalling. a, Expression levels of wild-type or the EXE motif (QSE, DED or AAA) mutants of β3 in complex with wild-type αIib in CHO cells, as determined by flow cytometry. Mouse IgG was used as a negative control. b, Spreading of CHO-1b9 cells expressing wild-type αIibβ3 and QSE, DED or AAA mutant αIibβ3 on fibrinogen for 1 h. Quantification of surface areas of individual cells (mean ± s.e.m.). c, Typical microscopy images. d, Flow cytometric analysis of wild-type αIibβ3, AAA or Y747A mutant αIibβ3 expression in CHO cells. Mouse IgG was used as a control. e, CHO cells expressing wild-type, AAA or Y747A β3 without (top panels) or with (bottom panels) co-expression of recombinant THD were solubilized and immunoprecipitated with anti-β3 or pre-immune serum. 10% lysates and immunoprecipitates were immunoblotted with anti-talin, anti-Gα13 or anti-β3 antibodies. f, Typical western blots for Fig. 3g. Wild-type or AAA-mutant-αIibβ3-expressing CHO-1b9 cells were allowed to adhere to immobilized fibrinogen, solubilized at various time points, and analysed for RHOA activation and SRC Tyr 416 phosphorylation.
Extended Data Figure 7 | mP6 selectively inhibits integrin outside-in signalling without affecting inside-out signalling. a–d. Washed human platelets were stimulated with 0.025 U ml\(^{-1}\) α-thrombin in the absence or presence of 250 μM myristoylated peptides, mP13 (a, b) and mP6 (c, d) with stirring (1,000 r.p.m.) at 37 °C, and then solubilized at various time points. Lysates were immunoprecipitated with anti-β₃ rabbit serum or equal amounts of pre-immune serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-Gα₁₃, anti-talin or anti-β₃ antibodies. a, c, Typical western blot results. b, d, Typical turbidity changes in platelet suspension indicating integrin-dependent platelet aggregation. e, Quantification of human platelet spreading on immobilized fibrinogen for 1 h, without or with treatment with DMSO, mP₆Scr, or mP₆ as shown in Fig. 4b (mean surface area ± s.e.m.). f, Flow cytometric analysis of PAR4-AP-induced Oregon Green-labelled soluble fibrinogen binding to human platelets pre-treated with 100 μM mP₆Scr or 100 μM mP₆ stimulated with increasing concentrations of PAR4-AP. Integrilin-treated platelets were used as a negative control. g, Flow cytometric analysis of 100 μM PAR4-AP-induced PAC1 binding to human platelets pre-treated with 100 μM mP₆Scr or mP₆. Integrilin-treated platelets were used as negative control. h, Flow cytometric analysis of PAR4-AP-induced Oregon Green-labelled soluble fibrinogen binding to human platelets pre-treated with solvent DMSO, mP₁₃Scr or mP₁₃. Resting platelets were used as a negative control.
Extended Data Figure 8 | The in vivo effect of mP6: selective inhibition of thrombosis but not haemostasis. a, Representative images of laser-induced mouse cremaster arteriolar thrombosis (red) in the context of the bright-field microvascular histology, visualized by infusion of nonblocking rat anti-mouse GP Ibα antibody conjugated to DyLight 649. The C57BL/6 mice were injected with 5 μmol kg$^{-1}$ micellar formulated mP6 or mP6Src (negative control), 12 μmol kg$^{-1}$ Integrillin or buffer, 3 min before laser-induced arteriolar wall injury. White arrows indicate the directions of the blood flow. b, The mean platelet fluorescence intensity for 30 thrombi (performed in three mice) for each treatment at selected time points (mean ± s.e.m., n = 30, t-test). Fluorescence in mP6- and Integrillin-treated mice is minimal. c, Comparison of mP6 (5 μmol kg$^{-1}$) with the same dose of Integrillin and their respective controls in occlusion time of FeCl$_3$-induced carotid artery thrombosis in mice. Typical arterial blood flow charts of FeCl$_3$-induced occlusive thrombosis are shown. d, Comparison of mP6 (5 μmol kg$^{-1}$) with the same dose of Integrillin and controls in mouse tail bleeding analysis. Released haemoglobin levels were used as a parameter to assess blood loss (mean ± s.d., n = 10).
Extended Data Figure 9 | Platelet uptake of mP6 and mP6Scr, and no effect of mP6 on hemogram.  a, Estimation of intracellular levels of 1-pyrenyl diazomethane (PDAM)-conjugated mP6 and mP6Scr following incubation with platelets for 5 min. Platelets were pelleted by centrifugation, and the amounts of PDAM-conjugated peptides in platelet lysates were estimated (mean ± s.d., n = 3). b, Haemogram of mouse whole blood before or 1 h after injection of mP6 or mP6Scr (5 μmol kg⁻¹), showing no significant differences.