Supporting Online Materials

Materials and Methods

Reagents

Myristoylated $G\alpha_{13}$ SRI peptide, Myr-LLARRPTKGIHEY (mSRI), and myristoylated-scrambled peptide (Myr-LIRYALHRPTKEG) were synthesized and purified at the Research Resource Center at University of Illinois, Chicago or kindly provided by Dr. G. Le Breton, University of Illinois, Chicago (*S1*). Expression and purification of recombinant $G\alpha_{13}$ was described previously (*S2*). Anti-RhoA antibody and cell permeable C3 transferase (C3 toxin) were purchased from Cytoskeleton, Inc.; anti-$G\alpha_{13}$(SC410), anti-c-Src (sc-18) and anti-mouse integrin β_3 (sc-6627) antibodies were from Santa Cruz Biotechnology, Inc; anti-phospho-Src Y^{416} antibody was obtained from Cell Signaling; anti-human integrin β_3 antibody, MAb 15 and anti-$\alpha_{IIb}\beta_3$ antibody, D57, were kindly provided by Dr. Mark Ginsberg (University of California, San Diego, La Jolla, CA); anti-GPIb monoclonal antibody LJP3 was kindly provided by Dr. Zaverio Ruggeri, the Scripps Research Institute, La, Jolla, CA); anti-tubulin and anti-flag antibodies were purchased from Sigma-Aldrich; lipofectamine 2000, viraPower lentivirus expression system, Alexa Fluor 546-conjugated phalloidin, Alexa Fluor 633–conjugated phalloidin, and Alexa Fluor 546–conjugated anti-mouse IgG antibody were from Invitrogen; Y27632 was purchased from Calbiochem.

Platelets preparation, platelet spreading and clot retraction.

Studies using human platelets were approved by the Institutional Review Board of University of Illinois at Chicago. Human washed platelets were prepared from freshly drawn blood of healthy

volunteers and resuspended in modified Tyrode's buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10 mM HEPES, pH 7.4)(*S3, S4*). Animal studies were approved by the institutional Animal Care Committee of University of Illinois at Chicago. Blood was freshly drawn from the inferior vena cava in isoflurane-anethetized mice. Mouse platelets were isolated and washed using methods previously described (*S4, S5*). For analyzing platelet spreading on integrin ligand fibrinogen, washed platelets were allowed to spread on 100 μg/ml fibrinogen-coated coverslips at 37°C for 90 minutes, stained and viewed with a Leica RMI RB microscope or Zeiss LSM510 META confocal microscope as previously described (*S5*). Clot retraction was analyzed with the previously described method (*S6, S7*). Briefly, mouse platelets (6×10^8/ml) were resuspended in platelet-depleted human plasma, and 0.4 U/ml α-thrombin was added to initiate coagulation. The clots were photographed at various time points. Sizes of retracted clots on photographs were quantified using NIH Image J software. Statistical significance was determined using t-test.

Co-immunoprecipitation and binding assays

Human platelets or CHO cells expressing recombinant integrin $\alpha_{IIb}\beta_3$ were solubilized in modified RIPA Buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF), or RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and complete protease inhibitor cocktail tablets (1 tablet/5 ml buffer, Roche). As previously described(*S7*), cell lysates were incubated with 2 μg/ml of D57 (antibody to integrin $\alpha_{IIb}\beta_3$), LJP3(antibody against GPIb), or mouse IgG, and further incubated with protein G-conjugated Sepharose beads. Cell lysates were also incubated with

rabbit anti-Gα_{13} IgG antibody (1.5 μg/ml) or an equal amount of rabbit IgG and further incubated with protein A-conjugated Sepharose beads. After 3-6 washes with lysis buffer, immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blots with antibodies against β_3 (MAb15), GPIb (anti-IbαC (*S8*)) or Gα_{13}. In some experiments, 1 μM GDP, 1 μM GTPγS or 30 μM AlF_4^- were added to the reaction to assess the effect of Gα_{13} activation on integrin binding. In other experiments, 250 μM mSRI or scrambled control peptide was incubated with platelet lysates prior to immunoprecipitation. GST bead pull down analysis was previously described (*S7*). Purified Gα_{13} was incubated with glutathione beads-bound to GST, GST-β_1CD or GST-β_3CD at 4°C overnight. Bead-bound proteins were analyzed by immunoblotting. For GST-β_3CD cDNA construction, integrin-β_3 cytoplasmic domain (716-762) cDNA was generated by PCR and cloned into pGEX-4T2 vector using Bam HI and Xho I restriction sites. The forward primer is 5'-CGTGGATCCAAACTCCTCATCACCATCCACGACC-3'; the reverse primer is 5'-GCGCTCGAGTTAAGTGCCCCGGTACGTGATATTG-3'. For GST-β1CD cDNA construction, β_1 cytoplasmic domain (752-798) cDNA was amplified by PCR and cloned into pGEX-4T1 vector using EcoRI and Xho I restriction sites. The primer sequences are: (1) forward: 5'-GCGAATTCAAGCTTTTAATGATAATTCATGAC-3'; (2) reverse: GCGCTCGAGTCATTTTCCCTCATACTTCGGATT-3'. GST, GST-β_1CD and GST-β_3CD were purified from BL21 (DE3) *E. coli* using glutathione-conjugated beads.

Expression of wild type Gα_{13} and truncation mutants for β3 binding

Human $G\alpha_{13}$ cDNA (*S9*) was tagged with Flag-epitope at N-terminus using PCR with the Flag cDNA sequence incorporated into the forward primer, and then subcloned into pCDEF3 vector using Kpn I and Not I restriction sites. The forward primer sequence is 5'-GCGGGTACCGCCATGGACTACAAGGACGACGATGACAAGGCGGACTTCC-TGCCGTCGCGGTCCGT-3'; The reverse primer sequence is 5'- GGCCGGCGGCCGCTCACT-GTAGCATAAGCTGCTTGAGGTT-3'. Truncation mutants of $G\alpha_{13}$ were generated using PCR with reverse primer sequences 5'-GGCCGGCGGCCGCTCAAATATCTTGTTGTGATGGAAT-ATAATCTGGTTCTCCAAGTTTATCCAAG-3' for mutant 1-196; and 5'-GGCCGGCGGCCGCTCATTCAAAGTCGTATTCATGGATGCC-3' for mutant 1-212.

cDNA encoding Flag-tagged wild type $G\alpha_{13}$ or $G\alpha_{13}$ mutants were transfected into 293FT cells using lipofectamine 2000. Cell lysates were prepared 48 hours after transfection. Flag-tagged wild type or mutant $G\alpha_{13}$ in 293FT cell lysates were incubated with glutathione bead-bound GST or GST-β_3CD at 4°C overnight. After centrifugation and washing, bead-bound proteins were immunoblotted with anti-Flag antibody.

RhoA activity assay.

Platelets in modified Tyrode's buffer or adherent on immobilized fibrinogen were lysed quickly in 0.8 ml lysis buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 500 mM Nacl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 μg/ml each of aprotinin and leupeotin, 1 mM phenylmethylsulfonyl fluoride, and 200 μM sodium vanadate). Lysates were cleared by centrifugation at 18,000 g for 2 minutes at 4°C, and the supernatant was incubated for 1 hour with 30 μg purified GST-Rhotekin RhoA-binding domain fusion protein (GST-RBD) bound to

glutathione-Sepharose beads(*S10*). Samples were washed three times using washing buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100) and then immunoblotted with an anti-RhoA monoclonal antibody. Cell lysates were also immunoblotted with anti-RhoA as loading control.

Interference of $G\alpha_{13}$ expression with siRNA, rescue with siRNA-resistant $G\alpha_{13}$, and bone marrow transplantation.

Two different $G\alpha_{13}$ siRNA target sequences were used: siRNA #1, 5'-GTCCACCTTCCTGAAGCAG; siRNAi #2, 5'- GGAGATCGACAAATGCCTG. Scrambled siRNA sequence is: 5'- GAGGAGCCGACGCTTAATA-3'.These sequences are conserved in hamsters and mice. Lentivirus was prepared by co-transfection of pLL3.7-scrambled siRNA or pLL3.7-$G\alpha_{13}$ siRNA (#1 and #2) with pLP1, pLP2 and pLP/VSVG plasmids (Invitrogen) into ~90% confluent 293FT cells using Lipofectamine 2000. 48 hours after transfection, cell culture medium containing virus was filtered, titered and stored at -80°C. Bone marrow cells from 6-8 week old healthy C57/BL mice were isolated aseptically from femurs and tibias. Stem cells were negatively selected by MACS Lineage cell depletion kit (Miltenyi Biotec) and cultured in RPMI 1640 complete medium with 10 ng/ml interleukin-3, 10 ng/ml interleukin-6, 10 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), and 100 ng/ml stem cell factor (SCF). 50 multiplicity of infection (MOI) lenti-virus was used to infect mice bone marrow stem cells twice with 6 μg/ml polybrene. 48 hours after infection, 5×10^6 stem cells resuspended in PBS were transplanted by retrobulbar injection into lethally irradiated (10.5Gy) C57/BL mice one day after irradiation (*S11*). The siRNA-resistant mutants of $G\alpha_{13}$ were generated by PCR.

These mutants changed the $G\alpha_{13}$ siRNA #1 target sequence to 5'-GTCCACCTTttTaAAGCAG-3' or siRNA #2 target sequence to 5'- GGAGATCGAtAAgTGCCTG-3' without changing the amino acid sequence of $G\alpha_{13}$. The mutants were subcloned into pLenti6/V5-Dest vector using EcoR I and Sal I restriction sites in PCR fragments and EcoRI and Xho I restriction sites in the vector. The primer sequences are as follows: (1) Flag tagged forward primer: 5'-CGGAATTCG-CCATGGACTACAAGGACGACGATGACAAGGCGGACTTCCTGCCGTCGCGGTCCGT-3'; (2) reverse primer: 5'-GCCGTCGACTCACTGTAGCATAAGCTGCTTGAGGTT-3'; (3) mutation site forward primer for resistance to siRNA#1: 5'-GTCCAAGGAGATCGATAAG-TGCCTGTCTCGGGAA-3'; (4) mutation site reverse primer for resistance to siRNA #1: 5'-TTCCCGAGACAGGCACTTATCGATCTCCTTGGAC-3'; (5) mutation site forward primer for resistance to siRNA#2: 5'-CGGCAAGTCCACCTTTTTAAAGCAGATGCGGATC-3'; (6): mutation site reverse primer for resistance to siRNA#2: 5'-GATCCGCATCTGCTTTAAAAAGGTGGACTTGCCG-3'.

CHO cells expressing human $\alpha_{IIb}\beta_3$ cells (123 cells) were transfected with $G\alpha_{13}$ siRNA construct with or without cotransfection of Flag-tagged siRNA resistant-$G\alpha_{13}$ plasmid using lipofectamine 2000. After 30 hours, the cells were detached by 0.53 mM EDTA in phosphate-buffered saline, and allowed to spread on 100 μg/ml fibrinogen. For c-Src phosphorylation, cells were solubilized in SDS-sample buffer and immunoblotted with anti c-Src pY416 antibody. For immuno-staining experiments, 123 cells co-transfected with $G\alpha_{13}$ siRNA plasmid and siRNA-resistant $G\alpha_{13}$ plasmid were allowed to adhere to 100 μg/ml fibrinogen for 1 hour, fixed by 4% paraformaldehyde, and stained by anti-flag antibody and Alexa Fluor 546-conjugated secondary

antibody, and Alexa Fluor 633–conjugated phalloidin. Images were obtained using a Zeiss LSM510META confocal microscope.

Quantitation and statistics.

Western blot bands were scanned, and analyzed for uncalibrated optical density using NIH Image J software. Student *t*-test was used to determine statistical significance.

Additional Discussions

Our data show that thrombin-induced transient increase in RhoA activity is associated with a reciprocal decrease in $G\alpha_{13}$-β_3 interaction, and that an increase in $G\alpha_{13}$-β_3 interaction following ligand binding to $\alpha_{IIb}\beta_3$ is associated with a reciprocal inhibition of RhoA activity (Fig. 4). This phenomenon is possibly duo to competition between RhoGEF and β_3 for $G\alpha_{13}$ binding, because both RhoGEF and β_3 interact with the SRI in $G\alpha_{13}$. Thus, thrombin-induced activation of $G\alpha_{13}$ binding to RhoGEF inhibits $G\alpha_{13}$-β_3 interaction. Ligand binding to integrins greatly enhances integrin-$G\alpha_{13}$ interaction and possibly reduces $G\alpha_{13}$-RhoGEF interaction. This competition may be an additional mechanism contributing to integrin-mediated RhoA inhibition.

Supplementary Figures and Legends

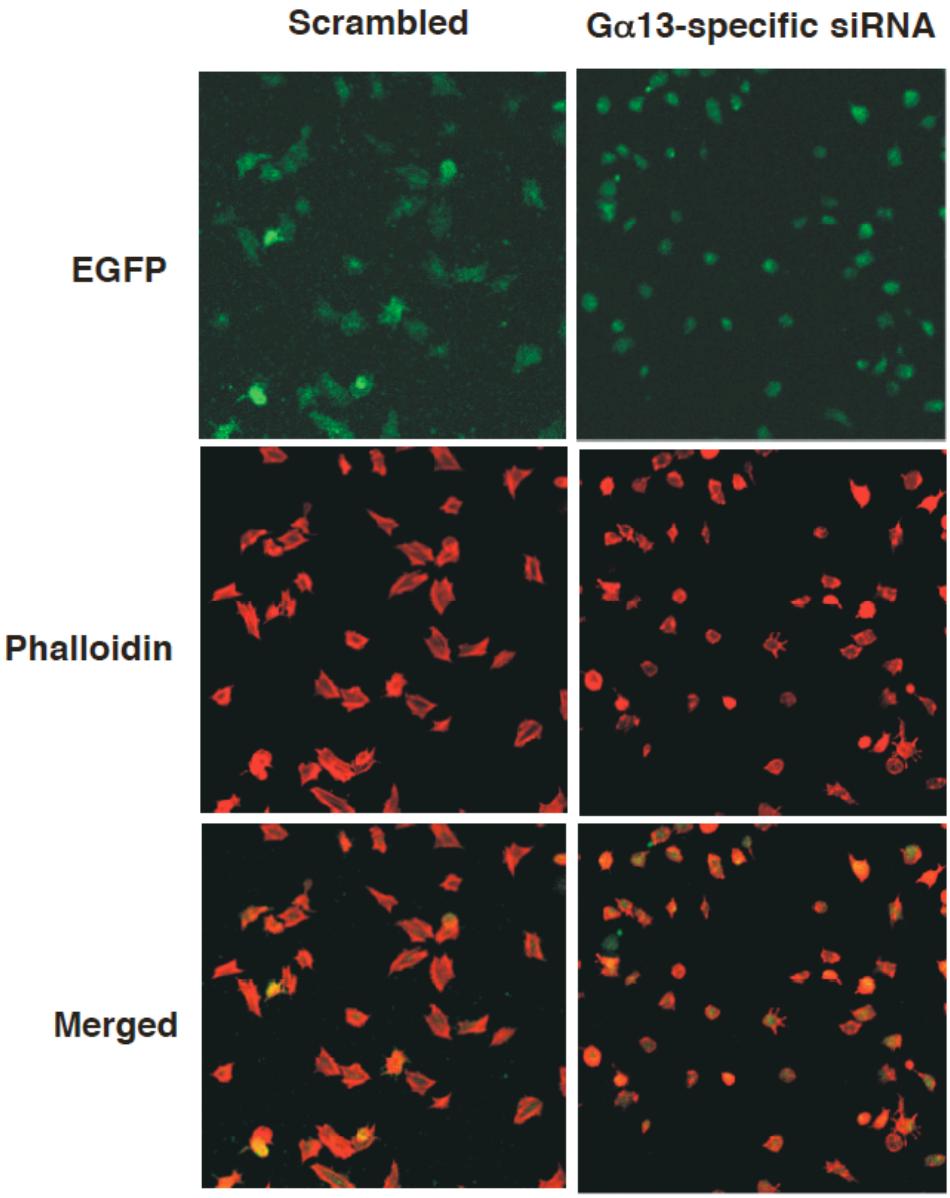

Fig. S1. Efficiency of platelet replacement by irradiation and transplantation of lentivirus-infected bone marrow stem cells. Five weeks after high dose irradiation and transplantation of bone marrow stem cells infected with scrambled siRNA- or $G\alpha_{13}$-specific siRNA#1- encoding lentivirus, platelets were isolated from recipient mice, and allowed to adhere to immobilized fibrinogen. Platelets were imaged using a Zeiss LSM 510 META confocal microscope. Green: EGFP fluorescence indicating that platelets are derived from transplanted stem cells. Red: Alexa Fluor 546-conjugated phalloidin staining indicating total platelets.

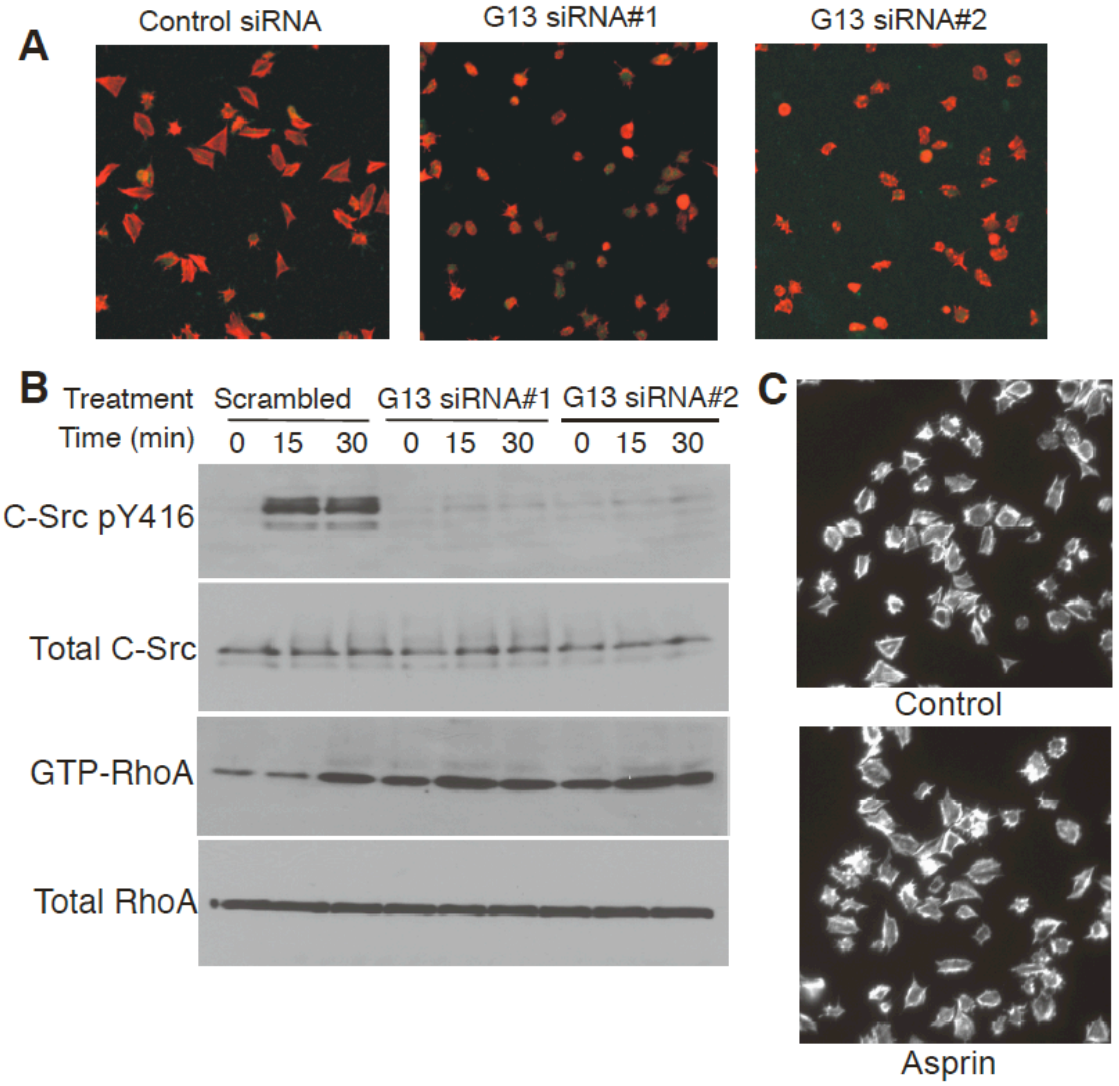

Fig. S2. Similar effects of two different Gα_{13} siRNA on platelet spreading, c-Src activation and RhoA activity, and the effect of aspirin on platelet spreading. (A) Confocal microscopy images of spreading mouse platelets transfected with scrambled siRNA, Gα_{13} siRNA #1- and Gα_{13} siRNA #2 on immobilized fibrinogen. Merged EGFP green fluorescence and Alexa Fluor 546-conjugated phalloidin red fluorescence. (B) Scrambled siRNA, Gα_{13} siRNA#1- and Gα_{13} siRNA#2-transfected platelets were allowed to adhere to immobilized fibrinogen for the indicated time, and analyzed for c-Src activation and RhoA activity. Note that two different siRNA similarly inhibited platelet spreading and c-Src activation, and accelerated activation of RhoA. (C) Mouse platelets were pre-incubated with or without 1 mM aspirin for 30 minutes at room temperature, and then allowed to spread on immobilized fibrinogen.

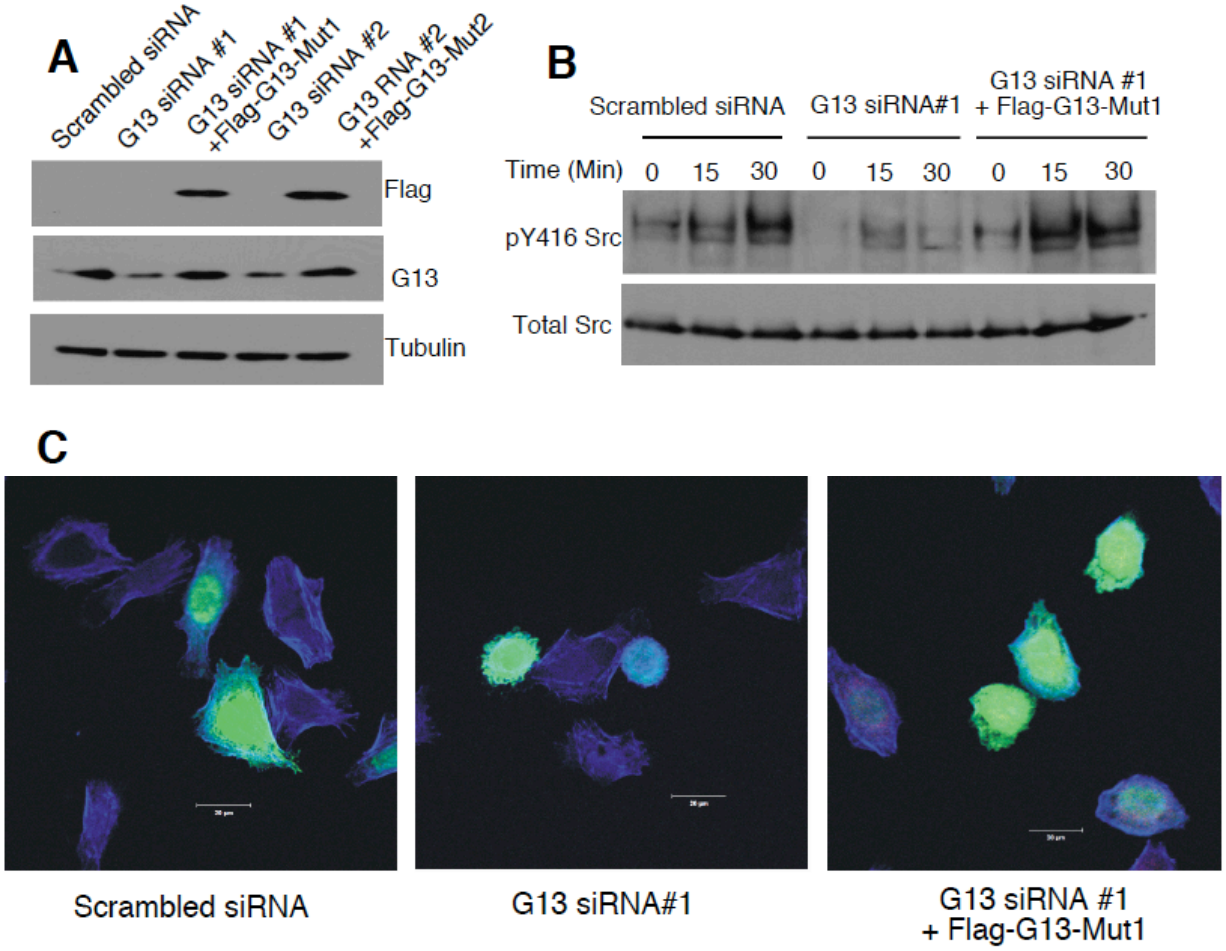

Fig. S3. Inhibitory effects of Gα_{13} siRNA on cell spreading and c-Src phosphorylation in CHO cells expressing integrin $\alpha_{IIb}\beta_3$ and its rescue by an siRNA-resistant mutant of Gα_{13}. Stable CHO cell line expressing integrin $\alpha_{IIb}\beta_3$ (123 cells) were transfected with cDNA constructs encoding EGFP and scrambled control siRNA or Gα_{13} siRNA with or without co-transfection of Flag-tagged siRNA-resistant mutants of Gα_{13} cDNA constructs (Flag-G13-Mut1). (A) Cells were solubilized and immunoblotted with anti-Gα_{13} antibody, anti- Flag (for detecting Flag-tagged Gα_{13}) and an antibody to tubulin (loading control). (B) Cells were plated on fibrinogen-coated surfaces for various lengths of time, solubilized, and lysates were immunoblotted for c-Src phosphorylation at Y416 to indicate c-Src activation, or total amount of c-Src. (C) Cells adherent to fibrinogen were stained with Alexa Fluor 633-labeled phalloidin (artificial blue color) and Alexa Fluor 546-conjugated anti-Flag antibody (Red), and imaged using a Zeiss LSM 510 META confocal microscope. Cells that were successfully transfected with siRNA constructs express EGFP and thus show green fluorescence. Note that Gα_{13} siRNA inhibited integrin-dependent c-Src activation and cell spreading on fibrinogen, which was rescued by expressing Flag-G13-Mut1.

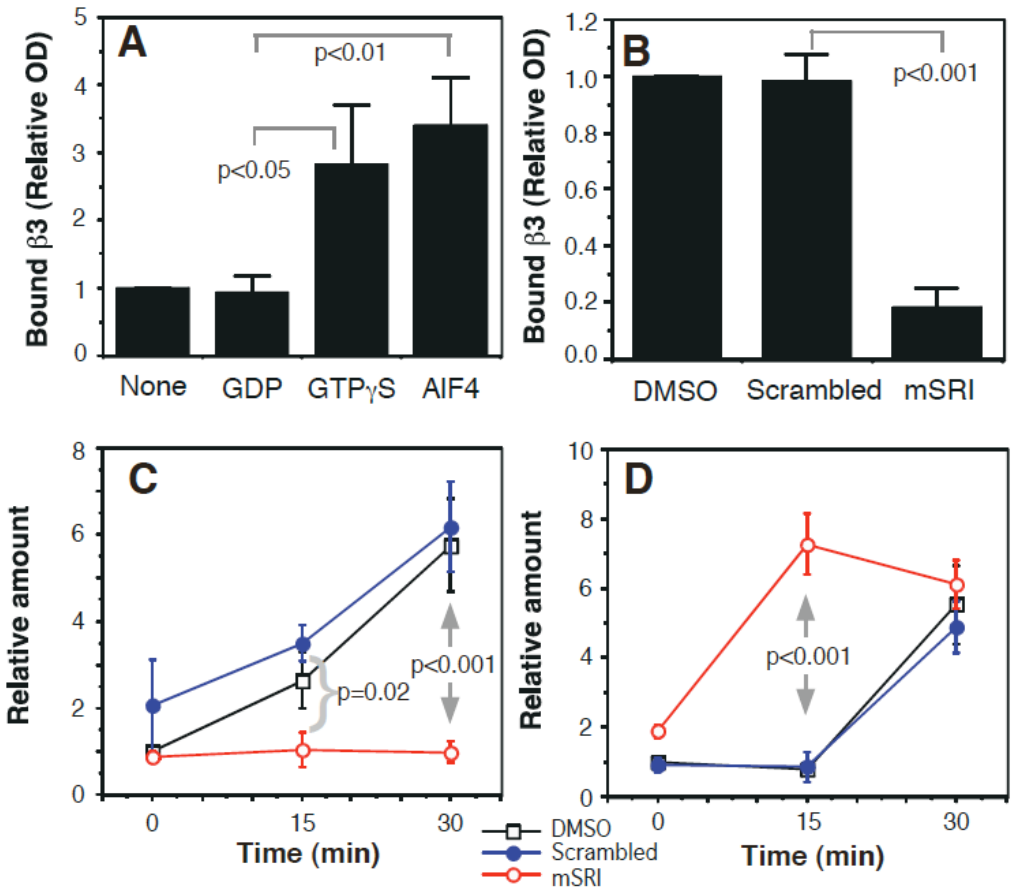

Fig. S4. Quantitative data from experiments shown in Fig. 2 and Fig. 3. (A) Quantitative data for Fig. 2A, showing that co-immunoprecipitation of β_3 with $G\alpha_{13}$ was enhanced by GTP and AlF_4^-. (B) Quantitative data for Fig. 2G, showing mSRI inhibited co-immunoprecipitation of β_3 with $G\alpha_{13}$. (C and D) Quantitative data from Fig. 3A showing that mSRI inhibited c-Src activation (C) and accelerated RhoA activation (D). All data are expressed as mean±SD from 3 experiments. Statistical significance was determined using Student t-test.

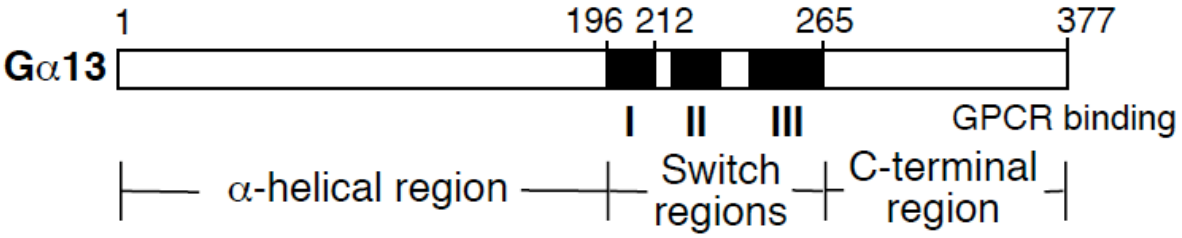

Fig. S5. A schematic of $G\alpha_{13}$ with switch regions indicated. Two $G\alpha_{13}$ truncation mutants were developed to map the β3 binding site (See Fig 2F): (1) the mutant encoding a $G\alpha_{13}$ fragment containing residues 1-196 lacking switch region I, and (2) the mutant encoding a $G\alpha_{13}$ fragment (residues 1-212) containing switch region I.

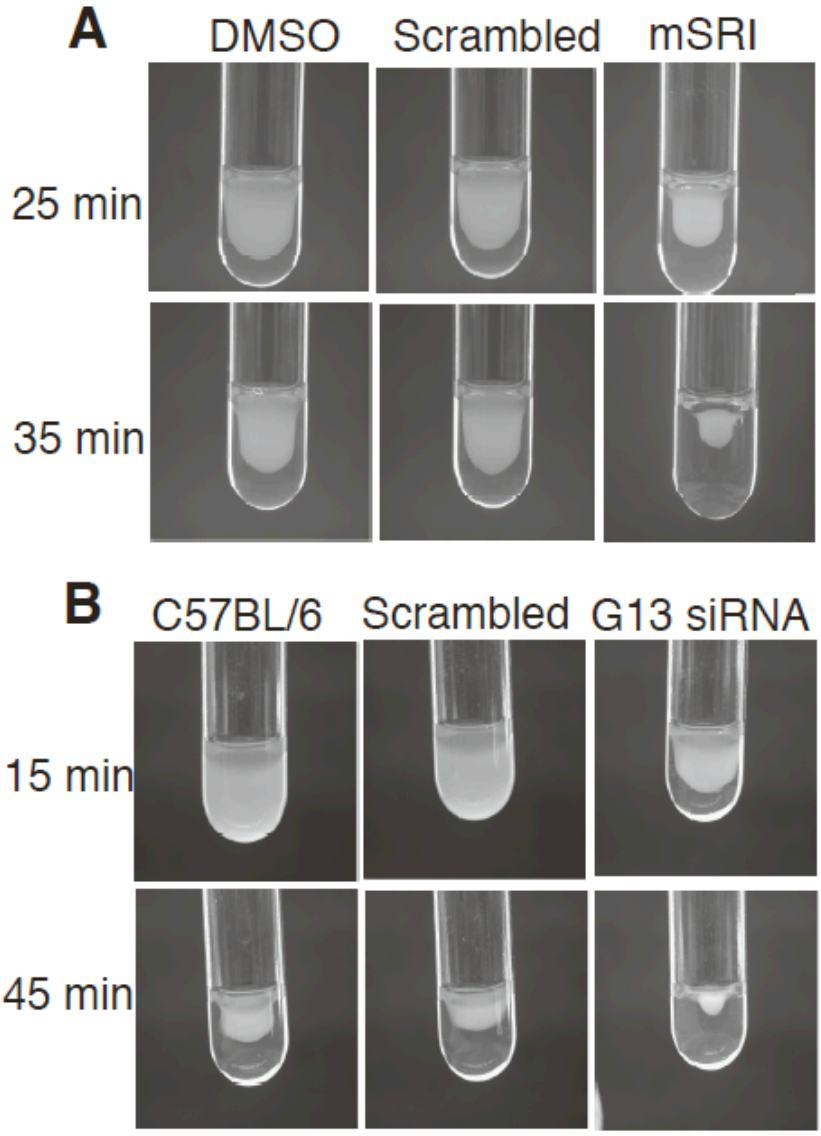

Fig. S6. Typical images of clot retraction showing the effects of mSRI and Gα13-knockdown on platelet-mediated clot retraction. (A) Effect of 250 μM mSRI peptide on clot retraction of human platelet-rich plasma compared with DMSO and scrambled peptide. Quantitative data are shown in Fig. 4A. (B) Comparison of clot retraction mediated by control siRNA platelets and Gα13-knockdown platelets. Quantitative data are shown in Fig. 4B.

References

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