Molecular Pharmacology

Pro32Pro33 mutations in the integrin β3 PSI domain results in αIIbβ3 priming and enhanced adhesion: reversal of the hypercoagulability phenotype by the Src inhibitor SKI-606

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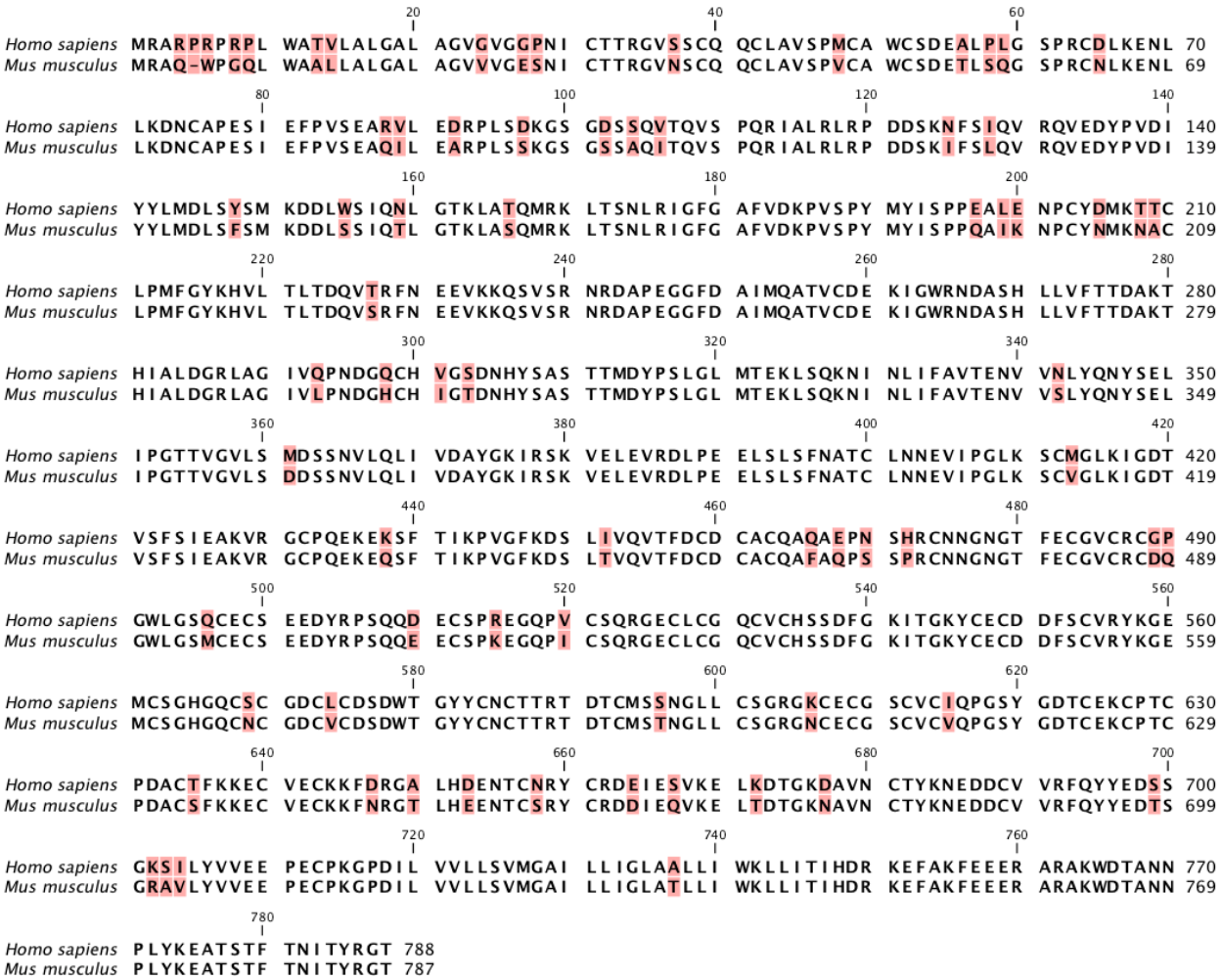
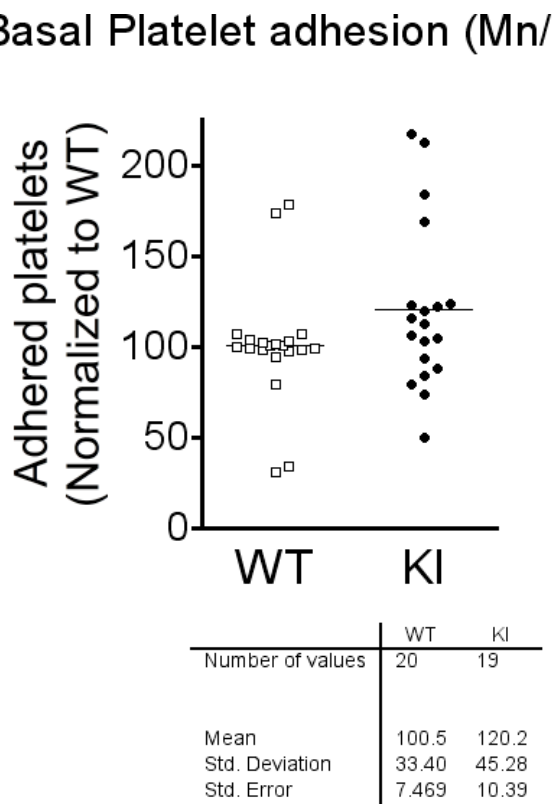
Supplemental Figures

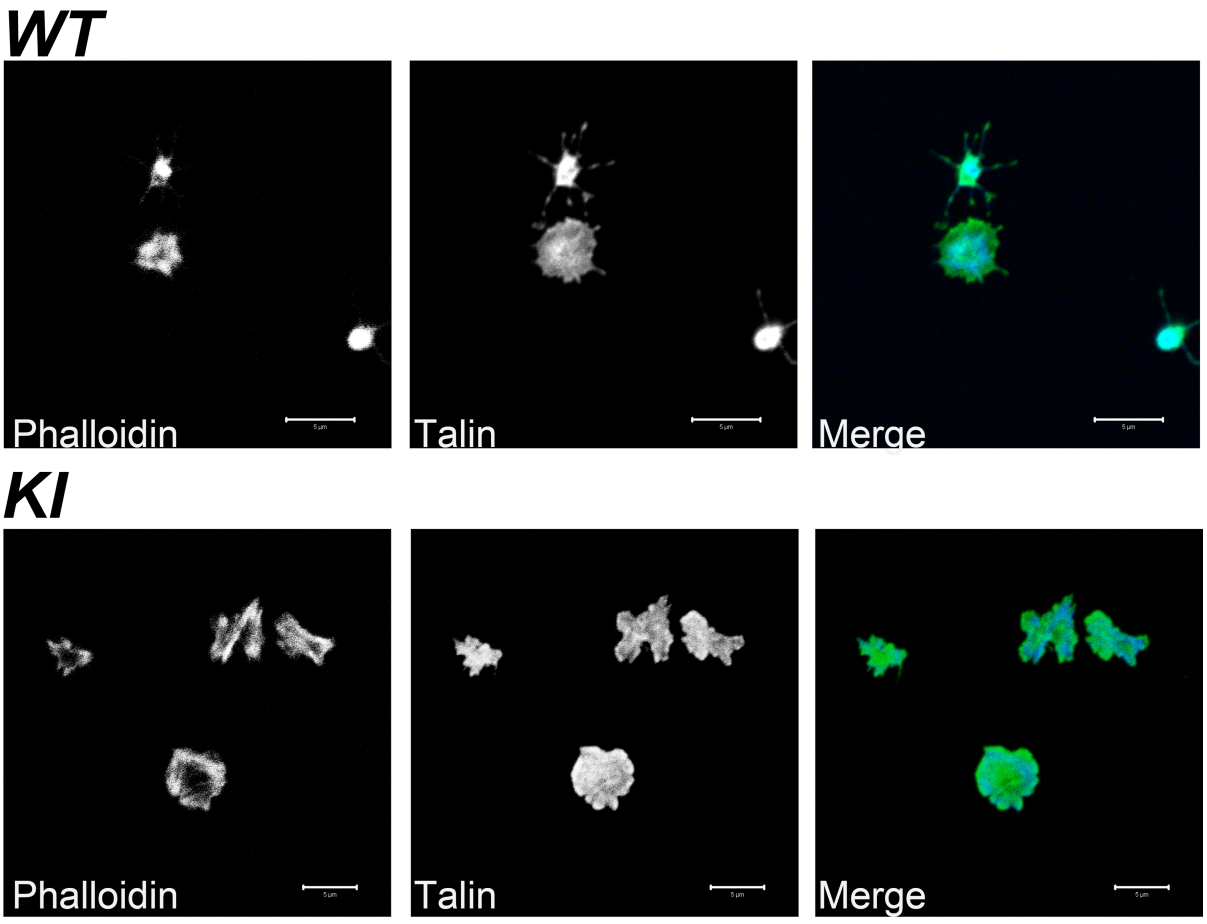


Supplemental Figure 1: Protein sequence alignment of the mature integrin β3 from humans (NP_000203) and mice (NP_058060). Non-identical residues are colored in pink.

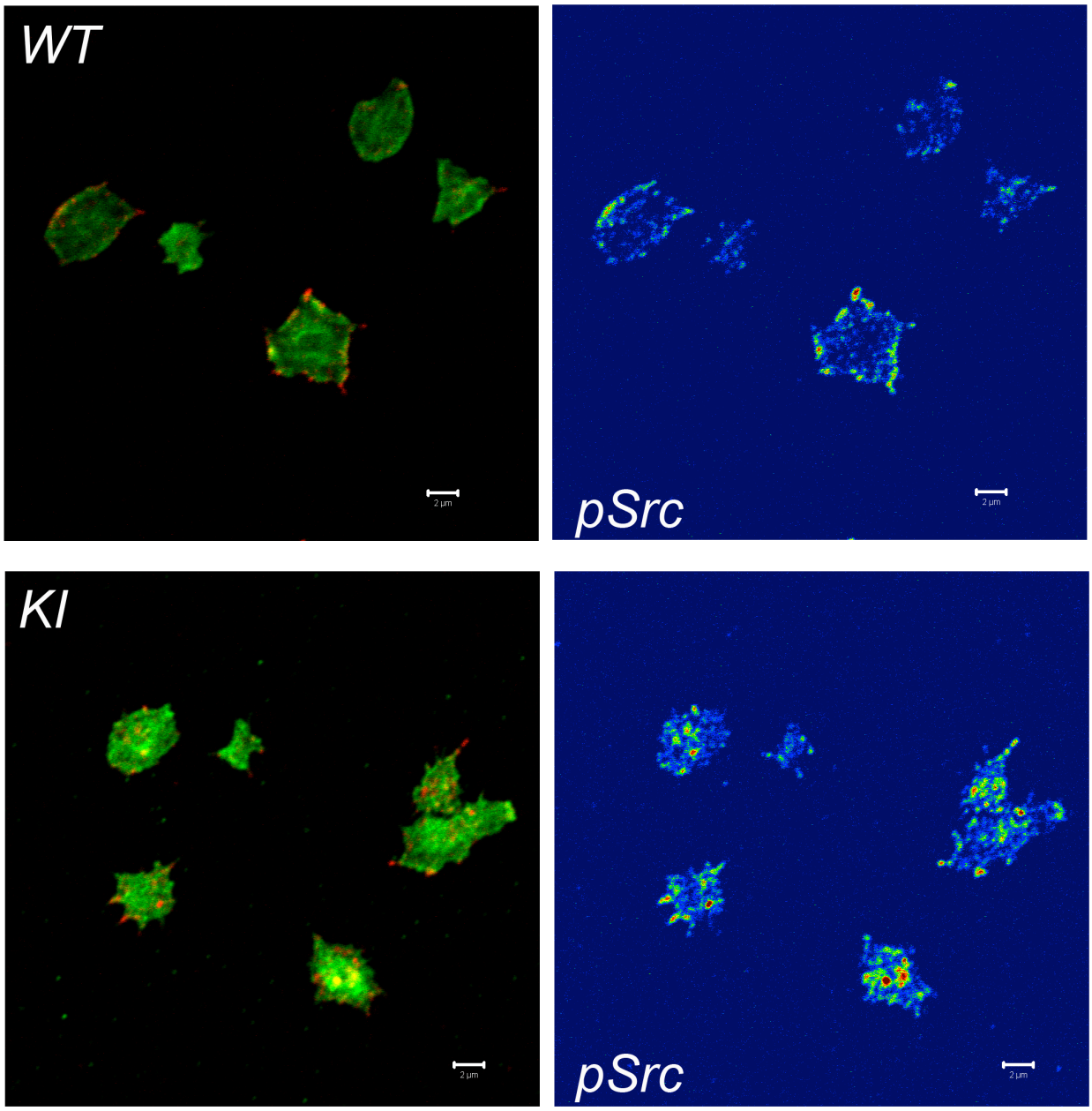


	WT	KI
Number of values	20	19
Mean	100.5	120.2
Std. Deviation	33.40	45.28
Std. Error	7.469	10.39

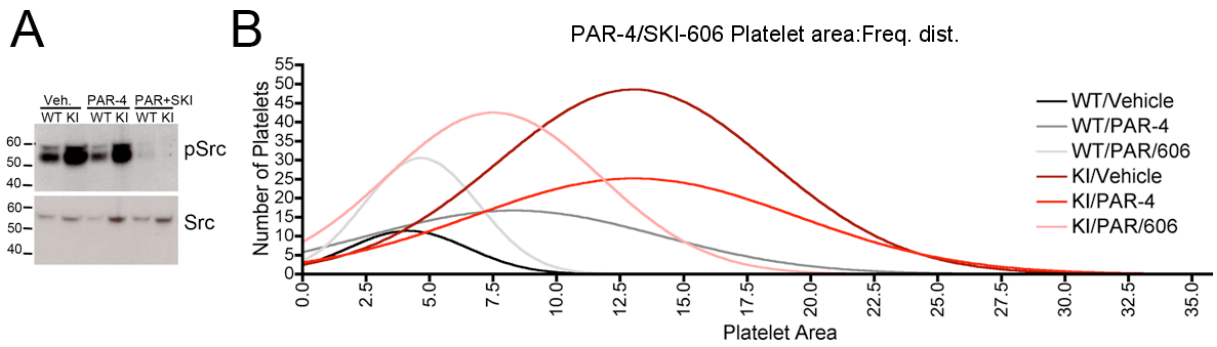
Supplemental Figure 2: Basal platelet adhesion onto fibrinogen: Platelets were resuspended in 1x PBS and seeded onto 25µg/ml fibrinogen for 15 minutes at 37ₒC. The number of adhered platelets was assessed indirectly by in cell western of β-actin.



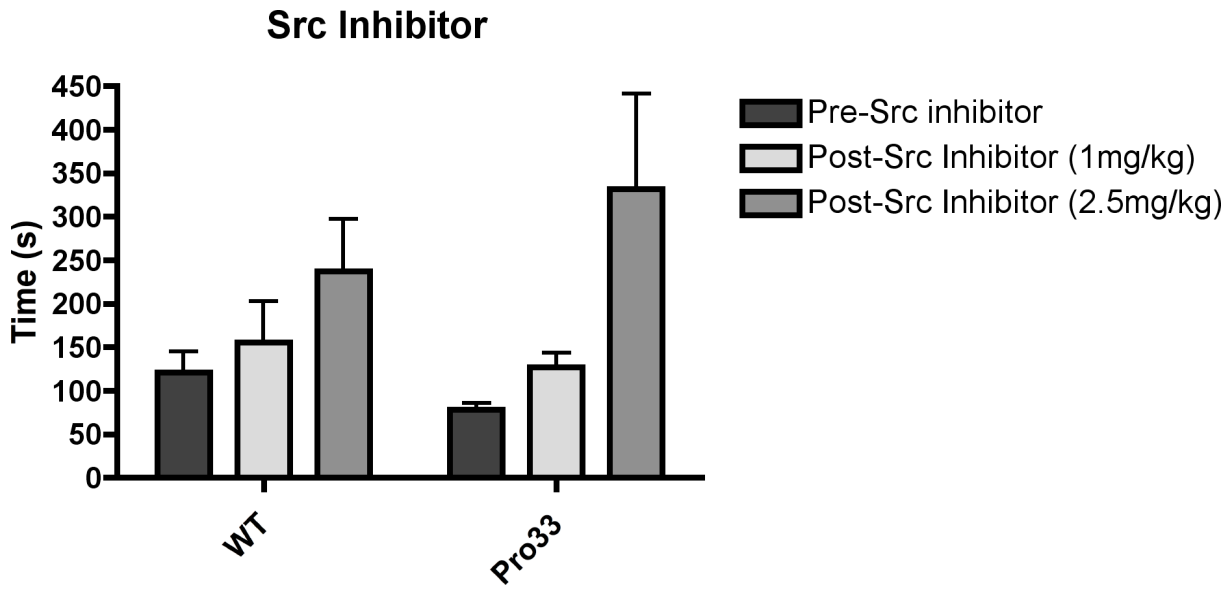
Supplemental Figure 3: Platelet adhesion onto fibrinogen: Platelets were resuspended in 1x KRH and seeded onto 25µg/ml fibrinogen-coated coverslips for 15 minutes at 37$_{o}$C. Platelets were fixed and permeabilized for immunocytochemistry and confocal imaging. Platelets were stained with Phalloidin (blue) and Talin (green). Talin staining was used to calculate platelet area in Image J software.



Supplemental Figure 4: Src phosphorylation in adhered platelets. Platelets were resuspended in 1x KRH and seeded onto 25µg/ml fibrinogen-coated coverslips for 15 minutes at 37^{o}C. Platelets were fixed and permeabilized for immunocytochemistry and confocal imaging. Platelets were stained with pSrc (red) and Talin (green). Gradient image for pSrc is shown in the right panels, with increased fluorescence in red.



Supplemental Figure 5: Inhibition of Src by SKI-606 reduces PAR-4-induced platelet spreading. A, western blot of Src and phospho-Src(Tyr$_{416}$) show significant reductions in c-Src phosphorylation in platelets activated with PAR-4 AP. B, Distribution of platelet area in WT and KI platelets treated with PAR-4 AP and PAR-4 AP + SKI-606. Data compiled from confocal imaging and image J analysis of talin immunocytochemistry.



Supplemental Figure 6: Clotting times after *in vivo* administration of SKI-606. Concentration curve showing increased clotting times in the presence of SKI-606.

Supplemental Table 1: Complete Blood Cell Count for Mouse Platelets

Cell Type	WT			KI		
	Mean	SEM	N	Mean	SEM	N
white blood cell count	8.49	0.74	13	9.71	0.83	6
neutrophils	1.49	0.13	13	2.55	0.60	6
lymphocytes	6.34	0.56	13	6.51	0.93	6
monocytes	0.63	0.07	13	0.63	0.10	6
eosinophils	0.02	0.01	13	0.02	0.01	6
basophils	0.00	0.00	13	0.01	0.00	6
band neutrophils	0.00	0.00	13	0.00	0.00	6
neutrophils%	18.24	0.93	13	27.05	7.09	6
lymphocytes%	74.13	0.97	13	66.32	7.21	6
monocytes%	7.39	0.30	13	6.41	0.73	6
eosinophils%	0.19	0.08	13	0.16	0.07	6
basophils%	0.06	0.03	13	0.06	0.02	6
band neutrophils%	0.00	0.00	13	0.00	0.00	6
hematocrit	37.15	1.66	13	38.50	1.91	6
red blood cells	8.53	0.42	13	8.57	0.36	6
hemoglobin	12.12	0.56	13	12.33	0.63	6
mean corpuscular volume	43.75	0.61	13	44.83	0.57	6
mean corpuscular hemoglobin	14.24	0.19	13	14.33	0.18	6
mean corpuscular hemoglobin concentration	32.58	0.18	13	32.03	0.36	6
red cell distribution width	14.32	0.29	13	14.10	0.55	6
reticulated platelets#	25.05	7.38	13	9.97	3.75	6
reticulated platelets%	0.32	0.09	13	0.13	0.05	6
platelet	1089.85	84.71	13	1125.00	142.90	6
mean platelet volume	5.38	0.04	13	5.53	0.11	6
platelet distribution width	41.82	0.30	13	40.70	0.68	6
plateletcrit	0.59	0.05	13	0.63	0.08	6

Detailed Materials and Methods

KI Mouse Line. To examine the biological function and possible pathological consequences of the Leu33/Pro33 substitution, we generated *Itgb3* Pro33 mice by homologous recombination. The mouse integrin β3 subunit consists of 762 amino acids with 86% overall homology to its human counterpart. The construct used to target the mouse *Itgb3* had the Ser23Gln33 mutated to Pro32Pro33, to induce a similar conformational alteration as the PI^{A2} antigen in humans (which contains Pro32Pro33) and contained a cassette for both selection of neomycin resistance (Neo, positive selection) and thymidine kinase resistance (TK, negative selection). The Cre cassette, expressed under the tACE (angiotensin-converting enzyme161) testis-specific promoter was upstream of a Neo cassette {Bunting, 1999, 10385621}. Both Neo and Cre open reading frames were flanked by loxP sites for removal during germline transmission. Arms for homologous recombination were obtained by PCR with complete sequencing to ensure against unanticipated mutations (construct was generated at Gene Dynamics LLC, Tigard, OR). Full sequencing of the targeting construct insured 100% homology with the Celexa C57BL/6 *Itgb3* gene, with the exception of three highly repetitive sequences, one in the 5' arm and two downstream of exon 3 (all located in intronic regions). The construct was injected onto C57BL/6J embryonic cells and implanted onto C57BL/6J blastocysts and therefore no backcrossing was needed to achieve a pure genetic background (inGenious Targeting Laboratory, Inc. Ronkonkoma, NY). Screening of clones was tested by two complementary PCR/RFLP approaches (Figure 1). PCR1 (Figure 1C): primer A (5'-GCTAACGTCGCTGGTC-3') and B (3'-CACTTGGTCGTGGCAGCCCGGACC-3') generated a 8.5Kb band in KI allele only. In PCR 2 (Figure 1C): Primer C (5'-AGCCAGCTCATTCTTGGGCTCTTA-3') and Primer D (5'-AAACGCTCTACCACACAGCTCACT-3') generated a 4121bp band. The digestion of the 4121bp with MspI generated two fragments (879 and 4121) in WT and three fragments (4121, 608 and 271bp) in KI allele. Southern Blotting confirmed positive clones. Mice were genotyped by PCR (Figure 1D) using genomic DNA extracted from tails and primer A (5'-GCTAAC GTCGCTGGTC-3′) and primer F (5′-AAGGGGAAAAGTCACCCTTG-3′) as depicted in Figure 1D. Sequencing of the genomic region between Exons 2 and 3 demonstrated full excisions of the Neo/Cre cassettes and presence of the coding polymorphism, with no other changes in the mouse *Itgb3* KI gene.

Animals and Housing. Pro32Pro33 KI mice were born at Mendelian ratios, independently of the genotype of the parents, and were fertile with no obvious developmental or behavioral effects. Experimental mice were obtained by wt/ki X wt/ki crosses or by ki/ki x ki/ki and wt/wt x wt/wt crosses. Neither maternal effects nor significant phenotypic differences were found between C57BL/6J mice and our wt mice. All mice were group housed in temperature and humidity controlled conditions under a 12 hour light-dark cycle with food and water available ad libitum. All studies were performed in accordance with humane guidelines established by the Vanderbilt Institutional Animal Care and Use Committee under an approved protocol (M/11/065). Age and sex-matched mice were used in all experiments (both males and females

ranging from 8-20 weeks of age). The colony manager determined experimental cohorts and experimenters were blinded to the genotypes. All experiments were run with either wt/wt (WT) or ki/ki (KI) homozygous mice.

Blood Collection. For terminal blood collection, cardiac puncture was performed using a 25-gauge needle/1 mL syringe containing 0.03 mL of 10X sodium citrate on mice that were euthanized (5% isoflurane; cervical dislocation). For platelet isolation, whole blood was layered onto 1.5ml of Fico/Lite Platelets (Atlanta Biologicals, Inc., Lawrenceville, GA) and spun for 15 minutes at 700 x g. Platelets were collected and washed in 1ml modified Tyrodes-HEPES buffer (10mM HEPES, 11.9mM $NaHCO_3$, 127.2mM NaCl, 5mM KCl, 0.4mM NaH_2PO_4, 1mM $MgCl_2$, 5mM glucose, pH 7.4) and collected by centrifugation at 5,000 x g for 5 minutes. Platelets were counted in a Coulter counter (Beckman Coulter).

Whole Blood Flow Cytometry. Briefly, 250μL of whole blood was added to 750mL of Tyrodes-HEPES buffer (15mM HEPES, 0.138M NaCl, 2.7mM KCl, 0.4mM NaH_2PO4, 1mM $MgCl_2$, 5mM D-Glucose, pH7.4) that had been pre-warmed to 37^{o}C. Samples were then added to tube containing pre-aliquots of JON/A-PE and P-selectin-FITC (EMFRET Analytics & Co. KG, Würzburg, Germany) or integrin αIIb (CD41, BioLegend, San Diego, CA) or integrin β3 (CD61 BioLegend, San Diego, CA). Suspensions were mixed briefly and allowed to sit for 15min at room temperature in dark to avoid quenching of fluorescent probes. Activation was stopped by addition of 500μL of 2% paraformaldehyde in phosphate-buffered saline (PBS, 0.138M NaCl, 0.0027M KCl, pH 7.4). Samples were analyzed at the Nashville VA Medical Center flow cytometry core. Un-stimulated samples were run in duplicate to determine basal activity. Data shown is representative of four to eight independent experiments (PAR-4 AP in Figure 6A is at 250μM).

Tail Bleed. Mice were anesthetized using 5.0% isoflurane (JD Medical Distributing Co., Inc., Phoenix, AZ) and 1.0 L/min oxygen until unresponsive. The mice were then mask-ventilated with 5.0% isoflurane and 1.0 L/min oxygen on a surgical stage for maintenance anesthesia, which was decreased to 2.0% isoflurane and 1.0 L/min oxygen once unresponsiveness was confirmed. A transverse incision was made with a scalpel over a lateral vein at a position where the diameter of the tail was 2.25 to 2.5 cm. The tail was immersed in normal saline (37^{o}C) in a hand-held test tube. The time from the incision to the cessation of bleeding was recorded as the bleeding time. Data (WT=15, KI=20) was analyzed using unpaired Student's t-Test.

Whole Blood Clot Formation (PTT). The time necessary for clot formation was examined by using a Diagnostica Stago clot formation reader. For these experiments, 90μL of whole blood was added to a single well containing a small metal bebe. 10μL of $CaCl_2$ was added to a final concentration of 1.64mM $CaCl_2$ and recording was started. The read-out corresponded to the number of seconds necessary for substantial fibrin network formation to occur within each sample resulting is interruption of the small magnetic bead movement within the miniwell. Blood samples (WT=14, KI=20) were run in duplicate and averages analyzed using unpaired Student's t-Test.

Whole Blood Aggregation. Whole Blood aggregation was measured by determining electrical impedance in a multiplate analyzer (Dynabyte GmbH, Munich, Germany). Briefly, 175μL of 37°C 2X $CaCl_2$ was added to miniwells seated and connected to the electrode plug. 175μL of citrated whole blood was then added and allowed to incubate for 3mins. After the incubation period, agonist (200μM PAR-4 AP) was added to each well followed by immediate initiation of electrical measurements. From the program recording, aggregation and the velocity of aggregation was determined over a 6 minute period. Data (WT=6, KI=6) were analyzed with unpaired t test with Welch's correction (unequal variances).

Aggregation in washed platelets. Aggregation was performed as described previously{Carneiro, 2008, 18317590}. Briefly, samples were spun for 10 minutes at 1000rpm. 500uL of PRP was collected from the top layer of the supernatant and transferred into a separate tube. Pelleted platelets were suspended in modified Tyrodes-HEPES buffer and adjusted to a concentration of 3×10^8 platelets/ml. After stimulation with 0.05 U/ml thrombin the change in light transmission was monitored with an aggregometer (Chrono-log Corporation, Havertown, PA). Result shown is representative of six independent experiments.

Thromboembolism. Mice were matched by age (12-16 weeks) and were anesthetized with inhaled isoflurane. The right jugular vein was exposed by a lateral neck incision, and a 27G needle used to collect 100μl of whole blood in sodium citrate. The left jugular vein was exposed to inject a coagulation solution containing 100μg/ml ADP (Sigma-Aldricht, St. Louis, MO), 200μg/ml collagen (equine tendon type I fibrillar collagen; Chronolog, Haventown, PA) and 200μg/ml epinephrine (Sigma-Aldricht), in sterile saline (0.9% NaCl) at a dose of 5μl/g during 10 seconds. One minute after the injection was complete; a sample of blood was collected into sodium citrate. Mice were euthanized by rapid decapitation. Platelet counts in whole blood were obtained by diluting the samples 1:10,000 in Tyrodes-HEPES buffer. Data (WT=10, KI=11) were analyzed by two-way repeated measures ANOVA (drug vs. genotype effects) with Bonferroni post-tests.

Platelet attachment and in cell western blots. Whole blood was diluted 1:8 and seeded onto 96-well clear-bottom black plated previously coated with fibrinogen. Plates were coated with fibrinogen (5-500μg/ml) overnight at 4°C and washed twice with PBS before blocking with 5% BSA for 30min at R.T. BSA was then removed and washed twice with PBS immediately before adhesion experiments. After adding 45μl diluted blood/well, $MnCl_2$ (0.2mM) was added to all wells and plates were incubated at 37°C for 15 minutes. Alternatively, plates were coated with 25μg/ml fibrinogen and platelets exposed to 200μM PAR-4 AP. Plates were washed three times with PBS and adhered platelets fixed with 4% paraformaldehyde. After fixation, platelets were permeabilized with 0.2% Triton in PBS for 10min at R.T. and blocked with 1%BSA and 5% normal donkey serum in PBS. Primary antibodies (mouse anti-actin, or mouse anti-GAPDH; rabbit anti- Src, pSrc, FAK, pFAK, ERK or pERK) were added at a 1:1,000 dilution in BSA/goat serum buffer overnight at 4°C. Primary antibodies were washed and secondary antibodies (IRDye 800CW Donkey anti-Rabbit IgG and IRDye 680RD

Donkey anti-Mouse IgG, LI-COR Biosciences, Lincoln, NE) were added at a 1:250 dilution for 1 hour at R.T. Plates were scanned and data obtained with the in cell western macro in an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE). Adhesion was measured in the actin channel and analyzed with a two-way repeated measures ANOVA (genotype vs. concentration) and Bonferroni posttests. Kinase activation was measured in kinase channel and ratios of phospho-kinase/kinase calculated. All samples were normalized to WT vehicle conditions and tested using non-parametric t-tests.

Platelet Spreading. Isolated and washed platelets (10^{10}/ml) were resuspended in Krebs-Ringer's HEPES (KRH) buffer (130mM NaCl, 1.3mM KCl, 2.2mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8g/L glucose, 10mM HEPES, pH 7.4) and seeded onto 12-well (5mm diameter) glass printed slides (Thermo Scientific Cell-Line® Specialty Printed Microscope Slides, SSG Braunschweig, Germany) previously coated with 25μg/ml fibrinogen and blocked with 1% BSA. Each well received 4μl of washed platelets and 1μl of a solution containing: 1mM PAR-4, 1mM PAR-4 + 0.5μM SKI-606 (Bosutinib, dual Src/Abl tyrosine kinase inhibitor, Sigma-Aldricht, St. Louis, MO) or 1μl KRH buffer alone (vehicle). Slides were incubated at 37°C for 15min, washed once with 1x PBS and fixed with 4% paraformaldehyde. Platelets were permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA and 5% normal goat serum. Slides were incubated with primary antibodies at 1:1.000 dilution for 1 hour at R.T. (mouse anti-talin from Sigma-Aldricht, rabbit anti-pSrc from Cell Signaling). Slides were washed three times with 1x PBS and incubated with secondary antibodies (goat anti-mouse Cy2 and goat anti-rabbit Cy3, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and phalloidin (Molecular Probes®, Life Technologies Corporation) at 1:200 dilution in 1% BSA for 1 hour at R.T. Images were captured with a Zeiss LSM510 META Inverted Confocal Microscope located at the Vanderbilt Cell Imaging Shared Resource (CISR). Platelet number and area were quantified by a blinded experimenter using ImageJ analysis software in the talin channel (Cy2). Platelet number is defined as the number of platelets per image. Data are presented as means ± SEM of 4-5 images per mouse for 2-3 mice per experiment (indicated in the results section). Data was analyzed by unpaired Student's t-Test for basal attachment or two-way ANOVA (genotype vs. treatment) with Bonferroni posttests for agonist-induced attachment.

Western Blotting and co-Immunoprecipitations. To analyze kinase phosphorylation, washed platelets were resuspended in 1 x KRH, incubated at R.T. for 30min, lysed by addition of one volume of 1% Triton X-100 in PBS (containing protease inhibitor; Roche) and clarified by centrifugation at 13,000 g for 10 minutes at 4°C. Lysates were collected for input (10μg) and 200μg of protein extract was incubated with 30μl integrin β3 antibody (2C9.G2 Hamster anti-β3, BioLegend, San Diego, CA) covalently attached to protein A magnetic beads (Dynabeads, Life Technologies/Invitrogen, Grand Island, NY) for 1 hour at 4°C. Beads were isolated magnetically and washed 2 x with 1ml 0.5% Triton X-100 in PBS. Co-immunoprecipitated proteins were eluted with 1x NuPAGE LDS sample buffer (Life Technologies/Invitrogen, Grand Island, NY). Protein was separated on 4%–20% Tris-HEPES gels (NuSep, Inc.; Bogart, GA) and transferred to PVDF membranes for probing with mouse anti-αIIb, anti-β3, anti-Src, anti-p-Src^{416}, FAK and

pFAK^{397} and ERK and pERK (all antibodies were purchased at Cell Signaling Technology, Inc., Danvers, MA). Proteins were detected by chemiluminescence and exposed to Hyperfilm though multiple exposures to ensure linear distribution of signal. Films were scanned and band densities were established using ImageJ software. Data was normalized to WT vehicle control and analyzed by non-parametric Student's t-Tests.

Src *In Vivo* Inhibition. A 10 mM stock of SKI-606 (Sigma-Aldricht, St. Louis, MO) in dimethyl sulfoxide (DMSO) was diluted in sterile saline (0.9% NaCl) to 0.1mg/ml immediately prior to administration. Isoflurane anesthetized mice were transferred to a nose ventilator maintained at 2% and confirmed to be unresponsive. The jugular vein was exposed for collection of 300μL of blood in sodium citrate. After this initial blood draw, SKI-606 at was administered intraperitoneally at 1mg/kg and mice were maintained with 2% isoflurane for 30 minutes. After 30 minutes, cardiac puncture was performed as previously described for collection of post-inhibitor administration blood samples. Blood samples were used to perform time to clot experiments (ran in duplicates) and in-cell westerns for phosphorylation levels.

Data analysis and statistics. All data was analyzed in Prism 4.0c (Graphpad Software, Inc., LaJolla, CA) using Student's t-Tests or two-way ANOVA with Bonferroni posttest, where appropriate. Welch's correction parameters were used in samples with unequal variances (indicated in the results section). Non-parametric t-tests were used when each WT/KI pair was normalized to the WT data (all WT = 100). A P value of less than 0.05 was considered statistically significant. All data are shown as mean ± standard error of the mean (SEM, represented by error bars).