Supplemental Materials and Methods

Antibodies

The following antibodies were used in this study: anti-GPIbα (Xia.G5), anti-Integrin α2 (Sam.G4), anti-GPVI (JAQ1), anti-P-selectin (Wug.E9), anti-high affinity conformation of CD41/CD61 (JON/A), rat IgG, anti-GPIbα for platelet depletion (R300), and anti-GPIbβ for in vivo mouse platelet labelling (X488) from emfret ANALYTICS; anti-CD41 (MWReg30) and Streptavidin from BD Pharmingen; anti-ADAM10 ectodomain (139712) from R&D system; anti-CLEC-2 (17D9) and rat IgG2b from AbD Serotec; anti-Csk (C-20), anti-Ctk (Chk, C-20), anti-SH-PTP1 (C-19), anti-SH-PTP2 and anti-rat IgG-HRP from Santa Cruz; anti-Src (pY418), anti-Src (pY529), annexin V Alexa Fluor 647 conjugate, and Alexa Fluor 488 and Alexa Fluor 647 antibody labelling kit from Invitrogen Life Technologies; anti-Lyn (pY507), anti-Syk (pY525/526), anti-Syk (D1I5Q) and anti-Src from Cell Signaling; anti-Fyn (pY530) and anti-syrian hamster IgG-HRP from Abcam; anti-PTP-1B, anti-Fc epsilon RI gamma subunit, and anti-phophotyrosine (4G10) from Millipore; anti-α-tubulin (DM1A) from Sigma; anti-mouse and anti-rabbit ECL HRP conjugates from GE Healthcare; anti-CD148 (8A-1) antibody has been previously described;[1] anti-G6b-B (68-3), (N24) and anti-GPVI antibodies have been previously described.[2] Anti-fibrin antibody (clone 59D8), a generous gift from Dr. Timothy J. Stalker (University of Pennsylvania), has been previously described,[3] and labeled using Alexa Fluor monoclonal antibody labeling kits from Invitrogen.

Platelet clearance

Mice were injected intravenously with 150 μl of biotin-*N*-hydroxysuccinamide (4 mg/ml in saline, Sigma-Aldrich). The percentage of biotin$^+$αIIb$^+$ platelets in blood was measured in αIIb$^+$ cells co-stained with streptavidin daily post-injection and the percentage of labelled platelets remaining in the circulation was determined by flow cytometry. The rate of platelet

clearance was determined by a proportionate slope from linear trend lines between days 1 to 3.

Immunohistochemistry

Spleens and femurs were fixed in buffered formalin and embedded in paraffin. Sections (5 μm) were hematoxylin and eosin (H&E) or reticulin stained. Images were obtained by Slide Scanner Axio Scan.Z1 brightfield microscope and Axio Zen software (Zeiss). Analysis of MK counts in spleen and femur per view was performed in a double blinded manner.

Isolation of bone marrow cells and *in vitro* differentiation of megakaryocytes

Bone marrow was flushed using DMEM media supplemented with 10 % FBS, glutamine and penicillin/streptomycin using a 25 gauge needle from femora and tibiae of mice and the cells were spun down. Bone marrow cells were resuspended in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) in order to lyse erythrocytes and filtered by a 70 μm nylon mesh and subsequently resuspended in P2 buffer (PBS, 5 % FBS, 1 % BSA) for flow cytometry assay. Surface protein expression was measured in bone marrow cells with indicated FITC- or PE-conjugated antibodies by flow cytometry (BD FACSCalibur). Median fluorescence intensity (MFI) were measured in αIIb^+ gated population, and analysed at least 50,000 events for bone marrow cells. To culture megakaryocytes (MKs) for biochemical assay, cells expressing one or more of the lineage specific markers on their surface ($CD16/CD32^+$, $Gr1^+$, $B220^+$, $CD11b^+$) were depleted using immunomagnetic beads (sheep anti-rat IgG Dynabeads). The remaining population was cultured in 2.6% serum-supplemented Stempro medium with 2 mM L-glutamine, penicillin/streptomycin and 20 ng/ml murine SCF at 37°C and 5% CO_2 for 2 days. Cells were then cultured for a further 4 days in the presence of 20 ng/ml SCF and 50 ng/ml Tpo. After 4 days of culture in the

presence of Tpo, mature MKs were enriched using a 1.5%/3% BSA gradient under gravity (1 ×*g*) for 45 minutes at room temperature. MKs were starved for 3 hrs at 37°C in serum-free media to obtain whole-cell lysates as previously described.[4]

Total thrombus-formation analysis system (T-TAS)

Blood treated with 25 μg/ml hirudin was perfused with collagen-coated chip with shear rate 1000 s^{-1} for 10 minutes. Blood treated with 3.2% sodium citrate, 12 mM $CaCl_2$ and 50 μg/ml corn trypsin inhibitor was perfused with collagen plus tissue thromboplastin (tissue factor)-coated chip with shear rate 240 s^{-1} for 30 minutes. Individual time-dependent flow pressure curves, time to onset (T10), time to occlusion (T80), rate of thrombus growth (T10-80) and total thrombogenicity (Area under the curve: AUC) were measured by the total thrombus-formation analysis system (T-TAS, Fujimori Kogyo, Japan).

Whole-blood thrombus formation and platelet adhesion under flow

Mouse blood anticoagulated with 5 Unit/ml heparin, 40 μM PPACK and 50 Unit/ml fragmin was perfused for 3.5 minutes at 1,000 s^{-1} over glass coverslips coated with microspots of agonists; 100 μg/ml collagen type I, 12.5 μg/ml vWF-binding peptide (VWF-BP), 50 μg/ml laminin, and 250 μg/ml rhodocytin. Brigtfield and three-color fluorescence images were captured with an EVOS microscope (60x oil) (Life Technologies) and analysed using Fiji as previously described.[5]

Platelet aggregation and secretion

Aggregation and adenosine triphosphate (ATP) secretion were performed in a lumi-aggregometer (Chrono-Log, Havertown, PA) with continuous stirring at 1200 rpm at 37 °C for the times shown, and monitored by measuring changes in light transmission.

Static adhesion spreading assay

Washed platelets were pre-incubated with or without thrombin (0.1 Unit/ml, 5 minutes) placed on fibrinogen-coated cover-slips (100 μg/ml, 45 minutes, 37°C) and imaged (63x oil immersion lens), as previously described.[6]

Clot retraction

Platelet-rich plasma (PRP) was prepared from blood treated with 4% (w/v) sodium citrate by centrifugation at 200 g for 8 minutes at room temperature in the presence of prostacyclin (0.1 μg/ml) and adjusted to a concentration of 2 x 10^8 platelets/ml by platelet-poor plasma (PPP) which was prepared by centrifugation 1000 g for 10 minutes at room temperature. Fibrin clot retraction was performed by incubating 200 μl of PRP (4 x 10^7 platelets) in the presence of 1 Unit/ml thrombin and 2 mM $CaCl_2$ for 2 hours at room temperature in an aggregometer cuvette. A paper clip was added to facilitate clot removal at the termination of the experiment. The volume of residual clot-free plasma was determined, and clot volume was taken as 200 μl minus this value. Clot volume was expressed as a percentage of the original 200 μl plasma volume.

Platelet stimulation for biochemical analysis

For biochemical analysis of GPVI and CLEC-2 signaling pathways, platelets were pre-incubated with lotrafiban (10 μM), apyrase (2 Unit/ml) and indomethacin (10 μM) for 10 minutes. Afterwards, platelets were stimulated under stirring conditions (1200 rpm, 37°C) with collagen, CRP or CLEC-2 antibody for indicated times.

For biochemical analysis of the integrin αIIbβ3 outside-in signaling pathway, platelets were pre-incubated with apyrase (2 Unit/ml) and indomethacin (10 μM) for 10

minutes, and then placed on fibrinogen-coated plates (100 μg/ml) for 45 minutes at 37°C. Non-adherent platelets were used as controls.

After stimulating GPVI, CLEC-2 or αIIbβ3 outside-in signaling pathways for the specified times, platelets were lysed with an equal volume of ice cold 2 x lysis buffer (2% Nonidet P-40, 300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2 mM Na_3VO_4, 200 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstasin A, pH 7.4). In case of fibrinogen-adherent platelets, ice cold 1 x lysis buffer was used to lyse cells for 15 minutes on ice, which was followed by scraping platelets off the plates. Insoluble cell debris was removed by centrifugation for 5 minutes at 13,000 x g, 4°C, and whole cell lysates (WCLs) were precleared using protein G- or A-Sepharose (Sigma-Aldrich) for 30 minutes. G6b-B was immunoprecipitated from collagen-stimulated WCLs with anti-G6b-B antibody as previously described.[2]

Immunoblotting

WCLs were either boiled in SDS-loading buffer, and analyzed by SDS-PAGE and traditional Western blotting or analyzed on an automated capillary-based immunoassay platform; Wes (ProteinSimple, San Jose, USA) for quantitative analysis. WCLs to be analyzed on the Wes system were prepared according to manufacturer's instructions and were not precleared. Protein concentrations were determined using the Bio-Rad RC DC protein assay. Diluted samples and primary antibody were added to pre-filled microplates with Split Running Buffer (PS-MK14). Before analyzing WCLs from transgenic mouse platelets, optimal lysate and antibody concentrations were determined on *WT* WCLs. Briefly, for each antibody used, a lysate dilution experiment was performed to confirm the optimal dynamic range of the protein on Wes. This was followed by an antibody optimization experiment to compare a range of dilutions and select an antibody concentration near to saturation level to allow a

quantitative comparison of signals between samples. In each run, twenty-four samples were analyzed in parallel as triplicates of eight samples from the same experiment for either Src, Src p-Tyr418 and Lyn p-Tyr507 on one microplate (plate A) or Src p-Tyr529, Fyn p-Tyr530 and Syk p-Tyr525/6 on another microplate (plate B). Compass Software (ProteinSimple, San Jose, USA) was used to operate Wes and analyze results. Separation time was set to 31 minutes, stacking loading time to 21 seconds and sample loading time to 9 seconds for both microplates. Primary antibody incubation time was 60 minutes for plate A and 30 minutes for plate B, and exposure times were 5; 15; 30; 60; 120; 240 and 480 seconds for plate A and 2; 5; 10; 20; 40; 80 and 160 seconds for plate B. The defined antibody and lysate concentrations were as follows: anti-Src antibody (1:50 dilution), anti-Src p-Tyr529 antibody (1:100 dilution) and anti-Fyn p-Tyr530 antibody (1:50 dilution) were used for 0.025 mg/ml platelet and 0.1 mg/ml MK lysate concentration; anti-Src p-Tyr418 antibody (1:10 dilution) was used for 0.05 mg/ml platelet and 0.1 mg/ml MK lysate concentration; anti-Lyn p-Tyr507 antibody (1:10 dilution) was used for 0.2 mg/ml platelet and 0.4 mg/ml MK lysate concentration; anti-Syk p-Tyr525/6 antibody (1:50 dilution) was used for 0.1 mg/ml platelet lysate concentration. The only exception from the above described concentrations were CLEC-2-stimulated samples in Figure 6 B in which case Syk p-Tyr525/6 antibody (1:50 dilution) was used in combination with 0.05 mg/ml platelet lysate concentrations.

Statistical analysis

Quantification of data is indicated in the figures, figure legends and methods sections. Statistical parameters including the exact value of n, the definition of center, dispersion and precision measures (mean ± SEM) and statistical significance are reported in the figures and figure legends. Sample number (n) indicates the number of independent biological replicates in each experiment in most figures, the number of images of H&E-stained spleen and femur

sections in Figure S6A-B, the number of individual platelets in Figure 3Dii and 6Bii, and the number of injury induced in Figure 2Ciii. For multiple group comparisons, one-way or two-way ANOVA followed by *post-hoc* tests was used to determine statistical significance. Data from the Wes system were analyzed using repeated measures (RM) one-way ANOVA (Figure 4Ai and Figure S8A) or RM two-way ANOVA (Figures 5A-C and 6C-D) to control for variability in different experiments and Wes runs or by t-test (Figure S9D). RM two-way ANOVA was also used to analyze data from traditional Western blots (Figure 5Civ). Ordinary one- or two-way ANOVA was used to analyze the rest of the figures. P values < 0.05 were considered significant. In figures, asterisks denote statistical significance compared to *WT* (Figure 1-5) or DMSO-incubated control samples (Figure 6). Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software, La Jolla, CA), except statistical analysis of platelet clearance and recovery, which were performed in Excel.

References

1. Lin J, Weiss A. The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. *J Cell Biol*. 2003;162(4):673-682.

2. Mazharian A, Wang YJ, Mori J, et al. Mice lacking the ITIM-containing receptor G6b-B exhibit macrothrombocytopenia and aberrant platelet function. *Sci Signal*. 2012;5(248):ra78.

3. Stalker TJ, Traxler EA, Wu J, et al. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. *Blood*. 2013;121(10):1875-1885.

4. Smith CW, Thomas SG, Raslan Z, et al. Mice Lacking the Inhibitory Collagen Receptor LAIR-1 Exhibit a Mild Thrombocytosis and Hyperactive Platelets. *Arterioscler Thromb Vasc Biol*. 2017;37(5):823-835.

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Table S1. Hematological analysis of *Csk KO, CD148 KO* and *DKO* mice.

Parameter	*WT* (n = 42-67)	*Csk KO* (n = 26)	*CD148 KO* (n = 12 -18)	*DKO* (n = 24-27)
PLT ($10^3/\mu L$)	825.1 ±83.1	289.9 ±125.15***	772.0 ±96.2	809.3 ±197.5
MPV (fL)	5.65 ±0.38	7.14 ±1.36***	5.78 ±0.46	6.60 ±0.53***
RBC ($10^6/\mu L$)	9.68 ± 1.07	9.03 ± 0.67	9.63 ± 1.40	9.37 ± 0.86
HCT (%)	28.07 ± 3.94	27.29 ± 2.07	26.31 ± 5.02	27.36 ± 2.64
WBC ($10^3/\mu L$)	7.39 ± 2.86	8.40 ± 3.80	8.37 ± 4.32	11.61 ± 4.98***
LYM ($10^3/\mu L$)	6.56 ± 2.53	6.91 ± 3.36	7.02 ± 3.78	8.84 ± 4.09*
MON ($10^3/\mu L$)	0.28 ± 0.24	0.67 ± 0.40***	0.48 ± 0.49	1.58 ± 0.70***
NEU ($10^3/\mu L$)	0.53 ± 0.38	0.74 ± 0.33	0.76 ± 0.62	1.05 ± 0.44***
EOS ($10^3/\mu L$)	0.008 ± 0.03	0.01 ± 0.03	0.02 ± 0.05	0.04 ± 0.07*
BAS ($10^3/\mu L$)	0.03 ± 0.06	0.07 ± 0.07	0.03 ± 0.04	0.10 ± 0.19*

PLT, platelets; MPV, mean platelet volume; RBC, red blood cells; HCT, haematocrit, WBC, white blood cells; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; EOS, eosinophils; BAS, basophils
Mean ± standard deviation, *$P < 0.05$, ***$P < 0.001$, one-way ANOVA with Tukey's test

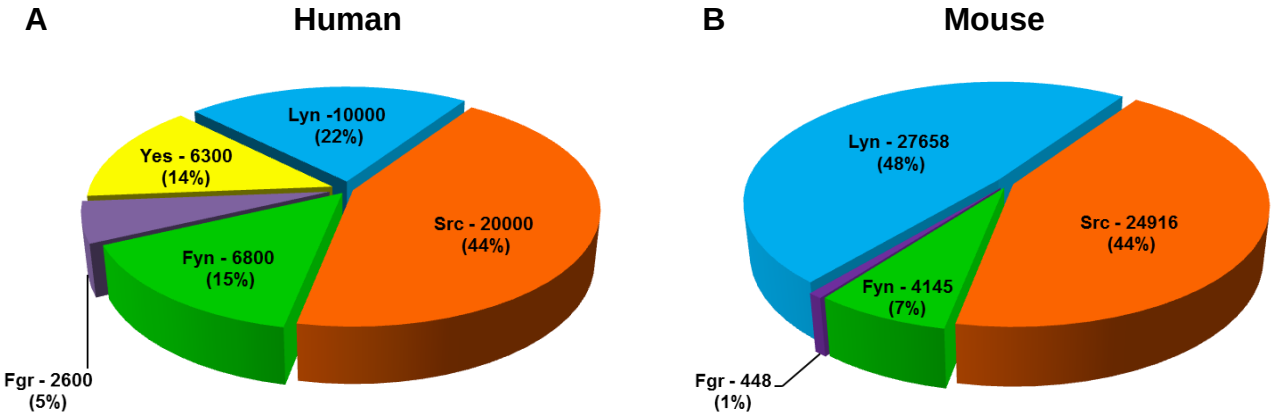


Figure S1. Expression levels of SFKs in platelets.
Number of copies of SFKs' expression in (A) human platelets[3] and (B) mouse platelets[4] are displayed as percentage values in a pie chart.

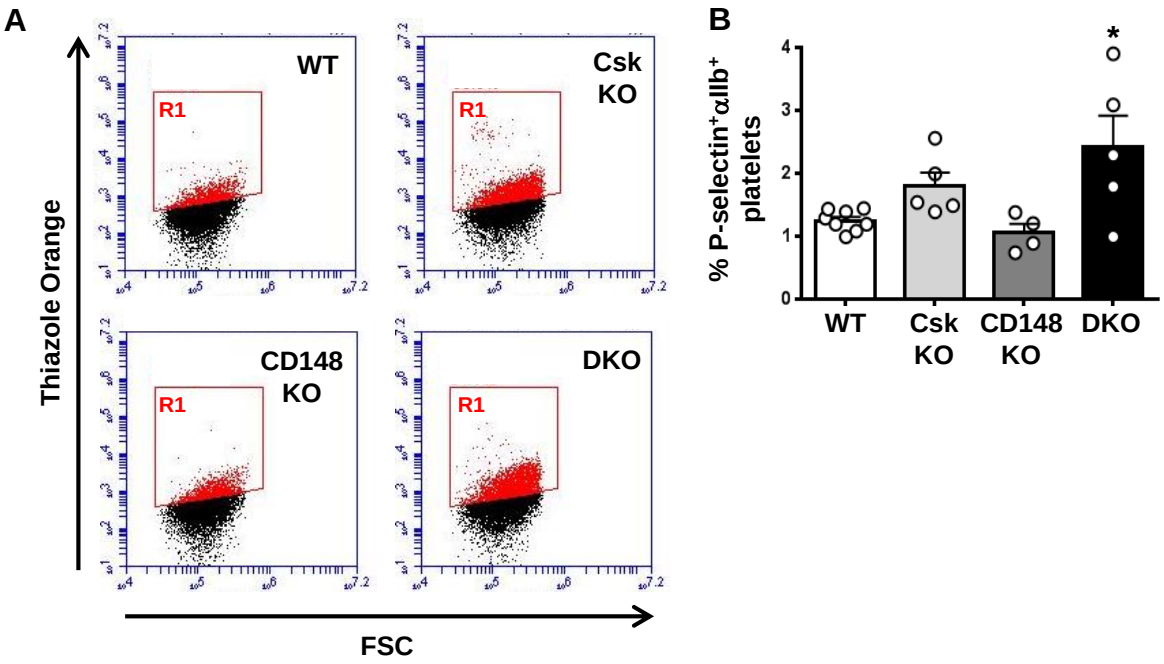


Figure S2. Increased immature platelet fraction but normal platelet P-selectin expression in *Csk KO* mice. (A) Representative image of reticulated platelet population in flow cytometry. Reticulated platelets in whole blood were measured in thiazole orange/αIIb double-positive cells detected by flow cytometry. αIIb positive platelets in whole blood are gated and the percentage of reticulated platelets was determined in region 1 (R1; in red) in dot plot diagram forward scatter (FSC) vs. RNA dye (Thiazole Orange). See also Figure 1C. (B) Percentage of P-selectin$^+$αIIb$^+$ platelets in blood. *P < 0.05, one-way ANOVA with Tukey's test, mean ± SEM.

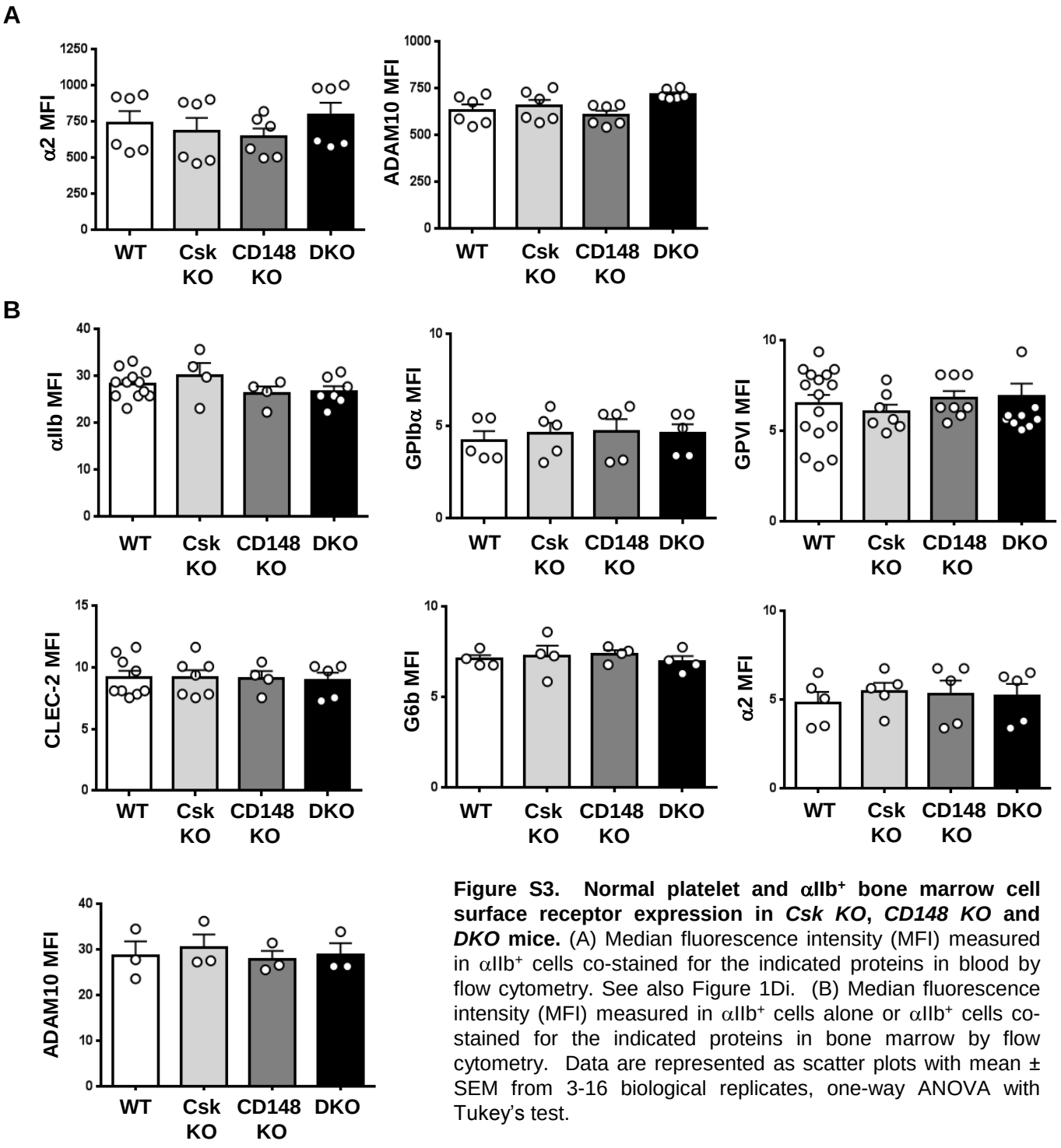


Figure S3. Normal platelet and αIIb⁺ bone marrow cell surface receptor expression in *Csk KO*, *CD148 KO* and *DKO* mice. (A) Median fluorescence intensity (MFI) measured in αIIb⁺ cells co-stained for the indicated proteins in blood by flow cytometry. See also Figure 1Di. (B) Median fluorescence intensity (MFI) measured in αIIb⁺ cells alone or αIIb⁺ cells co-stained for the indicated proteins in bone marrow by flow cytometry. Data are represented as scatter plots with mean ± SEM from 3-16 biological replicates, one-way ANOVA with Tukey's test.

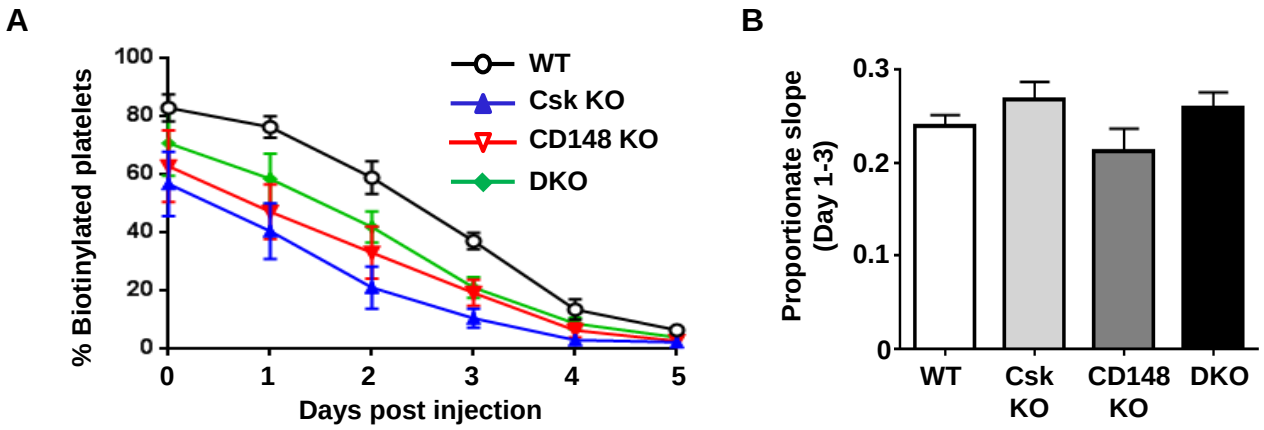


Figure S4. Normal platelet clearance in *Csk KO* mice. (A) Percentage of biotin$^{+}\alpha$IIb^{+} platelets in blood of post-injection of 150 µl biotin-*N*-hydroxysuccinamide. (B) The rate of platelet clearance determined by a proportionate slope from linear trend lines between day 1 to 3 from (A), n = 6 mice/time point/genotype. One-way ANOVA with Dunnett's test; vs *WT*, mean ± SEM.

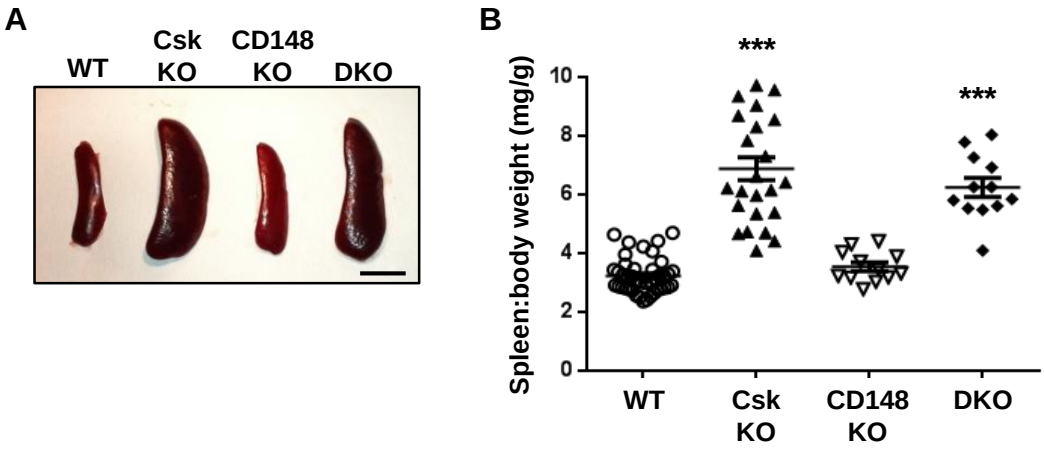


Figure S5. Splenomegaly in *Csk KO* and *DKO* mice. (A) Gross morphology of representative spleens (scale bar: 5 mm) and (B) spleen/body weight ratio.
****P* < 0.001, one-way ANOVA with Tukey's test

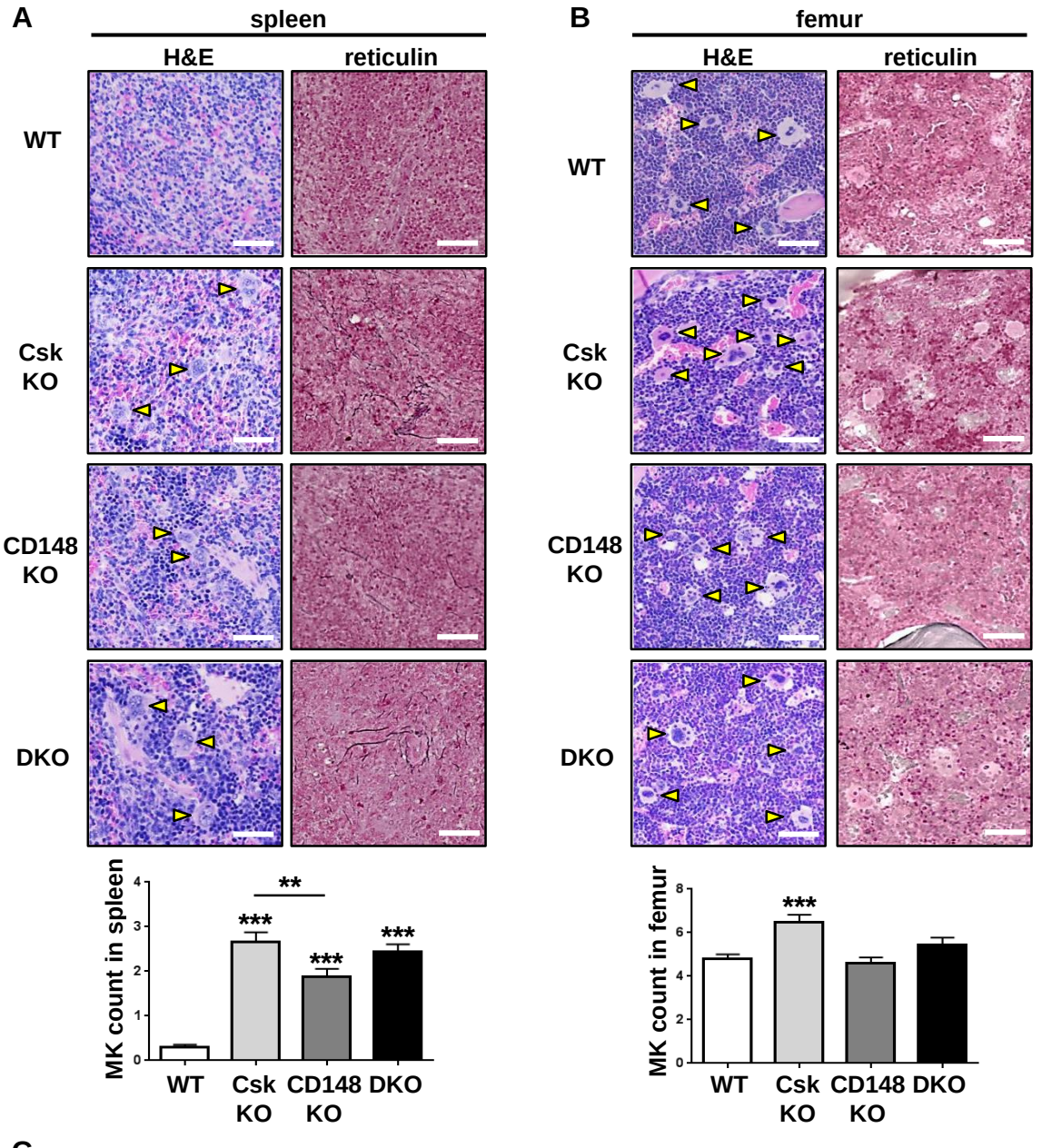


Figure S6. Extramedullary hematopoiesis and myelofibrosis in *Csk KO* and *DKO* mice. (A-B) Representative Haematoxylin and eosin (H&E) and reticulin staining in (A) spleen and (B) femur (scale bar: 50 μm). Analysis of megakaryocyte (MK) count per view was performed in a double-blinded manner., n = 75-100 images from 5 mice/genotype. (C) Percentage of P-selectin$^+\alpha$IIb$^+$ bone marrow (BM) cells. $^{**}P < 0.05$, $^{***}P < 0.001$, one-way ANOVA with Tukey's test , mean ± SEM.

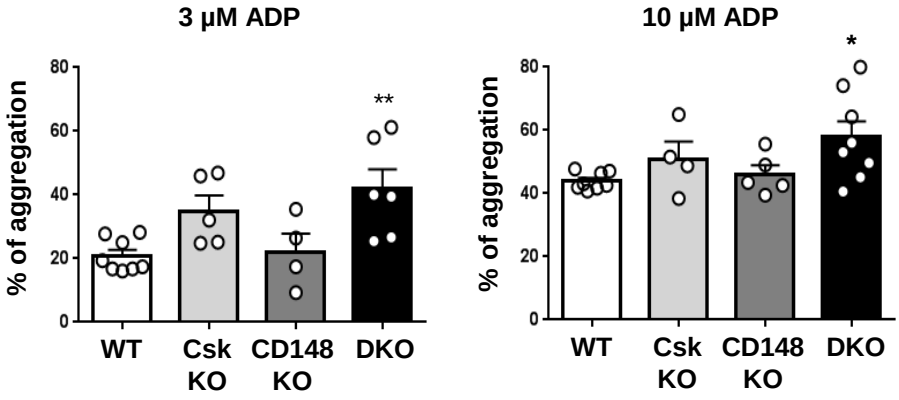


Figure S7. Increased ADP response in *DKO* mice. ADP-sensitive washed platelets were prepared in the presence of ADP scavenger apyrase and used at 2×10^8/ml. % of platelet aggregation in response to indicated dose of ADP were analyzed. See also Figure 3Cvii. Data are represented as scatter plots with mean ± SEM from 4-8 biological replicates, one-way ANOVA with Tukey's test.

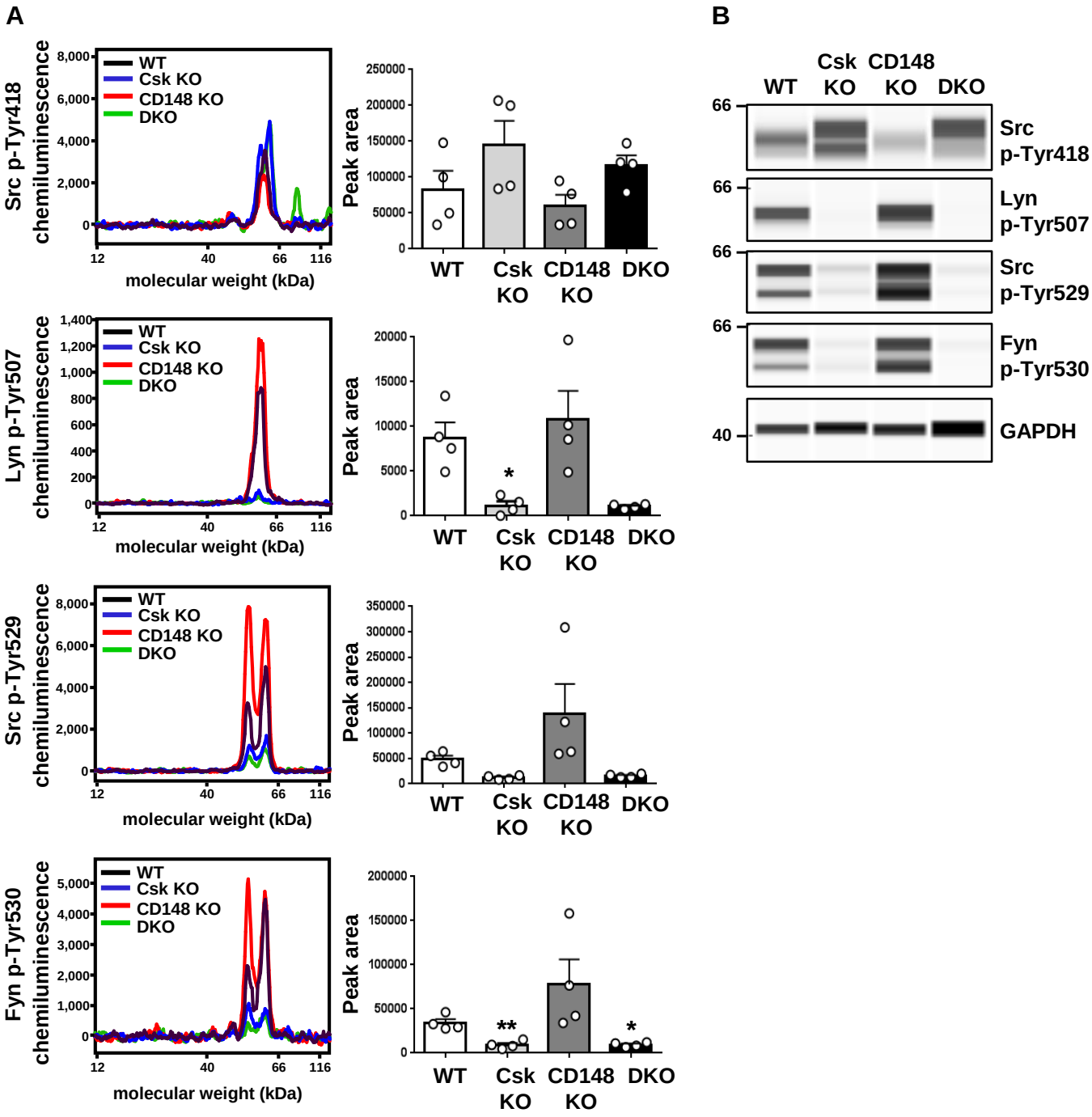
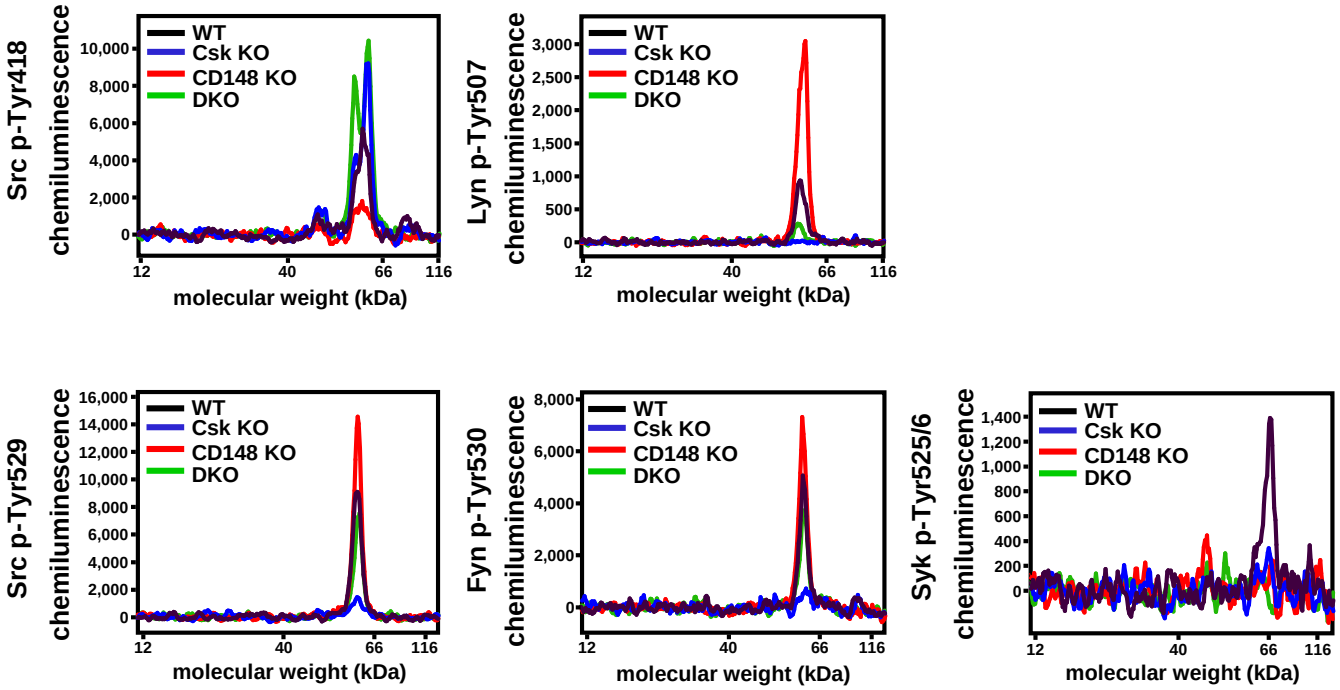
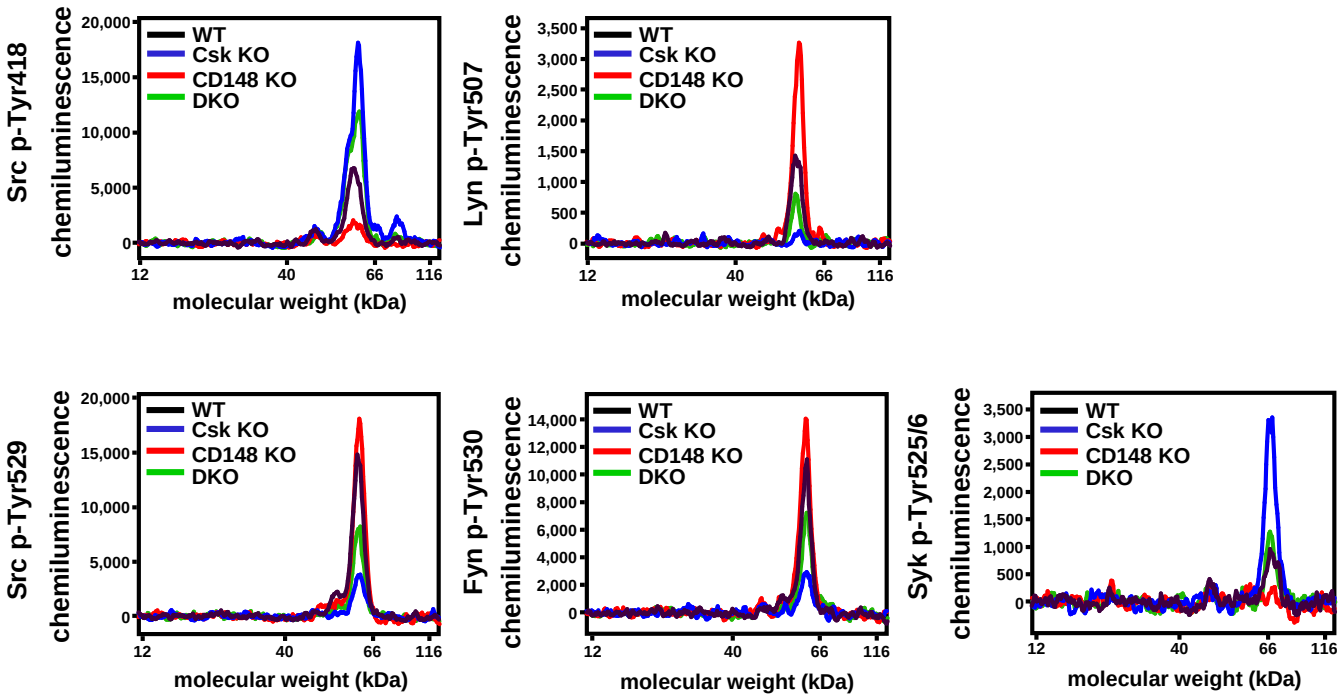


Figure S8. Increased megakaryocyte SFK activity in *Csk KO* mice. SFK activation loop and inhibitory tyrosine phosphorylation levels in bone marrow-derived megakaryocytes. (A) Representative electropherograms of capillary-based immunoassays on *ex vivo* differentiated megakaryocyte lysates with the indicated antibodies and the quantification of peak areas, n=4 mice/genotype. (B) Representative data from i) displayed as blots. **P* < 0.05, ***P* < 0.01, RM one-way ANOVA with Tukey's test, mean ± SEM.

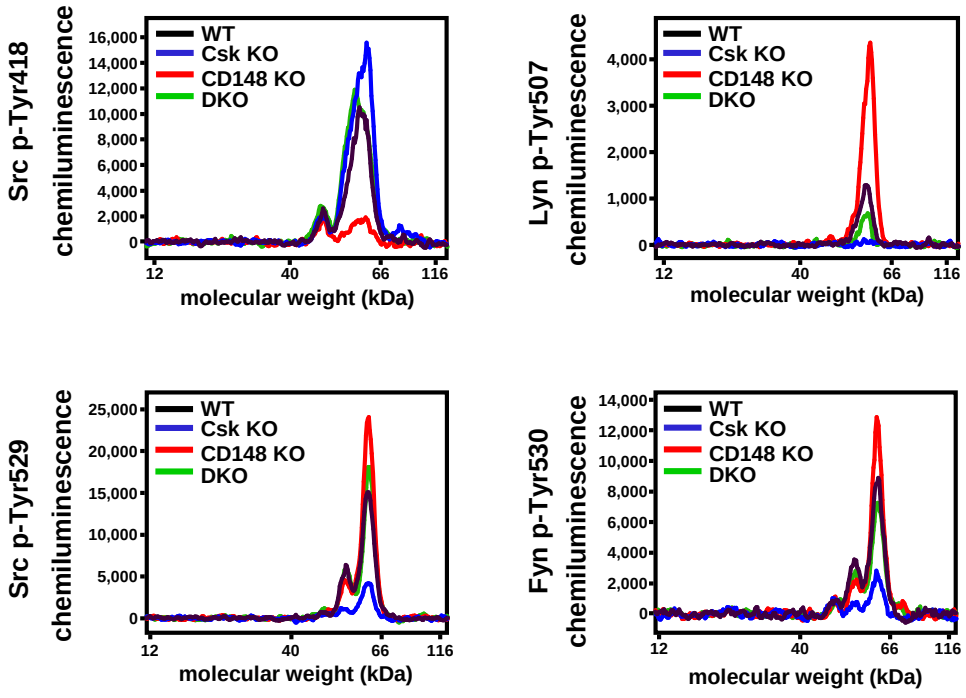
A Collagen stimulation



B CLEC-2 antibody stimulation



C Fibrinogen stimulation



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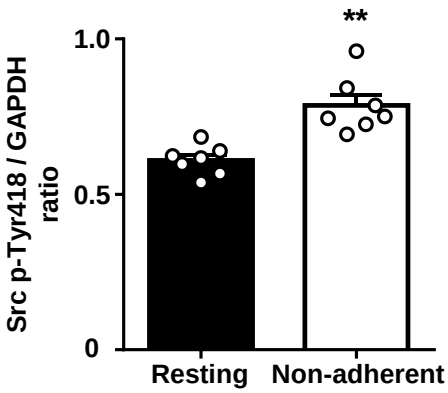
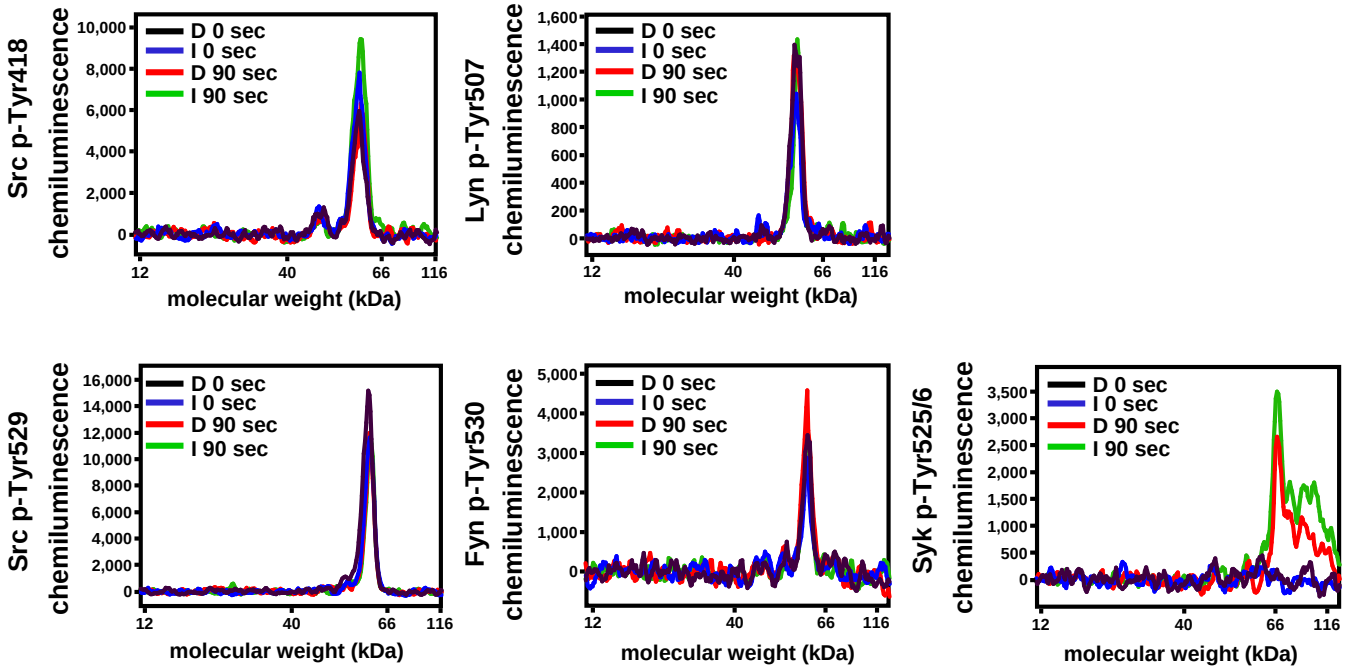


Figure S9. SFK activation loop and inhibitory tyrosine phosphorylation levels in activated platelets in *Csk KO*, *CD148 KO* and *DKO* mice. (A-C) Representative electropherograms of capillary-based immunoassays on platelet lysates with the indicated antibodies. Platelets were stimulated with A) collagen (30 μg/ml, 90 seconds), B) CLEC-2 antibody (10 μg/ml, 5 minutes) or C) were allowed to spread on fibrinogen-coated plates (100 μg/ml, 45 minutes, 37°C). (D) Src p-Tyr418 / GAPDH peak area ratio of resting and non-adherent *WT* platelets from fibrinogen-coated plates (100 μg/ml, 45 minutes, 37°C). Lysates were analyzed by capillary-based immunoassays for Src p-Tyr418 and GAPDH, n = 7 mice. **P < 0.01, paired, two-tailed t-test, mean ± SEM.

A CRP stimulation (*Csk*^{AS} platelets)



B CLEC-2 antibody stimulation (*Csk*^{AS} platelets)

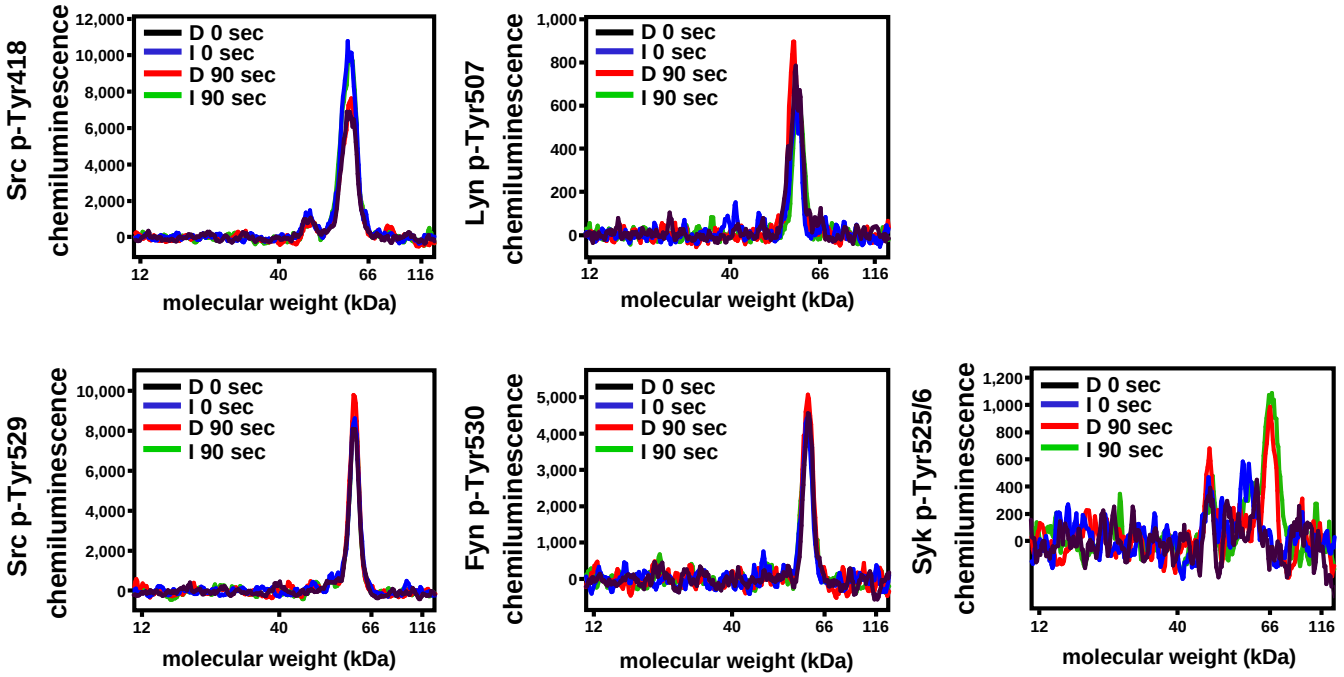


Figure S10. SFK activation loop and inhibitory tyrosine phosphorylation levels in activated platelets in *Csk*^{AS} mice. (A-B) Representative electropherograms of capillary-based immunoassays on platelet lysates with the indicated antibodies. Platelets from *Csk*^{AS} mice were pre-treated with either DMSO (D) or 10 μM 3-IB-PP1 (I) for 10 minutes and stimulated with A) CRP (30 μg/ml) or B) CLEC-2 antibody (10 μg/ml) for indicated times.

Videos

Video 1. Representative laser-induced thrombus formation in *WT, Csk KO, CD148 KO and DKO* mice.

Video 2. Representative laser-induced fibrin formation in *WT, Csk KO, CD148 KO and DKO* mice.

Video 3. Representative ferric chloride-induced thrombus formation in *WT*, *Csk KO, CD148 KO and DKO* mice.