

## Supplemental Methods

**Materials.** Monoparin heparin was obtained from CP Pharmaceuticals (Wrexham, United Kingdom); D-Phe-Pro-Ala-chloromethylketone (PPACK) from Calbiochem (San Diego, CA) and cross-linked collagen-related peptide (CRP) was prepared as previously described [32], fibrillar-type I equine tendon collagen was from Nycomed (Zurich, Switzerland); human plasma fibrinogen from Enzyme Research Laboratories (South Bend, IN, USA); DiOC<sub>6</sub> from Molecular Probes (Eugene, OR, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse  $\alpha$ 2, anti-mouse GPVI, and anti-mouse GPIIb $\alpha$  antibodies were from Emfret Analytics (Würzburg, Germany); FITC-conjugated anti-mouse GPIIb antibody from BD Pharmingen (San Diego, CA, USA). Anti-Src and Rabbit polyclonal anti-human SFK-pY418 antibody were obtained from Biosource (Camarillo, CA, USA), anti-Lyn from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-Fyn (FYN3) antibody, rabbit polyclonal anti-Fgr (M-60) antibody, rabbit polyclonal anti-Blk (K-23) antibody and rabbit polyclonal anti-Hck (N-30) antibody and rabbit anti-PLC $\gamma$ 2 antibody were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Rabbit polyclonal anti-Yes antibody and mouse monoclonal anti-Lck antibody and rabbit anti-Syk (BR15) polyclonal antibody were a gift from Mike Tomlinson and Joe Bolen (DNAX, USA). Mouse anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. Horseradish peroxidase (HRP) conjugated secondary antibodies were from GE Healthcare. All other reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

**Mutant mice.** Mice were housed under specific pathogen-free conditions in accordance with institutional guidelines approved by United Kingdom Home Office. *fyn*<sup>-/-</sup>, *lyn*<sup>-/-</sup> and *src*<sup>-/-</sup> mice were from Jackson Laboratories (Bar Harbor, ME, USA). *fgr*<sup>-/-</sup> mice have been described [33]. *fyn*<sup>-/-</sup>*lyn*<sup>-/-</sup> and *fgr*<sup>-/-</sup>*lyn*<sup>-/-</sup> mice were obtained from breeding of dual heterozygous

deficient mice. Mice were used between 8 and 12 weeks of age. *lyn<sup>-/-</sup>src<sup>-/-</sup>* and *fyn<sup>-/-</sup>src<sup>-/-</sup>* mice were generated as radiation chimeras ([34,35] and Supplemental Data) and used for experiments at 8 weeks after irradiation.

**Generation of chimeric mice.** 8 week-old C57BL/6 mice received two doses of irradiation, each of 450 Gy given 3 hours apart. The bone marrow from these mice was reconstituted with an intravenous injection of  $1.5 \times 10^6$  foetal liver cells obtained from E14-16 days [34] *lyn<sup>+/+</sup>src<sup>+/+</sup>*, *fyn<sup>+/+</sup>src<sup>+/+</sup>*, *lyn<sup>-/-</sup>src<sup>-/-</sup>* and *fyn<sup>-/-</sup>src<sup>-/-</sup>* mouse embryos. The genotype of the reconstituting foetal liver cells was assessed by PCR and the loss of protein expression was confirmed by western blot [35].

**Preparation of mouse platelets and aggregation.** Blood was drawn into acid citrate dextrose (10%) from the inferior vena cava of CO<sub>2</sub> asphyxiated mice following isoflurane anesthesia. Washed platelets were prepared as previously described [36]. Platelet aggregation and adenosine triphosphate (ATP) secretion were measured simultaneously under continuous stirring at 1000 rpm at 37°C using a Born lumi-aggregometer (Chrono-Log).

**Flow cytometry.** Platelets were stained with FITC-conjugated anti-mouse  $\alpha_2$ , anti-mouse GPVI, anti-mouse  $\alpha$ IIb and anti-mouse GPIb $\alpha$  antibodies for 30 min and analyzed using a FACScalibur flow cytometer (Becton Dickinson) and Summit software (DAKO).

**Western blotting and Immunoprecipitation Studies.** Protein samples from lysed mouse platelets, homogenised spleen and lung tissue were quantitated and normalized using Biorad DC protein assay kit. Syk and PLC $\gamma$ 2 immunoprecipitations were performed as described previously [35]. Whole cell lysates or immunoprecipitates were resolved on 4-12% NuPAGE Bis-Tris gradient gels, transferred to PVDF membrane and immunoblotted with primary antibodies and horseradish peroxidase-conjugated secondary antibody. Proteins were detected by enhanced chemiluminescence and autoradiography.

**Generation of mouse Fgr N-terminus protein.** Fgr cDNA was obtained from IMAGE consortium (Source Bioscience, Nottingham, UK). PCR product generated by primers containing EcoRI and NotI sites and mutational primers complementary to sequence within the Fgr cDNA sequence was cloned into pGEX4T2 vector (GE Life Sciences). The N-terminal (unique) domain of Fgr was then generated from the above product using primers complementary to the 5'-end of cDNA and a site 210bps into Fgr coding sequence. After transformation into BL21 chemically competent *E.coli*, proteins were expressed using IPTG. GST-Fgr (N-terminus) was then purified by affinity chromatography on a glutathione column.

**Lipid raft isolation.** Washed platelets (500ml at  $2 \times 10^9$ /ml) were stimulated with 3  $\mu$ g/ml CRP for 5 min in the presence of the  $\alpha$ IIb $\beta$ 3 antagonist, lotrafiban (10 $\mu$ M)). Lipid rafts were isolated as described previously [37].

**Quantitation of SFKs in mouse platelets.** Recombinant protein for mouse Src, Fyn, Lyn (Enzo Life Sciences) and Fgr (see below) at final amounts of 1, 3, 10 and 30 ng were resolved on 10% SDS-PAGE alongside mouse platelet lysates. Proteins were quantitated by densitometry after immunoblotting with appropriate primary and secondary antibodies using Odyssey infrared imaging system (Li-Cor). Subsequently, densitometry data from recombinant protein was used to generate a standard curve and concentration of proteins within the platelet lysate was determined. In the case of Lyn, which runs as a doublet in platelets, both bands were used for quantitation.

**Static adhesion and spreading studies.** Washed platelets ( $2 \times 10^7$ /ml) were incubated on the fibrinogen (100  $\mu$ g/ml) -coated coverslips for 45 min at 37°C and subsequently fixed.. Surface area of spreading platelets were analysed from differential interference contrast (DIC) images using Image J software as described [38,39]. Spreading was normalised by comparison of the wild-type platelet response observed in the absence (100%) and presence (0%) of the SFK inhibitor PP2.

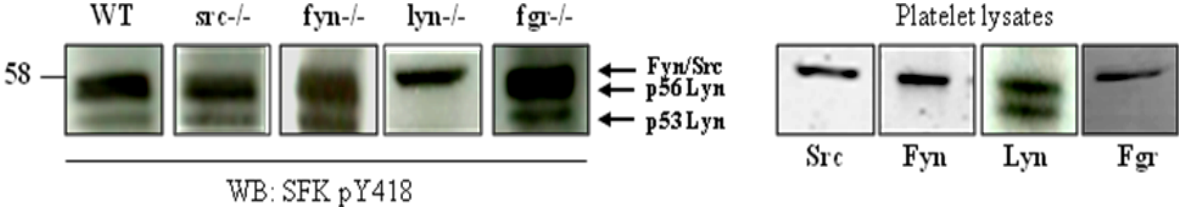
**Whole blood flow studies on immobilized collagen.** Anticoagulated (sodium heparin (10IU/ml) and PPACK (40 $\mu$ M)) mouse whole blood was fluorescently labeled with DiOC<sub>6</sub> (2 $\mu$ M, 10 min at 37°C)

and flowed through the type-I collagen from equine tendon (100 µg/ml) -coated capillary for 4 min at a shear rate of  $1000\text{s}^{-1}$ , followed by washing for 4 min at the same shear rate with modified Tyrode buffer before being fixed [38,40]. DIC image analysis was performed off-line using ImageJ as the percentage of surface area covered by platelets and surface area of thrombi formed.

***Laser-induced thrombus formation model.*** Laser-induced thrombi were generated at the luminal surface of arterioles of the cremaster muscle as previously described [7,41]. Real-time intravital brightfield and fluorescent real-time images of the developing thrombus were captured simultaneously and then analysed off line as described previously [7,41].

***Statistical analysis.*** Statistical analysis was performed using GraphPad Prism and significance was evaluated using One way Anova or Student's *t* test. *P value* < 0.05 was considered statistically significant.

**Supplementary Figure 1:** Identification of SFKs on phospho-tyrosine blots. *Left panel:* lysates from wild-type and SFK-deficient platelets were resolved on 10% SDS-PAGE gels before being transferred and blotted with either SFK pY418. *Right panel:* lysates from wt platelets only were immunoblotted with specific SFK antibodies. Gels were then aligned in order to determine the identity of the band of interest.



**Supplementary Figure 2:** Washed platelets ( $2 \times 10^8/\text{ml}$ ) prepared from *fyn*<sup>-/-</sup>, *lyn*<sup>-/-</sup>, *src*<sup>-/-</sup> or *fgr*<sup>-/-</sup>, *fyn*<sup>-/-</sup> *lyn*<sup>-/-</sup>, *fyn*<sup>-/-</sup> *src*<sup>-/-</sup> or *lyn*<sup>-/-</sup> *src*<sup>-/-</sup> mice and their respective litter-matched controls were stimulated with thrombin (0.5UI/ml). Platelet aggregation was measured as a change in light transmission using a lumi-aggregometer. Representative images are shown (n = 3 mice per condition).

