SUPPLEMENTAL DATA

Dok-2 regulates the shear-dependent adhesive function of platelet integrin $\alpha_{IIb}\beta_3$ in mice

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials - Murine-specific antibodies directed against GPVI (Six.E10 FITC), GPIbα (Xia.G5 PE), integrin α_2 (SAM.G4, FITC)) and integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa, [Leo.D2 FITC]), as well as the GPIb blocking antibody (Xia.B2) and either FITC- or PE- conjugated rat polyclonal IgG were purchased from Emfret Analytics (Emfret Analytics GmbH & Co., Germany). FITC-hamster anti-rat CD29 (integrin β_1) and PE-rat anti-mouse CD49e (integrin α_5) monoclonal antibodies were from BD Biosciences (Australia). Hexamethyldisilazane (HMDS) was from Sigma (St Louis, MO) and used according to Nesbitt et al (1). Dok-2 and Dok-1 antibodies have been previously described (2). All other reagents were from previously published sources (3-6).

In vitro perfusion studies - Perfusion assays were performed according to modifications of Maxwell *et al* (6), and Goncalves *et al* (7), using microslides (0.1 x 1.0 mm) coated with either fibrinogen (5-100 μg/ml) or type I collagen (10-100 μg/ml). Platelet adhesion was monitored using Differential Interference Contrast (DIC) Microscopy (inverted Leica DMIRB microscope, with 63X water objective (numerical aperture [NA] 1.2) or 100X PL APO objective (NA 1.40 to 0.7) (Leica Microsystems, Wetzlar, Germany), and recorded in real-time using a DAGE-MTI Charged-Coupled Device (CCD) camera 300 ETRCX (Dage-MTI, IN, USA), for off-line analysis.

(i) Reconstitution studies: HMDS-pretreated microslides (1) coated with immobilized fibrinogen ($600s^{-1}$) were blocked with Bovine Serum Albumin (BSA) (2%), followed by perfusion of mouse whole blood or mouse platelets reconstituted with RBCs (50% hematocrit). Non-adherent platelets were removed by perfusion of modified Tyrode's Buffer (10 mM HEPES, 12 mM $NaHCO_3$, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$ and 5 mM glucose, pH 7.2-7.3) and adhesion was monitored by DIC microscopy, as described above. Platelets were considered adherent if they remained attached to the matrix for at least 2 s.

(ii) Translocation dynamics and stationary adhesion: Platelet adhesion lifetime was determined by counting the number of frames for which a platelet was tethered to the matrix or an immobilized platelet, with an arbitrary cut-off of 250 frames (10 s). For analysis of initial platelet tethering, platelet displacement and average velocity, videos were digitized using DVTools (Pinnacle Systems) and each frame saved in TIFF file format using Adobe Premiere Pro v1.5 software (Adobe Systems). Adherent platelets were demarcated using ImageJ (ImageJ 1.38x, National Institutes of Health, USA) and the number, displacement and period of adhesion (expressed as 'number of frames') of incoming platelets was assessed on a frame-by-frame basis. Platelet behavior was classified as stationary (moving <1 cell diameter in 10 s), rolling (continuous rolling interactions over 10 s) or stop/start (distinct periods of stationary and rolling behavior over 10 s).

(iii) Two-stage analysis of platelet-platelet interactions: A modified method of Maxwell *et al* (6) was used for two-stage analysis of platelet-platelet interactions. Briefly, untreated washed platelets ($2x10^8$/ml) (in the absence of calcium) were perfused over HMDS-treated glass microslides ($1800\ s^{-1}$), and flow stopped to allow platelet adhesion and spreading . BSA (2%) was then perfused ($1800\ s^{-1}$) to block any uncoated glass, followed by perfusion of Tyrode's buffer. Anticoagulated murine whole blood was perfused over spread platelets monolayers at $1800\ s^{-1}$, followed by perfusion of modified Tyrode's buffer ($1800\ s^{-1}$) to remove non-adherent platelets. Platelet tethering to monolayers was imaged and recorded as described above, for off-line analysis. The number of platelets adherent to a single immobilized/nucleated platelet was determined, along with the lifetime of interaction. In some experiments, whole blood was treated with Xia.B2 (30 μg/ml) or GPI562 (10 μM) prior to perfusion.

(iv) Platelet:fibrinogen bond strength: A simplified flow perfusion method from Goncalves *et al* (8) was utilized. Briefly, whole blood was perfused over fibrinogen in the presence or absence of ADP receptor antagonists (MRS 400μM, 2MeSAMP 40μM) and Apyrase (0.02U/ml) at $600\ s^{-1}$ for 2 mins. The shear rate was increased to $1800\ s^{-1}$ for 3 mins using a programmable Harvard Syringe pump. Platelet adhesion was imaged and recorded for off-line analysis. Platelets were demarcated at 2 mins ($600\ s^{-1}$), and subsequently monitored until detachment (following an increase in shear rate to $1800\ s^{-1}$).

(v) Determination of thrombus surface area and volume: In studies evaluating platelet thrombus growth on immobilized bovine fibrillar type I collagen, microslides were coated with 5-100 μg/ml type I

collagen, for 2 h at room temperature. Mouse whole blood, in the presence or absence of the integrin $\alpha_{IIb}\beta_3$ inhibitor GPI562 (10 μM), was perfused for 3 mins at 1800 s^{-1}, followed by perfusion of modified Tyrode's buffer to remove non-adherent platelets.

a: Surface area – to determine rate of thrombus growth, a single field of view was imaged throughout the entire 3 min of flow, with additional fields also imaged at 3 and 13 mins. Surface area coverage was quantitated in pixels using ImageJ (1.38x) and converted to μm^2.

b: Thrombus volume - Thrombi were fixed in 4% PFA for 30 min, labeled with DiOC6 (1 μM), imaged via confocal sectioning (1 μm sections) (Leica TCS NT, Netherlands, 63X water objective, NA 1.2), and thrombus volume quantified offline (Metamorph 6, Universal Imaging Corp.; U.S.A).

(vi) Reciprocal flow studies: Whole Thrombi were performed by perfusion of WT or Dok-$2^{-/-}$ hirudinated mouse whole blood over a type I collagen matrix at 1800 s^{-1}. Whole blood from either WT or Dok-$2^{-/-}$ mice was then perfused over these preformed thrombi at 1800 s^{-1}. The number of platelets interacting with preformed thrombi of like-size was monitored using DIC microscopy and quantified off-line.

Electrolytic in vivo model of thrombosis - An electrolytic model of occlusive thrombus formation was performed as described by Sturgeon *et al* (5). An electrolytic injury was applied to an exposed carotid artery (4mA, 1.25 minutes, constant current), and blood flow monitored using a Doppler flow probe for 30-35 mins after delivery of electrical insult.

Intravital studies - **(i) Needle puncture model:** Intravital studies were performed according to a modified method of Denis *et al* (9). Mice (~18-20 g) were anaesthetized (60 mg/kg sodium pentobarbitone) and mesenteric venules (100 – 200 μm) were exteriorised via abdominal incision. Vessels were punctured using a microinjector needle with a standardized tip diameter of 4-6 μm (10). Microinjector needles (Glass capillaries, GD-1, 1x50 mm, Narishige, Japan) were prepared using a Narishige needle puller (PC-10 capillary puller, Narishige, Japan). Axial control of the microinjection needle was obtained using an Injectman micromanipulator (Eppendorf, USA). The tip of the microinjector needle was placed in the center of the vessel, 20 μm above the vessel bottom. Platelet aggregate formation was allowed to develop on the tip of the microinjector needle. Platelet interactions were visualized using DIC microscopy (Olympus IX81 inverted microscope; objective: 60X PlanSAPO, NA 1.35) and captured for off-line analysis using a digital EMCCD camera (QuantEM, photometrics), in combination with Metamorph 7.5 (Biostrategy, Australia). Platelet aggregate size was analyzed at 10 s intervals by 2D surface area analysis performed using ImageJ software (NIH, U.S.A.).

(ii) Rose Bengal injury model: The development of platelet aggregates and/or thrombi *in vivo* following Rose-Bengal induced injury was monitored using intravital microscopy. WT and Dok-$2^{-/-}$ mice (14-18 g) were prepared and maintained as described above. Vessel injury was achieved through photoactivation (excitation wavelength of 550 nm, 30s) of systemically administered Rose Bengal (10 mg/kg) (11). Platelet accrual to the vessel injury was imaged using DIC microscopy in real-time as described above. The number of platelets present in the field of view was determined off-line, at pre-determined time intervals (time zero = cessation of photoactivation) using ImageJ software for cell counting, with an arbitrary cut-off of 250 platelets, being applied.

Flow cytometric analysis of platelet glycoprotein expression levels, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression - For measurement of the levels of surface expressed platelet glycoproteins, mouse whole blood was collected in Clexane® (Enoxaparin, 40 U/ml) and diluted in platelet washing buffer (PWB) (1/20). Fluorescently conjugated murine specific antibodies (5 μl) Six.E10 FITC (anti-GPVI), Xia.G5 PE (anti-GPIbα), or Leo.D2 FITC (anti-GPIIb/IIIa or integrin $\alpha_{IIb}\beta_3$), were added to 25μl of diluted whole blood and incubated for 15 mins. For measurement of integrin $\alpha_{IIb}\beta_3$ activation, mouse whole blood was collected in Clexane® (40 U/ml) and diluted (as described above) or mouse washed platelets were isolated from WT or Dok-$2^{-/-}$ mice (5 x 10^7/ml). Whole blood/washed platelets were incubated with either Oregon-green fibrinogen, fluorescently conjugated JON-A, or the fluorescently conjugated IgG control antibody. Samples were allowed to rest or were stimulated with the indicated concentrations of CRP, Protease Activated Receptor 4 agonist peptide (PAR4AP) or ADP. For measurement of P-selectin expression, washed platelets isolated from WT or Dok-$2^{-/-}$ (5 x 10^7/ml) mice

were stimulated with thrombin (0.1-1.0 U/ml) in the presence of the FITC-conjugated anti-mouse P-selectin antibody. Samples were incubated for 30 mins. In all studies, platelet reactions were terminated by addition of 450 µl filtered PBS and samples analysed immediately by flow cytometry (FACScalibur, Becton Dickinson).

Platelet Aggregation - Washed platelets (3 x 10^8/ml) isolated from WT or Dok-$2^{-/-}$ mice were incubated with different concentrations of the indicated platelet agonists, in the presence of stirring (900 rpm). Aggregation was monitored in real-time using a four-channel automated platelet analyzer (AggRAM, Helena Laboratories), as previously described (4), and expressed as percentage (%) light transmission.

Clot retraction - Platelet Rich Plasma (PRP) was isolated from citrate anti-coagulated mouse whole blood. Platelet counts between mouse genotypes were normalized using autologous platelet poor plasma (PPP). Clot formation was initiated by the addition of exogenous calcium (1 mM), and platelet-mediated clot retraction initiated by addition of thrombin (5 U/ml), at 37^{o}C. Retraction was monitored visually at 15 min intervals for a maximum of 60 min, with digital images obtained using a Pentax K10D DSLR with 105 mm macro lens.

Static adhesion assays - Static adhesion assays were performed as previously described (7) with minor modifications. Brielfy, glass coverslips were treated with HMDS, according to Nesbitt *et al* (1), prior to coating with the indicated concentration of purified fibrinogen, and incubation with 2% BSA for 30 mins at room temperature, to block exposed glass. PRP platelet count was normalized to 1-2 x 10^7/ml with Tyrode's buffer, and platelets allowed to spread for 15, 30 and/or 45 mins before fixation and imaging, as described previously (7).

Dok-1 and Dok-2 protein expression - Platelets were isolated from WT or Dok-$2^{-/-}$ mice, stimulated, lysed in 2X reducing buffer, and analysed by SDS-PAGE using pre-cast gradient gels (4-12 %) as described previously (2). Proteins were transferred to PVDF and membranes immunoblotted with anti-Dok-2 antibody (H-192), stripped and then reprobed with anti-Dok-1 Ab (M-276).

Immunoprecipitation of Dok-2 from Lyn deficient platelets - Dok2 immunoprecipitation and western blotting was performed according to the method of Garcia *et al* (12). Briefly, washed $Lyn^{+/+}$ or $Lyn^{-/-}$ platelets ($8x10^8$/ml, 500 µl) were stimulated with thrombin (1 U/ml, 90 sec), and lysed with an equal volume of ice-cold 2x lysis buffer (2% NP-40, 20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 10µg/ml leupeptin, 10 µg/ml aprotinin, and 1 µg/ml pepstatin A, pH 7.3). Lysates were clarified by centrifugation (15,000 x*g*, 5 min, 4^{o}C), and incubated with 5 µg of 4G10 antiphosphotyrosine monoclonal antibody (Millipore) and 30 µl of protein A/G Plus –Agarose (Santa Cruz, CA) (4°C, 4 hrs). Protein A/G-Agarose beads were washed 3 times with 1x lysis buffer, subjected to 10% SDS-PAGE under reducing conditions, transferred to PVDF membranes. Membranes were probed with a rabbit anti-Dok-2 Ab (Santa Cruz). To ensure an equal protein loading, the same PVDF membranes were subsequently probed with 4G10 antiphosphotyrosine monoclonal antibody.

SAX-HPLC-based analysis of 3-phosphorylated phospholipids - Analysis of 3-phosphorylated phospholipids was performed as described previously (3,13). Briefly, washed platelets ($5x10^8$/ml) were labeled with 1.0mCi/ml inorganic ^{32}P, prior to stimulation with the indicated agonist. ^{32}P-labelled phospholipids were extracted and deacylated, followed by separation on a partisphere SAX-HPLC column using a NaH_2PO_4 (1.25 M) gradient (Whatman, 4.6 mmx125 mm).

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SUPPLEMENTAL VIDEO LEGENDS

Video 1. Dok-2 negatively regulates platelet adhesion to immobilized fibrinogen under flow. Anticoagulated whole blood from WT (WT, *left panel*) and Dok-2$^{-/-}$ (KO, *right panel*) mice was perfused across fibrinogen-coated (20 μg/ml) microslides at a shear rate of 600 s^{-1}. Direction of perfusion is indicated by the arrow. Video demonstrates the dynamics of mouse platelet adhesion on a fibrinogen substrate under flow conditions. Following whole blood perfusion, microslides were washed with modified Tyrode's buffer (period corresponds to the phase in the video marked *washout*). This video was taken from 1 experiment, representative of 4 independent experiments.

Video 2. Deficiency of Dok-2 enhances platelet aggregate development in a needle puncture model of thrombosis. This video demonstrates platelet aggregate formation in mouse mesenteric venules (imaged using intravital microscopy, as described under "Experimental procedures"), induced by a mechanical needle puncture (needle entering vessel from right-hand side of vessel). Left panel shows the rate and extent of thrombus growth in a WT mouse, whereas the right panel shows the rate and extent of thrombus growth in a Dok-2$^{-/-}$ mouse. These videos are taken from one WT and one Dok-2$^{-/-}$ mouse vessel, representative of 23-25 injuries.

Supplementary Figures and Legends

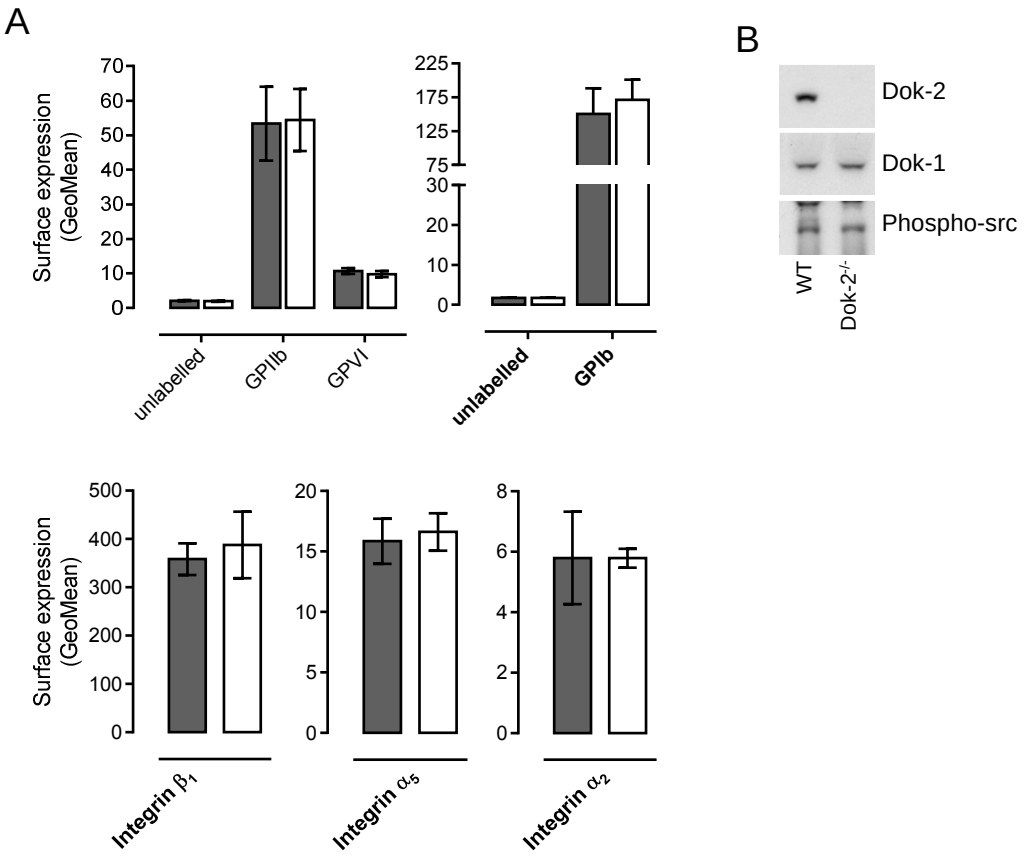

Figure S1. Dok-2 deficiency does not alter the surface expression of the major platelet glycoproteins or the protein expression of Dok-1. (A). Histogram depicting the surface expression of the major platelet glycoproteins GPIbα, GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), GPVI, integrin β_1, inetgrin α_5 and integrin α_2 (mean ± SEM, n=3). **(B)** Immunoblot analysis of Dok-1 and Dok-2 protein levels in stimulated platelet lysates from wild type (WT) or Dok-$2^{-/-}$ mice. Immunoblots were probed with an anti-Dok-2 Ab (H-192), stripped and reprobed with an anti-Dok-1 Ab (M-276). Blots were also probed for phosphotyrosine, and src (60 kDa) is represented as a loading control (lower panel). This image is taken from one immunoblot, representative of three independent experiments.

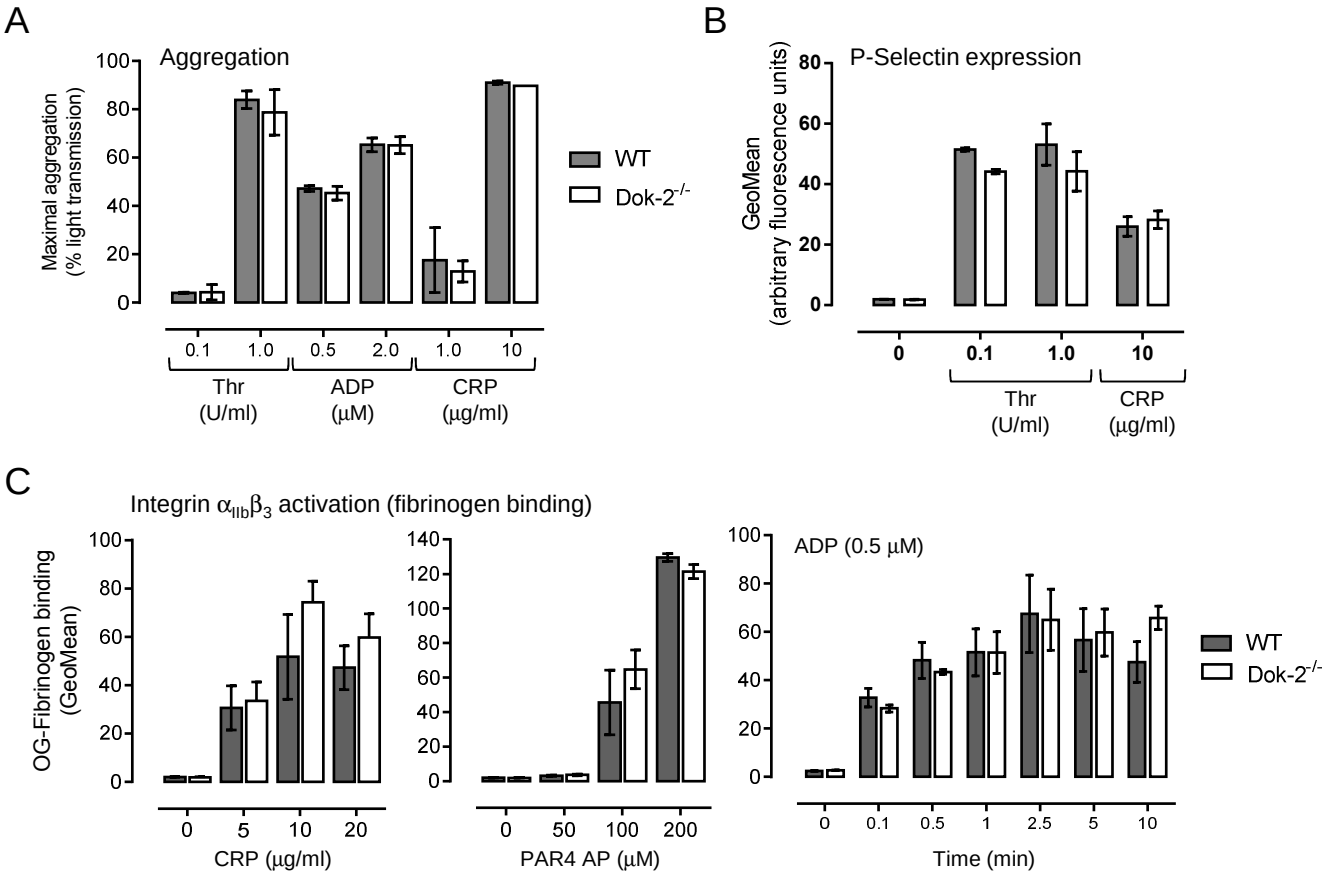

Figure S2. Dok-2 deficiency does not cause a global upregulation of integrin $\alpha_{IIb}\beta_3$ adhesive function. Histograms in this figure depict **(A)** maximal platelet aggregation (% light transmission), **(B)** levels of P-selectin expression and (C) Integrin $\alpha_{IIb}\beta_3$ activation, as measured through the binding of Oregon-Green (OG)-labeled fibrinogen, in WT and Dok-$2^{-/-}$ mouse platelets, following stimulation with the indicated agonists. In all experiments, platelets were stimulated for 30 minutes, unless otherwise indicated. **(A-C)** Data represents the mean ±SEM, n=3, with no statistical differences were found between WT and Dok-$2^{-/-}$ platelets.

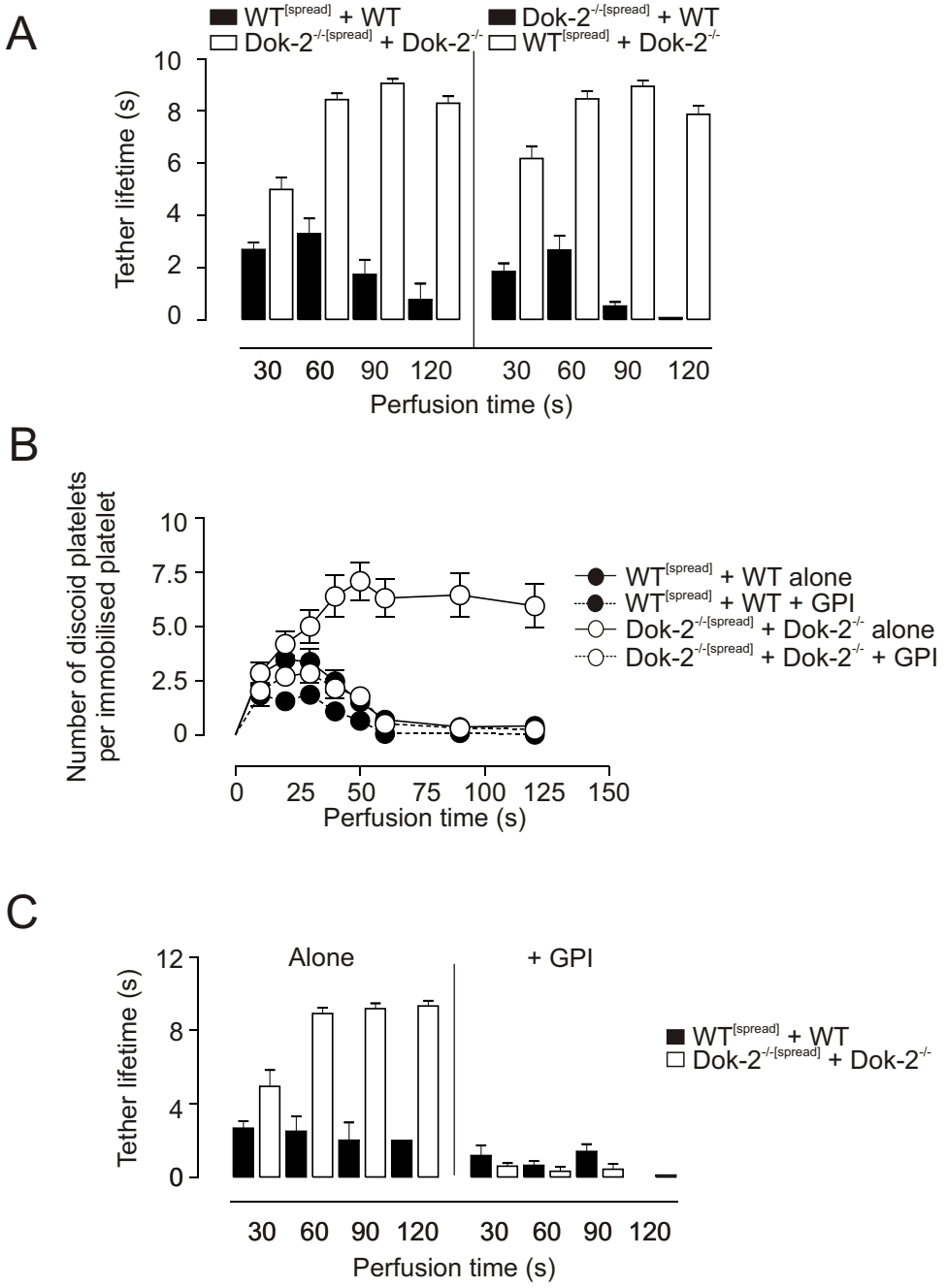

Figure S3. Dok-2 deficiency is associated with enhanced platelet-platelet adhesive interactions under flow. (A-C) Anticoagulated mouse whole blood was perfused over spread platelets for 2.5 min, and platelet-platelet interactions monitored in real-time using Differential Interference Contrast Microscopy. The total number of tethering discoid platelets (**B**) as well as their tether lifetimes (s) (**A, C**), defined by the number of frames for which the platelet remained attached to the spread platelet, was quanitifed off-line. (**C**) In some experiments; wild type (WT) blood was perfused over WT spread platelets (*$WT^{[spread]}$ + WT)*, and Dok-$2^{-/-}$ blood was perfused over Dok-$2^{-/-}$ spread platelets (*Dok-$2^{-/-[spread]}$ + Dok-$2^{-/-}$),* in the presence or absence of the integrin $\alpha_{IIb}\beta_3$ antagonist, GPI562 (GPI, 10 μM) (**B, C**) (mean ± SEM, n=3).

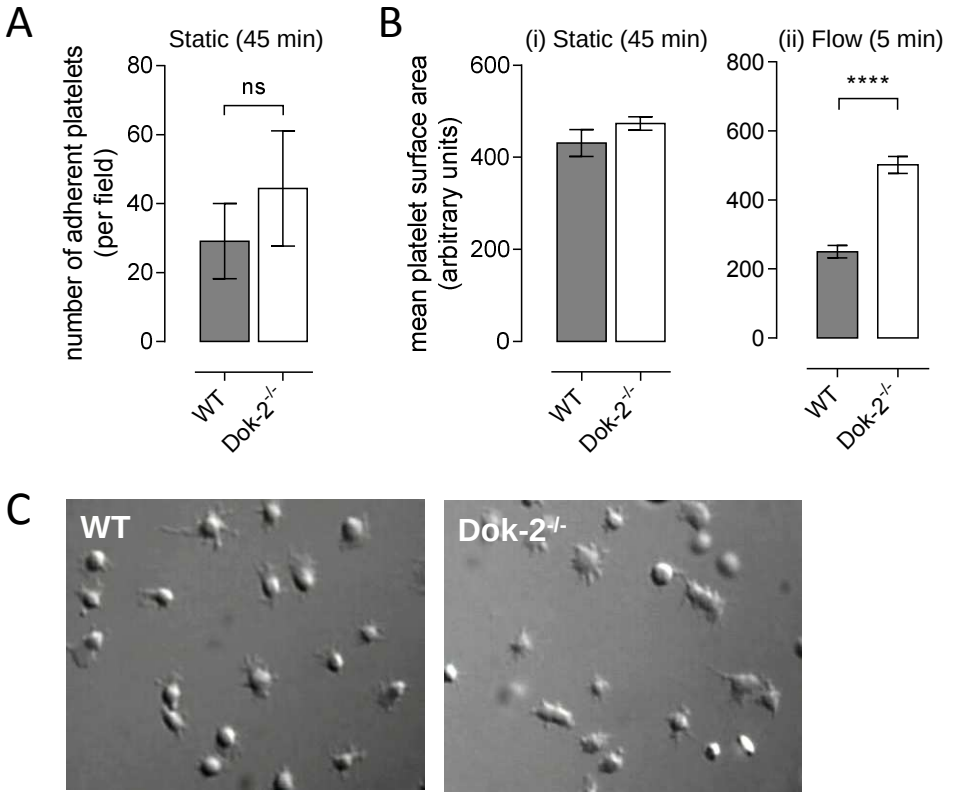

Figure S4. Dok-2 deficiency has no effect on platelet adhesion under static conditions. Histograms in this figure depict the number of wild type (WT) or Dok-2$^{-/-}$ platelets adherent to immobilized fibrinogen under static conditions **(A)**, as well as the mean surface area of individual platelets, under static **(i)** and flow **(ii)** conditions **(B)**. In all experiments, platelets were allowed to adhere for the indicated times, and data represents the mean ± SEM, n=3, where ^{ns} >0.05; ****, p<0.0001. **(C)** Representative images depicting adherent platelets on fibrinogen-coated coverslips, post 45-minutes application under static conditions. These images are taken from one representative of three independent experiments.

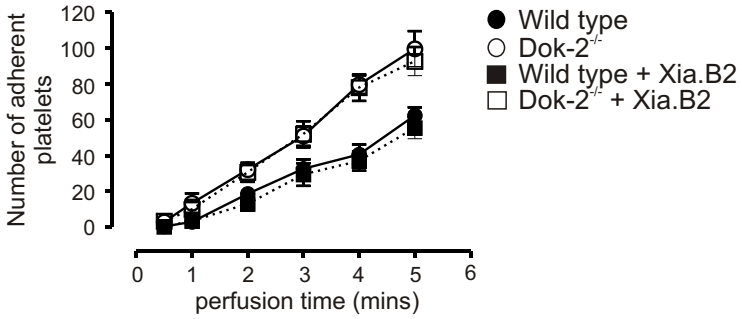

Figure S5. Mouse platelet translocation on fibrinogen is not mediated by the GPIb/V/IX-VWF interaction. Whole blood derived from wild type (WT) or Dok-$2^{-/-}$ mice was perfused across a fibrinogen matrix (20 μg/ml) in the presence (squares) or absence (circles) of the GPIb blocking antibody Xia.B2. The number of adherent platelets (per 25% of field) was quantified at the indicated time points (mean ± SEM, n=3).

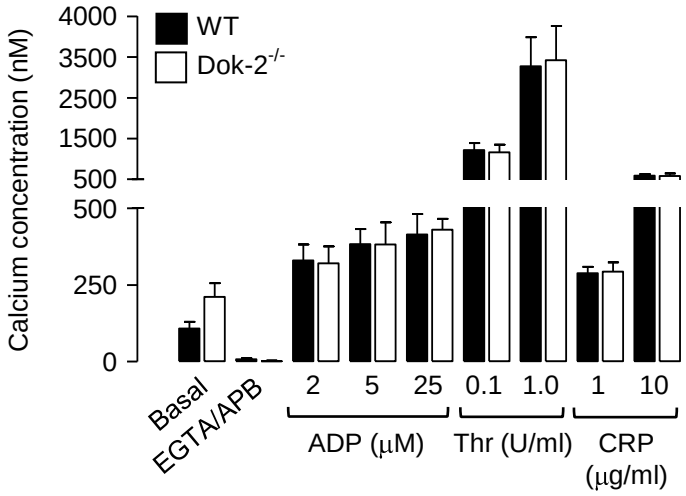

Figure S6. **Dok-2 deficiency is associated with normal soluble agonist-induced platelet calcium flux.** Washed platelets (2.5 x 10 /ml) isolated from wild type (WT) and Dok-2 mice were loaded with calcium dyes (Oregon-green BAPTA and FuraRed), and calcium concentrations (nM) determined under basal conditions, or following stimulation with the indicated concentrations of ADP, thrombin (Thr) or CRP for 10 min. Data are taken from 1 experiment, representative of 3 independent experiments.