The GPIbα intracellular tail - role in transducing VWF- and collagen/GPVI-mediated signaling

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SUPPLEMENTARY METHODS

Determination of complete blood counts, platelet counts, surface protein expression and platelet activation.

Mice were anaesthetized with ketamine/medetomidine and blood was collected retro-orbitally in 3.8% citrate. Blood was diluted with equal volume of saline and analyzed by the clinical pathology laboratory at Hammersmith Hospital to obtain full blood counts. Platelet counts were determined using precision count beads (Biolegend) and flow cytometry according to the manufacturer's instructions.

Platelets were washed as previously described with the following modifications.(1) Blood was diluted in an equal volume of modified Tyrode's buffer supplemented with prostaglandin E1 (PGE1) and apyrase (both from Sigma) and centrifuged at 150x*g* for 10 mins at room temperature (RT). PRP was subsequently centrifuged at 1000x*g* for 10 mins at RT and three additional centrifugation steps were performed to wash the platelets. Platelets were resuspended at $3x10^5$ platelets/µl in modified Tyrode's buffer. In experiments using plasma-free blood, red blood cells and leukocytes were separately washed twice in PBS, by centrifugation at 650xg for 10 mins at RT and resuspended in Tyrode's buffer. Washed platelets were subsequently added to obtain plasma-free blood.

Flow cytometry was performed to analyze the surface expression of GPIbα, GPIbβ, $\alpha_{IIb}\beta_3$ and GPVI in platelets from *GpIbα*$^{\Delta sig/\Delta sig}$ and wild-type littermates using the following antibodies (Abs; Emfret): XiaB2, X488, Leo.H4, and JAQ1, respectively. Whole blood was diluted with modified Tyrode's buffer (1/20) and stained with Abs for 15 mins at RT before being analysed. Mouse platelets were washed as above and incubated with varying concentrations of agonists - ADP (2-20µM; Labmedics), thrombin (0.02-0.2U/ml; Enzyme Research Laboratories [ERL]), CRP (1-10µg/ml; Cambcol Laboratories), rhodocytin (3 and 300nM; kindly provided by Professor Eble and Dr Hughes) in the presence of 2mM $CaCl_2$ for 10 mins at RT. Thereafter, platelets were incubated with JON/A-PE and Wug.E9-FITC Abs for 15 mins at RT to analyze the surface expression of activated $\alpha_{IIb}\beta_3$ and P-selectin. Samples were analyzed using a BD LSRFortessa X-20 flow cytometer.

Platelet aggregometry

Platelet aggregation was assessed by light transmission using the Chronolog 700 aggregometer with continuous stirring at 1,200 rpm at 37°C. Washed platelets were resuspended to a final concentration of $3x10^5$ platelets/µl in modified Tyrode's buffer and supplemented with 70µg/ml fibrinogen (ERL), 1mM $CaCl_2$ and different concentrations of ADP (1-10µM), α-Thrombin (10-50mU/ml) or CRP (0.5-10µg/ml). Platelet aggregation was monitored over 6 mins.

Platelet spreading

Coverslips were coated with fibrinogen (200µg/ml), CRP (100µg/ml), murine VWF (10µg/ml) or BSA (0.5mg/ml) overnight at 4°C. Coverslips were then blocked with PBS-BSA (5mg/ml) for 1 hour at RT. Washed *GpIbα*$^{+/+}$ or *GpIbα*$^{\Delta sig/\Delta sig}$ mouse platelets were added to the coverslips (150µl/coverslip, 25,000 platelets/µl) in the presence or absence of thrombin (1U/ml) or Botrocetin (2µg/ml) and allowed to adhere for 30 mins – 1 hour, at 37°C. When indicated, platelets were incubated with GR144053 (20µM) for 10 minutes to inhibit $\alpha_{IIb}\beta_3$ outside-in signaling prior to stimulation with Botrocetin. Coverslips were then washed with PBS, fixed with 10% formalin, and finally quenched with 50mM NH_4Cl-PBS. Platelets were then permeabilized in 0.1% Triton-PBS and stained with Flash Phalloidin™ Green 488 (2U/ml; Biolegend) for 1.5 hours, at RT. Finally, coverslips were mounted onto slides using ProLong™ Gold Antifade Mountant with DAPI (Thermofisher). Spread platelets were visualized using either a Vert.A1 inverted microscope (Zeiss; 40x and 63x air objectives) equipped with ExiBlue camera (Q Imaging) or a confocal microscope (SP5 Leica, 63x objective, z-stack, oil immersion). At least 3 fields of view were analyzed per condition. Surface area of spreading platelets was quantified using Slidebook software 5.0 (3i) and filopodia counted independently by two different researchers.

Western blotting

For analysis of GPVI and CLEC-2 tyrosine-mediated signaling pathways, washed platelets ($3x10^5$ platelets/µl) were stimulated for the indicated time points with 3µg/ml CRP or 30 and 300nM rhodocytin, respectively. Samples were lysed with an equal volume of RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (cOmplete, mini and PhosSTOP from Roche). The samples were run under reducing conditions with 4-12% Bolt™ Bis-Tris Plus or 4-20% Novex™ WedgeWell™ Tris-Glycine, 1.0 mm pre-cast gels and proteins were transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 3% BSA-TBS and incubated overnight at 4°C with the following primary antibodies: anti-phosphotyrosine 4G10 (Millipore), anti-phosphorylated SYK (pY525/526; Abcam), anti-SYK (D1I5Q; Cell signaling Technology), anti-PLCγ2 (Cell signaling Technology), anti-phosphorylated PLCγ2 (pY1217; Cell signaling Technology), anti-β-actin (Cytoskeleton Inc.), anti-GAPDH (1D4; Novus Biological). The membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse/rabbit secondary Abs (Dako) for 1h at RT and developed using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore). Detection and quantification of chemiluminescence intensities were quantified by using Chemidoc™ imaging system. and Image Lab 5.2.1 software (BioRad).

Flow assays

Murine VWF was expressed in HEK293T cells, purified and quantified as previously described.(2) VenaFluoro8+ microchannels (Cellix) were coated directly with murine VWF (36.75µg/ml) or collagen (200µg/ml; Labmedics) overnight, at 4°C, in a humidified chamber. Channels were blocked for 1 hour, at RT with HEPES Tyrode's buffer (134mM NaCl, 0.3mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5mM Glucose, 1mM $MgCl_2$, pH 7.3) supplemented with 1% BSA.

On the day of the experiments, blood was collected retro-orbitally from *GpIbα*$^{\Delta sig/\Delta sig}$ mice and wild-type littermates in 100µg/ml Hirudin (Refludan, CSL Behring GmbH) and labelled with Dy-Light 488-conjugated rat anti-mouse GPIbβ (Emfret Analytics, 6µg/ml). When indicated, blood was incubated 5 min prior perfusion with GR144053 (10µM), anti-GPVI JAQ1 or control Rat IgG (Emfret; 20µg/ml). Thereafter, whole blood or plasma-free blood was perfused through the channels at 200-1000s^{-1} using a Mirus pump (Cellix) for 3.5 mins and platelet adhesion/aggregate formation monitored in real-time by fluorescence microscopy (Vert.A1 inverted microscope, Zeiss), using an inverted CCD camera (ExiBlue from Q imaging) operated by the SlideBook^{TM}5.0 software. Quantification was performed using SlideBook 5.0 software (3i), to analyze platelet coverage, platelet velocity and thrombus build-up.

Tail-bleeding assay

Tail bleeding time was performed as described previously.(1, 3) Mice were anaesthetized with ketamine/medetomidine, placed on a heating pad (Harvard Apparatus) at 37°C and a 2 mm segment of the tail was sectioned with a sharp blade. The tail was immediately placed in warm PBS and the time taken for the stream of blood to stop for more than 60 seconds was defined as the bleeding time. To determine the extent of blood loss during the first 10 mins, hemoglobin content was determined by the colorimetric cyanmethemoglobin method using Drabkins reagent and bovine hemoglobin as a standard (Sigma).

Laser-induced thrombosis model

Thrombus formation was evaluated in the cremaster muscle microcirculation as previously described. (1, 3) Ketamine (75mg/kg) and medetomidine (1mg/kg) was initially given as an intraperitoneally injection. The anesthesia was maintained by giving additional ketamine (12.5mg/kg) every 40 mins. Briefly, Dy-Light 488-conjugated rat anti-mouse GPIbβ Ab (0.15µg/g;Emfret) and Alexa 647-conjugated fibrinogen (5% total fibrinogen; Invitrogen) were administered via a cannula inserted in the jugular vein. Vascular injury was induced by a pulse laser (Ablate!, 3i) focused through a 63X water-immersion objective (65-75% intensity, 5-15 pulses) leading to non-ablative/superficial injury.(4) No perforating injuries were performed under those conditions. Thrombus formation was

followed in real time for 3 mins after the injury. Median integrated fluorescence intensity over time from platelet or fibrin was determined and analyzed as detailed previously.(1, 3) The operator was blinded to the genotypes during both data acquisition and analysis.

Statistical analysis

Results are presented as mean ± SEM or median ± 95% confidence interval in accordance with their normality (Shapiro-Wilk) and analyzed using GraphPad Prism (8.01). Statistical analysis was performed using unpaired student t-test, the Mann-Whitney test or repeated measures ANOVA. Significance values are indicated in each figure legends.

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SUPPLEMENTARY TABLE

Supplementary Table S1 Haematological parameters

	Gp1bα$^{+/+}$	*Gp1bα*$^{\Delta sig/\Delta sig}$
PLT (10^3/µl)	1028 ± 187	818 ± 188****
RBC (10^6/µl)	9.0 ± 1.0	8.8 ± 0.7
HCT (%)	50.7 ± 5.2	49.7 ± 3.2
WBC (10^3/µl)	5.9 ± 1.8	6.7 ± 1.5

PLT, platelets; RBC, red blood cells; HCT, hematocrit, WBC, white blood cells; ****P <0.001, unpaired, two-tailed t-test, mean ± SD (n=10 per genotype)

SUPPLEMENTARY FIGURES AND LEGENDS

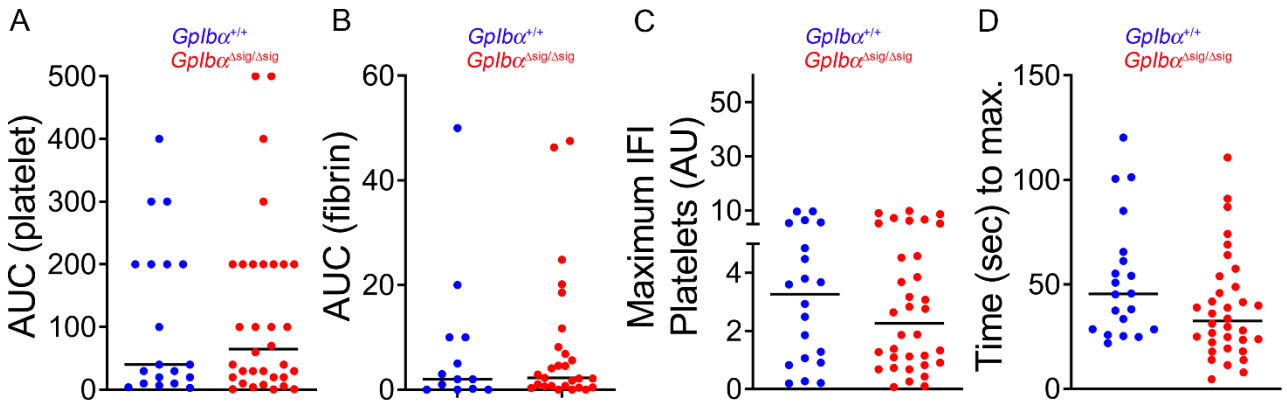

Figure S1: Thrombus formation and fibrin accumulation are similar in *Gplbα*$^{\Delta sig/\Delta sig}$ mice compared to *Gplbα*$^{+/+}$ mice. Mice were subjected to the laser induced thrombosis model as detailed in Figure 2. Graphs showing the area under curve values from the platelet IFI (A) or fibrin(ogen) IFI (B) vs time from individual thrombus. (C) Distribution of the maximal thrombus size expressed in IFI platelet arbitrary units (AU) and (D) the time to maximal thrombus size. Each symbol represents one thrombus. Horizontal lines intersecting the data set represent the median. Data was analyzed using Mann Whitney test; ns: $p>0.05$. Also see Video 1 and Fig. 2.

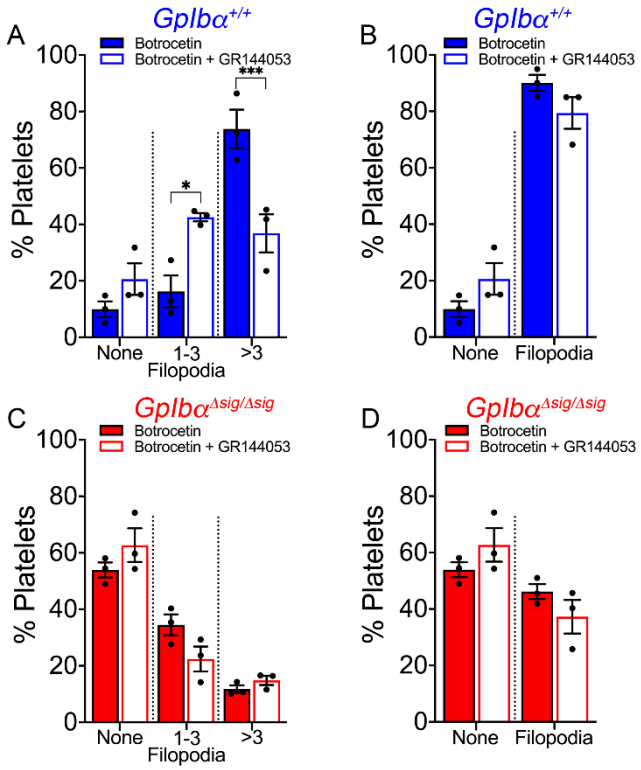

Figure S2: *Gplbα$^{Δsig/Δsig}$* platelets exhibit disrupted GPIbα-mediated signaling. *Gplbα$^{+/+}$* (A,B, blue bars) and *Gplbα$^{Δsig/Δsig}$* (C,D, red bars) platelets (n=3 for each genotype with individual data points representing the average of 3 fields of view) were spread on murine VWF and stained with Phalloidin-Alexa 488, in the presence of Botrocetin supplemented or not with GR144053. (A,C) Percentage of platelets from *Gplbα$^{+/+}$* and *Gplbα$^{Δsig/Δsig}$* mice with no filopodia, 1-3 filopodia or >3 filopodia formed on murine VWF upon stimulation with Botrocetin (511 *Gplbα$^{+/+}$* platelets and 547 *Gplbα$^{Δsig/Δsig}$* platelets analysed), or Botrocetin and GR144053 (359 *Gplbα$^{+/+}$* platelets and 480 *Gplbα$^{Δsig/Δsig}$* platelets analysed). (B,D) Percentage of platelets with or without filopodia formed on murine VWF upon stimulation with Botrocetin or Botrocetin and GR144053. All data is shown as mean ± SEM and was analyzed using two-way ANOVA followed by Sidak's multiple comparison test; *p<0.05, ***p<0.001. Also see Figure 2G-J.

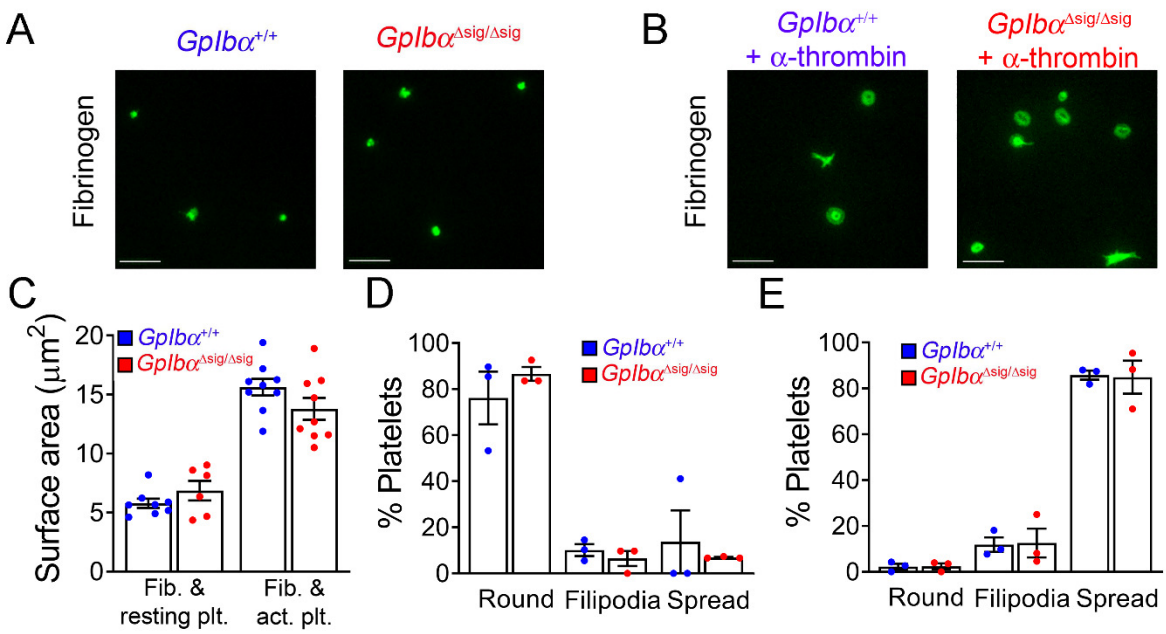

Figure S3: *Gplbα*$^{\Delta sig/\Delta sig}$ platelets spread normally on fibrinogen under basal and stimulated conditions. Representative micrographs (n=3 for each genotype; 3 fields of view analyzed per condition; scale bar 10 μm) of *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ platelets in the absence (A; 131 *Gplbα*$^{+/+}$ platelets and 86 *Gplbα*$^{\Delta sig/\Delta sig}$ platelets analysed) or presence of 0.2U/ml α-thrombin (B; 264 *Gplbα*$^{+/+}$ platelets and 497 *Gplbα*$^{\Delta sig/\Delta sig}$ platelets analysed) and spread on fibrinogen. Platelet spreading was visualized by Phalloidin-Alexa 488 staining. Bar graphs quantifying the surface area (C) and percentages of platelets that remained round, formed filopodia or spread on fibrinogen under basal conditions (D) or activated with α-thrombin (E). The data represent the mean ± SEM and was analyzed using two-way ANOVA followed by Sidak's multiple comparison test; p>0.05. Fib.:fibrinogen; plt.: platelets; act.: α-thrombin-activated. Also see Figure 4A-G.

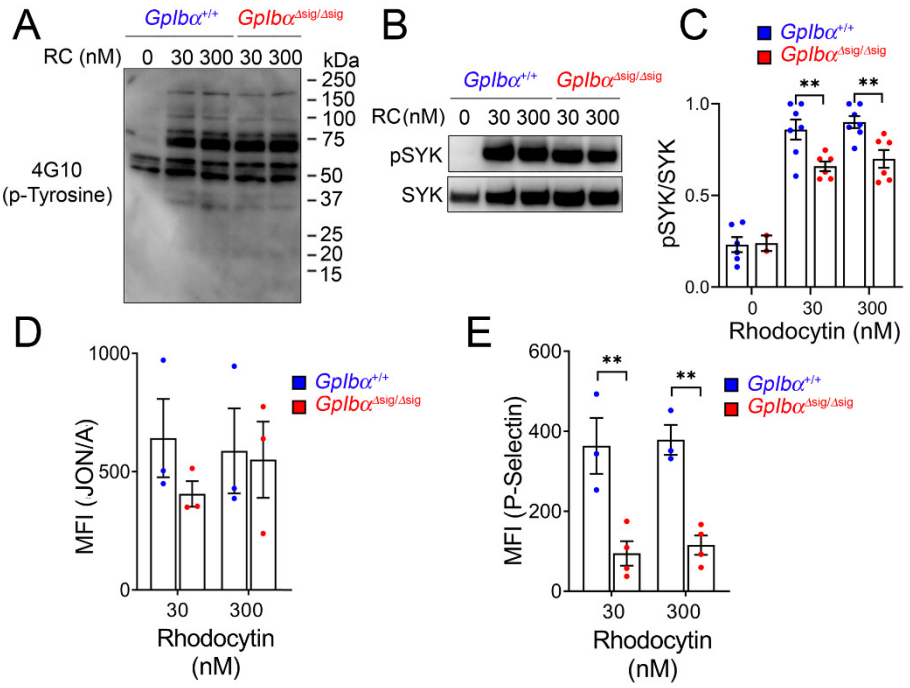

Figure S4: Truncation of the GPIbα intracellular tail ~~does not greatly~~ influences CLEC-2 mediated signaling. (A) Western blot analyzing tyrosine kinase phosphorylation in platelets from *GpIbα*$^{+/+}$ and *GpIbα*$^{\Delta sig/\Delta sig}$ mice, following 5 min stimulation with rhodocytin (RC; 30 and 300nM), (representative of n=3). (B) Western blot and (C) bar graph analyzing the levels of phosphorylated and non-phosphorylated SYK in platelets from *GpIbα*$^{+/+}$ and *GpIbα*$^{\Delta sig/\Delta sig}$ mice, after 5 mins stimulation with RC (representative of n=3). (D-E) Flow cytometric analysis of surface expression of activated $\alpha_{IIb}\beta_3$ (D) and P-selectin (E) in *GpIbα*$^{+/+}$ and *GpIbα*$^{\Delta sig/\Delta sig}$ platelets (n≥3) after stimulation with rhodocytin (RC, 30-300nM). Data is shown as mean ± SEM and analyzed using two-way ANOVA followed by Sidak's multiple comparison test; **p<0.001. Also see Figure 4H-L.

Video 1 (separate file). Laser-induced thrombus formation in a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse: Representative videos of fluorescently-labeled platelets (green) and fibrin(ogen) (red) accumulating at the site of laser-induced injury in a cremaster muscle arteriole of a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse. Thrombus formation was studied using a combination of brightfield and fluorescence microscopy. Results are presented in Figure 3. A timer is shown in the top left corner (hh:mm:ss:000) and a 10 µm scale bar in the bottom left corner.

Video 2 (separate file). Platelet capture on murine VWF-coated microchannels. Representative videos of hirudin-anticoagulated blood from a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse perfused over mouse VWF. Thrombus formation was visualized over 3 minutes of perfusion at 1000s-^{1}. Results are presented in Figure 2D-F. A timer is shown in the top left corner (hh:mm:ss:000) and a 10 µm scale bar in the bottom left corner.

Video 3 (separate file). Formation of platelet aggregates on collagen-coated microchannels at 3000s^{-1}. Representative video of hirudin-anticoagulated blood from a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse over fibrillar collagen type I. Thrombus formation was visualized over 3 minutes of perfusion at 3000s-^{1}. Results are presented in Figure 5. A timer is shown in the top left corner (hh:mm:ss:000) and a 10 µm scale bar in the bottom left corner.

Video 4 (separate file). Formation of platelet aggregates on collagen-coated microchannels at 1000s^{-1}. Representative video of hirudin-anticoagulated blood from a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse over fibrillar collagen type I. Thrombus formation was visualized over 3 minutes of perfusion at 1000s-^{1}. Results are presented in Figure 6. A timer is shown in the top left corner (hh:mm:ss:000) and a 10 µm scale bar in the bottom left corner.

Video 5 (separate file). Formation of platelet aggregates on collagen-coated microchannels at 200s^{-1}. Representative video of hirudin-anticoagulated blood from a *Gplbα*$^{+/+}$ and *Gplbα*$^{\Delta sig/\Delta sig}$ mouse over fibrillar collagen type I. Thrombus formation was visualized over 3 minutes of perfusion at 200s-^{1}. Results are presented in Figure 7. A timer is shown in the top left corner (hh:mm:ss:000) and a 10 µm scale bar in the bottom left corner.