Single platelet and megakaryocyte morpho-dynamics uncovered by multicolor reporter mouse strains *in vitro* and *in vivo*

Leo Nicolai,[1,2] Rainer Kaiser,[1,2] Raphael Escaig,[1,2] Marie-Louise Hoffknecht,[1,2] Afra Anjum,[1,2] Alexander Leunig,[1,2] Joachim Pircher,[1,2] Andreas Ehrlich,[1] Michael Lorenz,[1] Hellen Ishikawa-Ankerhold,[1] William C. Aird,[3] Steffen Massberg[1,2] and Florian Gaertner[4]

[1]Department of Medicine I, University Hospital, LMU Munich, Munich, Germany; [2]German Center for Cardiovascular Research (DZHK), partner site Munich Heart Alliance, Munich, Germany; [3]Department of Medicine, Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Boston, MA, USA and [4]Institute of Science and Technology (IST) Austria, Klosterneuburg, Austria

Correspondence:

Leo Nicolai
leo.nicolai@med.uni-muenchen.de

Florian Gaertner
florian.gaertner@ist.ac.at

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Suppl. Materials and Methods.

Mouse anesthesia. If not stated otherwise, anesthesia was performed by isoflurane induction, followed by intraperitoneal injection of Medetomidine (0.5 mg/kg body weight) Midazolam (5 mg/kg body weight) and Fentanyl (0.05 mg/kg body weight). Toe pinching reflexes and breathing pattern were used to determine adequate depth of anesthesia. Core body temperature was maintained by heating pads, and narcosis was maintained by repetitive injections of 50% of the induction dose, if necessary.

Antibody and reagents. *Antibodies*. For IVM, flow cytometry and histology/immunofluorescence staining the following antibodies were utilized: anti-mouse GpIb (X488/X649, derivatized, polyclonal, emfret, Cat No X649), anti-mouse CD41-AF647 (MWReg30, Biolegend, Cat No 133933), P-selectin-AF647 (RMP-1, Biolegend, Cat No 148303), MHC-II-AF647 (M5/114.15.2, Biolegend, 107617). Thrombin (Cat No T6884), Iron (III) chloride (Cat No 12322), Recombinant human albumin (HSA) (Cat No 1012595), Fibrinogen from human plasma, unconjugated and conjugated to AlexaFluor 647 (Cat F3879 and F35200), Adenosindiphosphate (ADP) (Cat No 0195), and Paraformaldehyde (PFA), Glutaraldehyde (GDA) were purchased from Sigma Aldrich. Horm Collagen was from Takeda, Prostaglandin I2 sodium salt from Abcam (Cat No ab120912), U46619 from Tocris (Cat. No. 1932).

$FeCl_3$-induced thrombus formation in the mouse carotid artery. Arterial thrombosis was performed as previously described [31]. Briefly, mice were anesthetized, and the left carotid artery was exposed. A DyLight 649 conjugated Gp1b-Antibody (X649, emfret, 50 µl) to visualize platelets was injected via the tail vein. Next, a filter paper (mm^3) was saturated with Ferric Chloride solution (Sigma, 10%) and was placed abounding the vessel laterally. The paper was removed after 3 minutes and the ensuing thrombus formation was imaged on a epifluorescence microscope (AxioScope; Carl Zeiss) for the duration of 40 minutes. The platelet signal was used to quantify thrombus area in relation to vessel area over time. For histology, thrombi were retrieved after vessel occlusion and snap-frozen. Cyrosections (10µm thickness) were imaged on a LSM 880 confocal microscope with Airyscan module from Carl Zeiss (40x/1.3 oil immers. obj.).

Tail bleeding assay. Tail bleeding was performed as described previously. Mice were anesthetized, 5 mm of the tail was cut with a precision scissor (MST) and the tail was immediately placed in 37 °C warm sodium chloride (0,9%) solution. Bleeding and Re-Bleeding times were recorded for 20 minutes.

Blood counts. If not stated otherwise, blood counts were obtained in EDTA anticoagulated blood with an ABX Micros ES60 (Horiba Diagnostics) cell counter.

Platelet isolation. Mouse blood was drawn intracardially or retroorbitally from anesthetized mice into a syringe containing 1/7 volume of Acid-Citrate-Dextrose (39 mM citric acid, 75 mM sodium citrate, 135 mM dextrose; ACD), diluted 1:1 with modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 12 mM NaHCO3, 5.5 mM glucose, 10mM HEPES, pH = 6.5) and centrifuged with 70 g for 35 min at RT with the break switched off. The supernatant consists of platelet rich plasma (PRP), either directly used for experiments or used for preparation of washed platelets (WP) by a 1:3 dilution in modified Tyrode's buffer (pH = 6.5) with PGI2 (0.1mg/ml) and a second centrifugation step (1200g, 10min, RT) and re-suspension of the cell pellet in Tyrode's buffer (pH = 7.2).

3D clot retraction. 3D clot retraction was assessed as previously described, with minor variations [33]. AF647-fibrinogen conjugate (15 µg/ml), and thrombin (1U/ml) were added to Platelet rich plasma (PRP), and clot retraction was imaged after 20 min on a LSM 880 confocal microscope in AiryScan mode (20x/0.8 obj., 2x zoom for overviews, and 5x zoom for detailed views, laser power: 0.96%).

Activation flow cytometry. Platelet activation was analyzed as previously described, with minor modifications [34]. Washed platelets (2.5*10^5/µl) were diluted 1:24 in modified Tyrode's buffer, and activated by addition of Calcium (1mM), and the stated compounds. 5 minutes after activation, CD42d-

APC, CD62P-PE and JON/A-FITC antibodies (1:200) were added. After 20 minutes, platelets were fixated by adding 1:4 cold PFA 4%. Measurement was carried out on a BD Fortessa flow cytometer and analysis was completed with FlowJo (V10).

Adhesion and spreading. Platelets were activated with U46619, ADP and Calcium, and pipetted onto fibrinogen (40 µg/ml) or collagen (10 µg/ml) coated surfaces. After 15 minutes, adherent cells were imaged with an Olympus IX83 Epifluorescence microscope.

Clot retraction. Clot retraction was assessed as previously described [35]. Briefly, platelet rich plasma pre-incubated for 20 minutes was recalcified, and 1U/ml thrombin added. After 30 min incubation at 37°C, clots were imaged and the remaining fluid quantified to assess degree of retraction.

Suppl. Fig. legends

Suppl. Fig 1.

a, overview image of PF4-Cre positive cells in the bone marrow (humerus). Scale bar= 100µm **b**, FarRed nuclear stain reveals multilobulated nuclei in megakaryocytes. Scale bar = 10 µm **c-d,** PF4 expression beyond the megakaryocyte lineage. Representative mesenteric (c) and cremaster (d) tissue stained from PF4-cre Rs-26 Confetti mice with Rs26-Confetti positive cells with macrophage morphology (arrows). scale bar = 10 µm.

Suppl. Fig. 2

a, platelet counts, hemoglobin content and mean platelet volume of Cre- and Cre+ littermates. n=4 mice, ttest. **b**, representative flow cytometry plots of platelets isolated from Cre- and Cre+ animals and **c**, and micrographs of platelets from Cre- and Cre+ animals **d**, baseline expression of surface markers and fibrinogen binding of platelets isolated from Cre- and Cre+ animals, n=4 mice, ttest. **e**-**g**, fold-change in expression of surface markers or fibrinogen binding upon stimulation with (**e**) ADP, (**f**) collagen, or (**g**) thrombin compared to baseline, n=4 mice, ttest. **h**, color based analysis reveals no difference in activation measured by fibrinogen binding upon thrombin stimulation, n=3 mice, ANOVA.

Suppl. Fig. 3

a, time to initial hemostasis and bleeding time in tail bleeding assay of Cre- and Cre+ littermates. n=4 mice, ttest. **b**, in vitro clot retraction assay of platelets isolated from Cre- and Cre+ animals, n=4 mice, ttest. **c**, **d**, FeCl-induced thrombosis model of the carotid artery, (**c**), thrombus size measured by accumulation of injected Gp1b-AF649 antibody labelling circulating platelets. (d), representative micrographs showing YFP, RFP and injected Gp1b-AF649 signals of growing thrombi in Cre+ and Cre- mice, n=3-4, ttests. **e**-**h**, spreading characteristics of Cre- and Cre+ platelets, (**e**), representative micrographs, (**f**), spread cells per field of view on fibrinogen, n=4 mice, ttest (**g**), size of adherent cells on fibrinogen, n=48 cells from 4 mice, ttest, and (**h**), spread cells per field of view on collagen, n=4 mice, ttest.

Suppl. Fig. 4

a-c, Rs26-Confetti platelets analyzed by color, (**a**) platelet size and (**b**) morphology depending on color (RFP, YFP and CFP) and (**c**) migration, n=31-33 mice. **d**, shape descriptors and representative micrograph of PF4cre; $R26R^{Confetti}$;$Myh9^{flox}$ platelets (n= 40) compared to PF4cre; $R26R^{Confetti}$ platelets (n=47). ttest. **e**, tracking of n=21 individual platelets from Mhy9 deficient mice in single thrombus allows for computation of single-cell velocity and covered distance. Data from PF4-cre Rs26-Confetti mice (n=118) for comparison derived from **Fig 7e**. ttest.. **f**, representative overview image of CD45+ leukocytes in a mesenterial ferric chloride thrombosis model. Scale bar = 20 µm.

Suppl. Video 1- Arterial thrombus formation in PF4cre-Rs26Confetti mice

Thrombus formation using a Ferric Chloride Injury model in Pf4-Cre, $R26R^{Confetti}$ mice. RFP and GFP represents intrinsic fluorescence, Channel 3 (FarRed) is used to depict overall platelet signal by injection of DyLight649 conjugated anti-Gp1b antibody.

Suppl. Video 2- *In vitro* thrombus under static conditions

Visualization of a thrombus formed under static conditions using thrombin and platelet rich plasma (PRP) isolated from Pf4-Cre, $R26R^{Confetti}$ mice.

Suppl. Video 3- *In vivo* thrombus formation in the mesentery

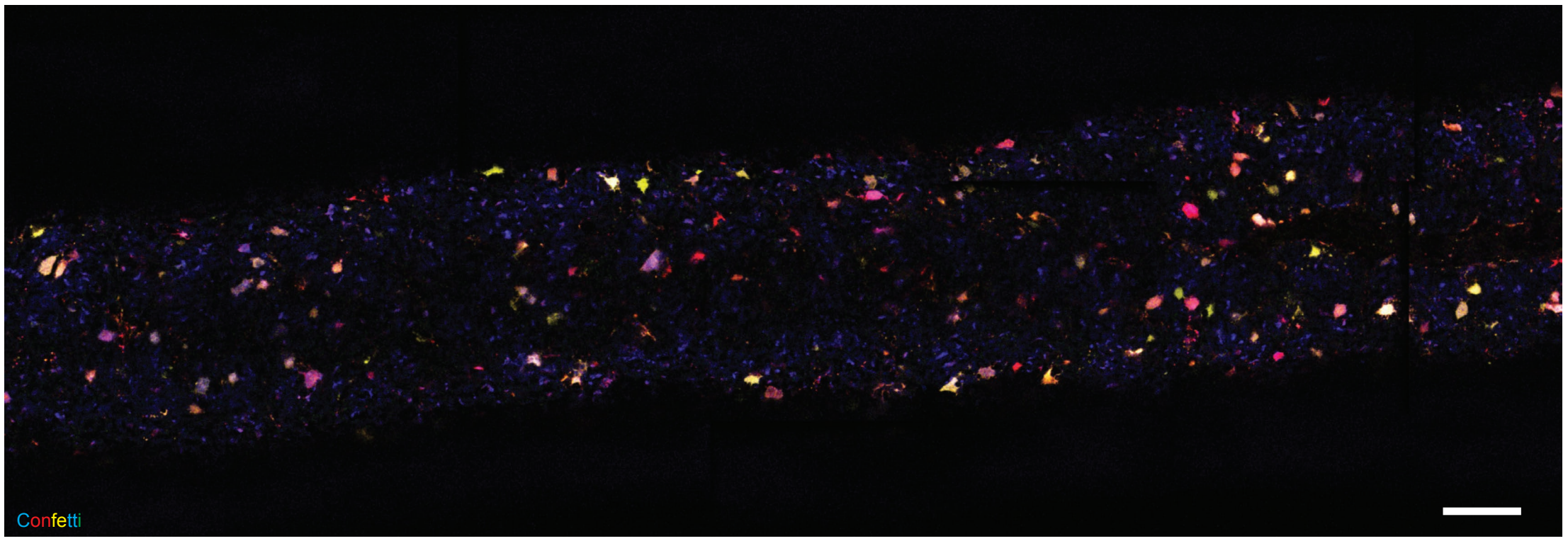
In vivo Ferric chloride thrombosis model in the mesentery allowing for the visualization of individual platelets in a growing thrombus.

Suppl. Video 4- *In vivo* thrombus formation in PF4cre-Mhy9-Confetti mice in the mesentery

In vivo thrombosis model in the mesentery allowing for the visualization of individual platelets in platelet targeted myosin II (MHY9 -/-) deficient mice.

Suppl. Fig. 1

a



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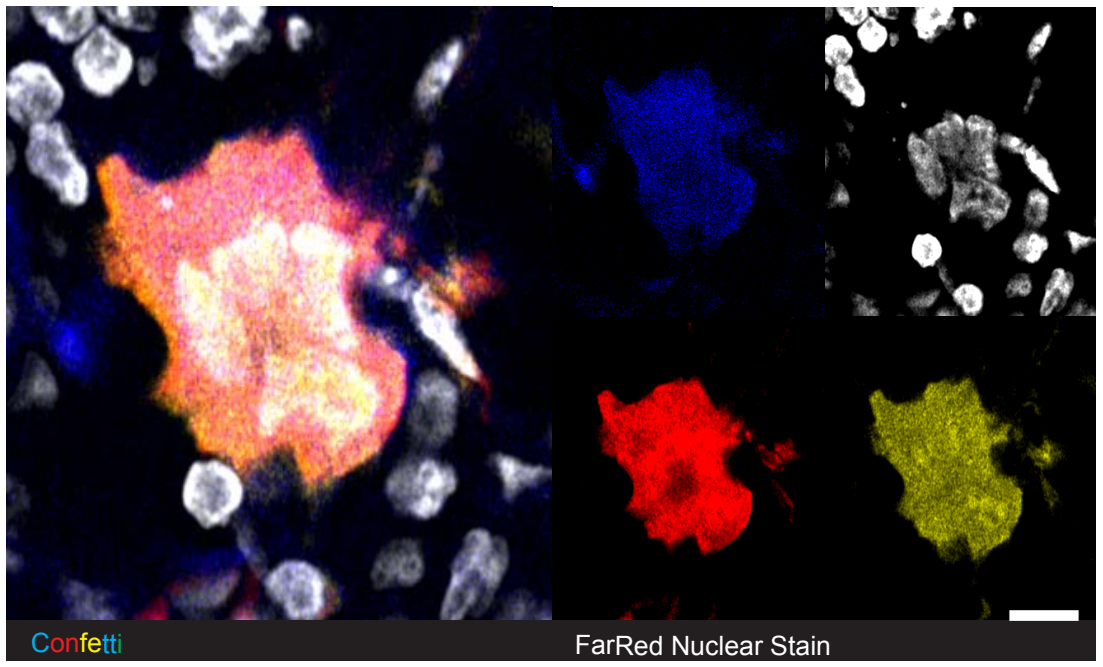
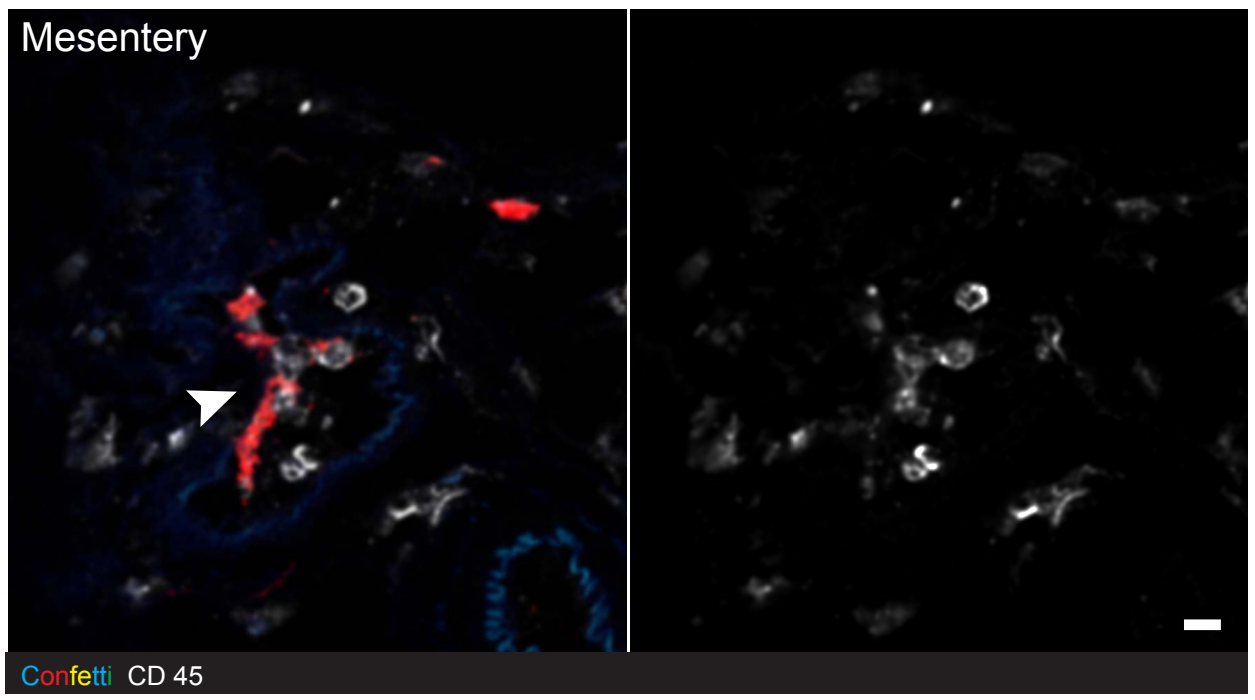
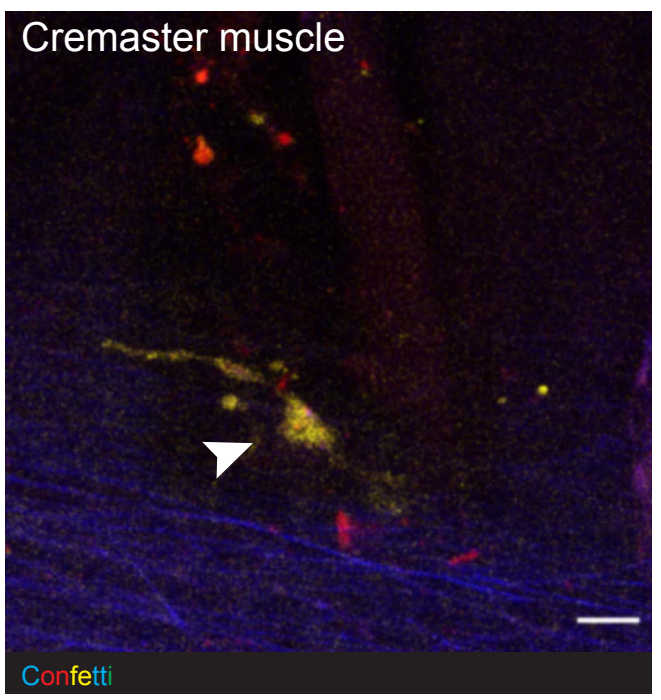


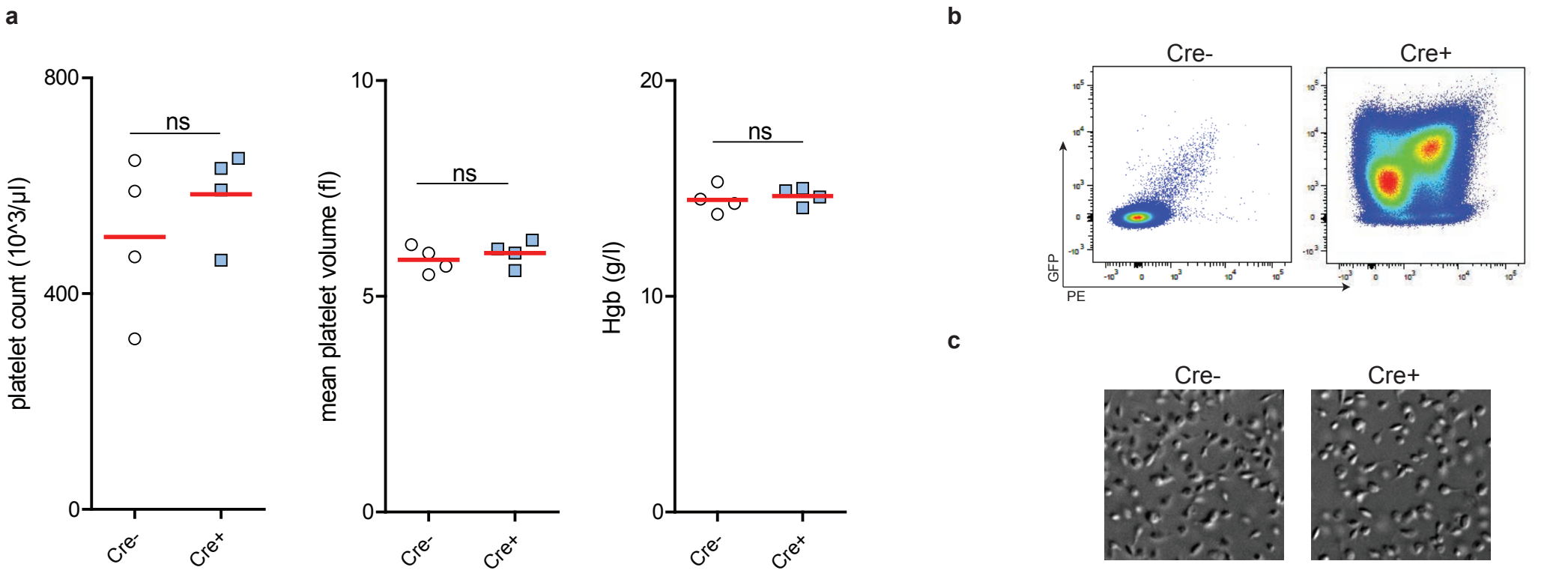
c



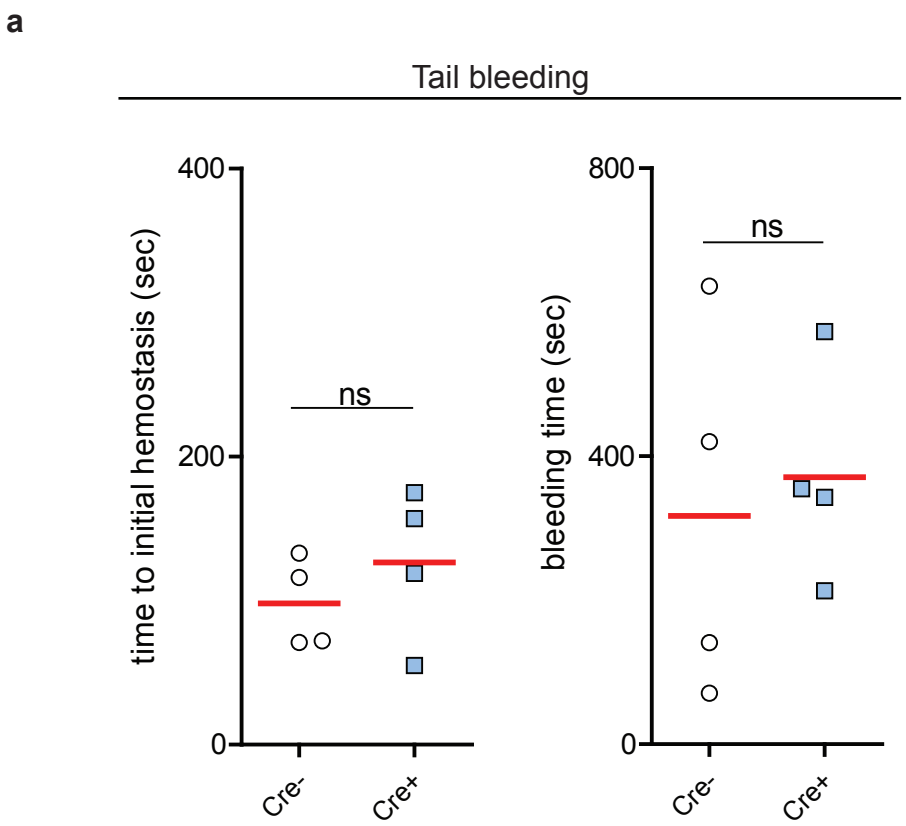
d

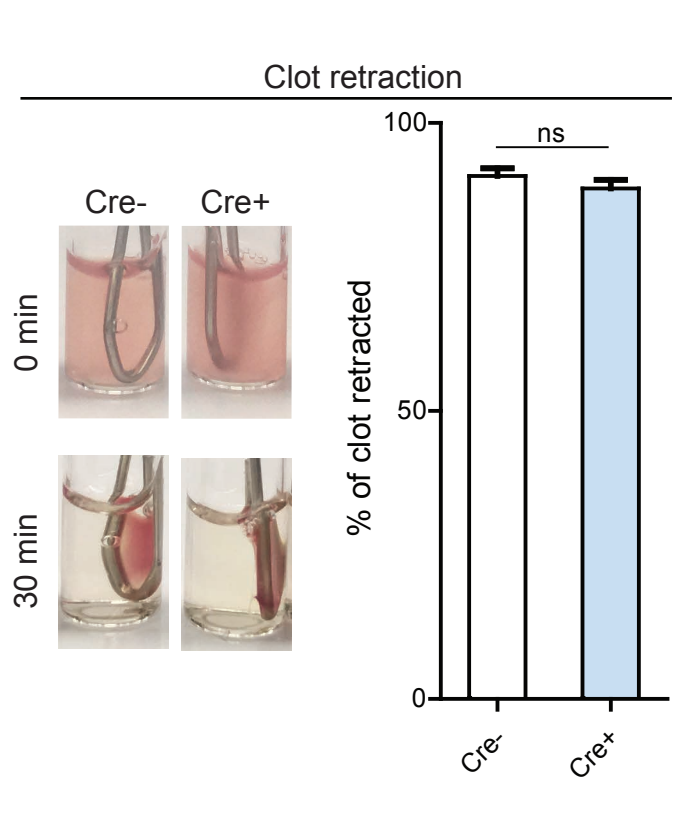


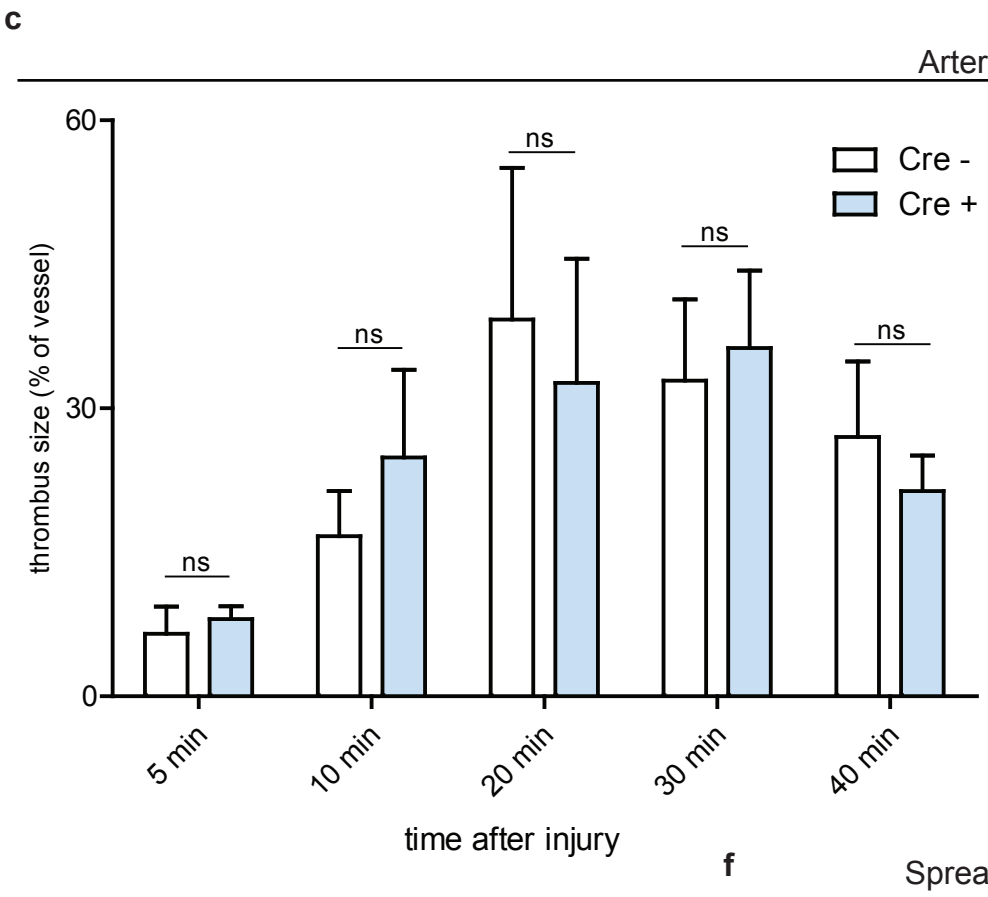
Suppl. Fig. 2

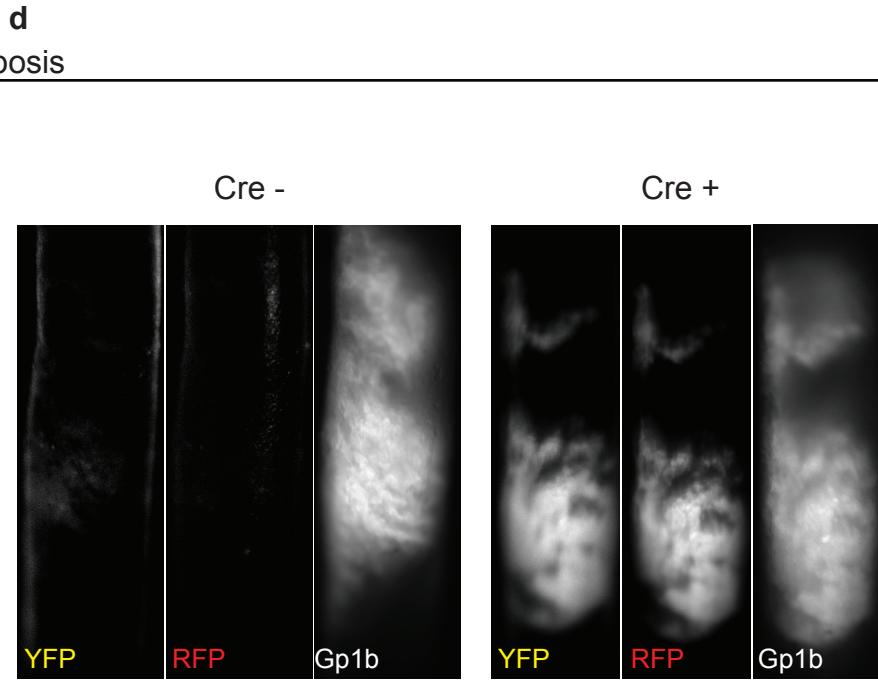

a
platelet count (10^3/µl)
800
400
0
ns
Cre-
Cre+
mean platelet volume (fl)
10
5
0
ns
Cre-
Cre+
Hgb (g/l)
20
10
0
ns
Cre-
Cre+
b
Cre-
Cre+
GFP
PE
c
Cre-
Cre+
d
Baseline
Platelet size (FSC - MFI)
1000
500
0
ns
Cre-
Cre+
Surface GPIIbIIIa (MFI)
400
200
0
ns
Cre-
Cre+
Surface GP1b (MFI)
1500
750
0
ns
Cre-
Cre+
P-Selectin expression (MFI)
400
200
0
ns
Cre-
Cre+
Fbg binding (MFI)
400
200
0
ns
Cre-
Cre+
e
ADP 20 µM
Fbg binding (Fold increase)
3
2
1
0
ns
Cre-
Cre+
f
Collagen 0.1µg/ml
Fbg binding (fold increase)
4
3
2
1
0
ns
Cre-
Cre+
P-Selectin expression (fold increase)
4
3
2
1
0
ns
Cre-
Cre+
g
Thrombin 0.1 U
P-Selectin expression (fold increase)
25
20
15
10
5
0
ns
Cre-
Cre+
h
YFP
RFP
Thrombin 0.1U
MFI Fibrinogen binding
80000
60000
40000
20000
0
ns
RFP+
RFP+YFP+
YFP+

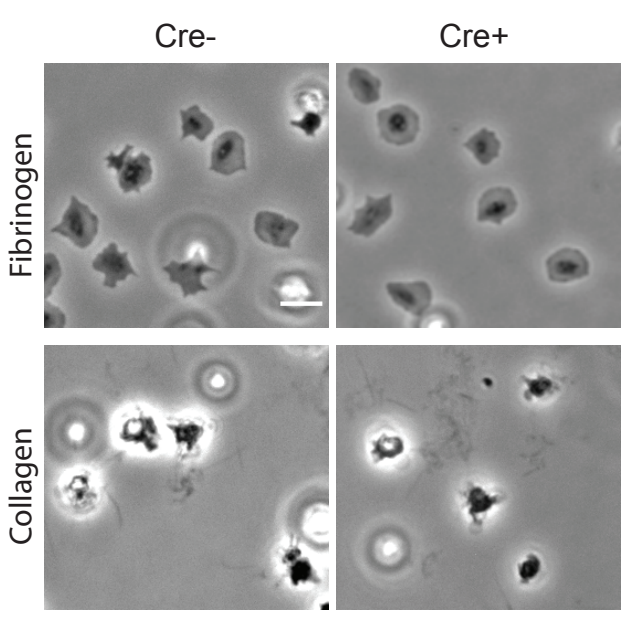
Suppl. Fig 3

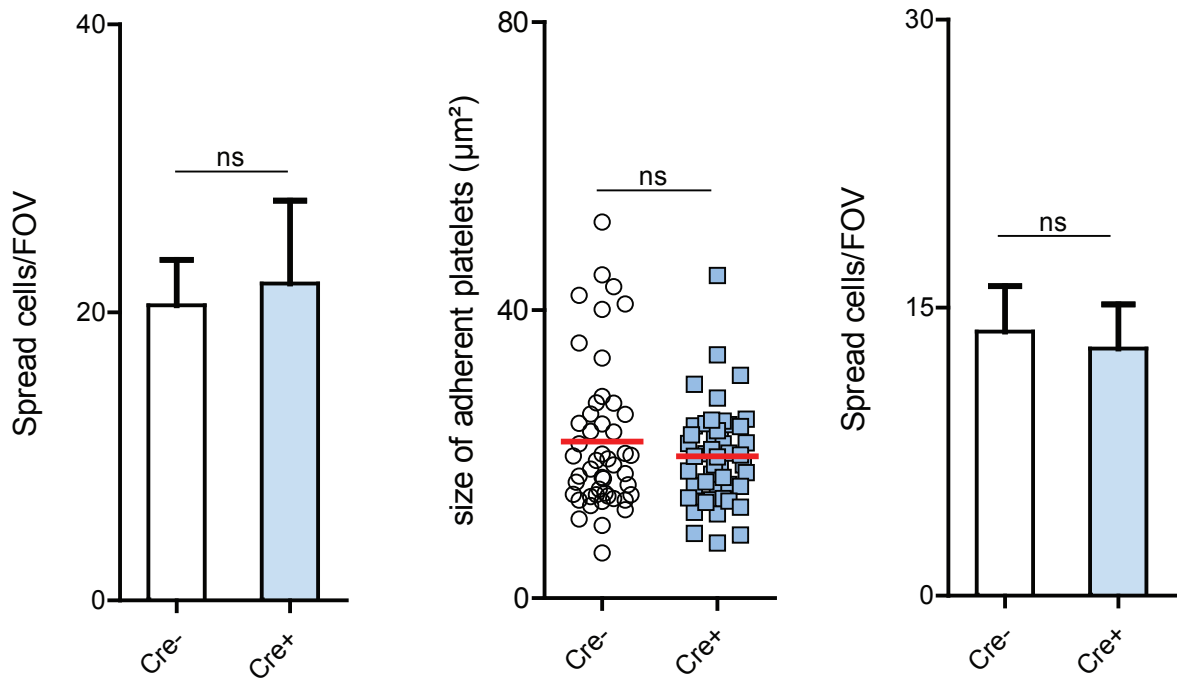

a
Tail bleeding
time to initial hemostasis (sec)
400
200
0
ns
Cre-
Cre+
bleeding time (sec)
800
400
0
ns
Cre-
Cre+


b
Clot retraction
Cre-
Cre+
0 min
30 min
% of clot retracted
100
50
0
ns
Cre-
Cre+

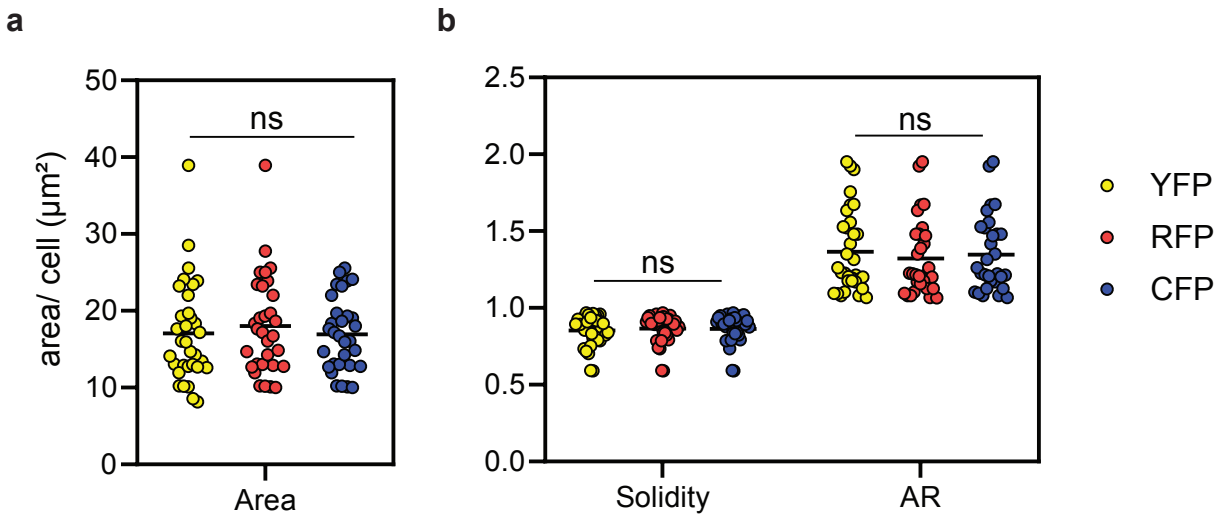

c
thrombus size (% of vessel)
60
30
0
ns
Cre -
Cre +
5 min
10 min
20 min
30 min
40 min
time after injury

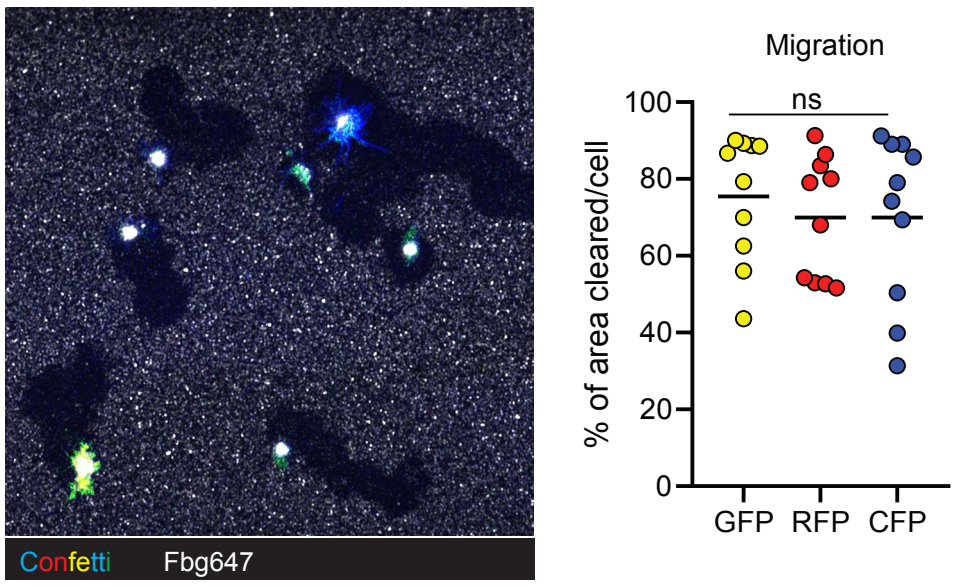

d
Arterial thrombosis
Cre -
Cre +
YFP
RFP
Gp1b
YFP
RFP
Gp1b

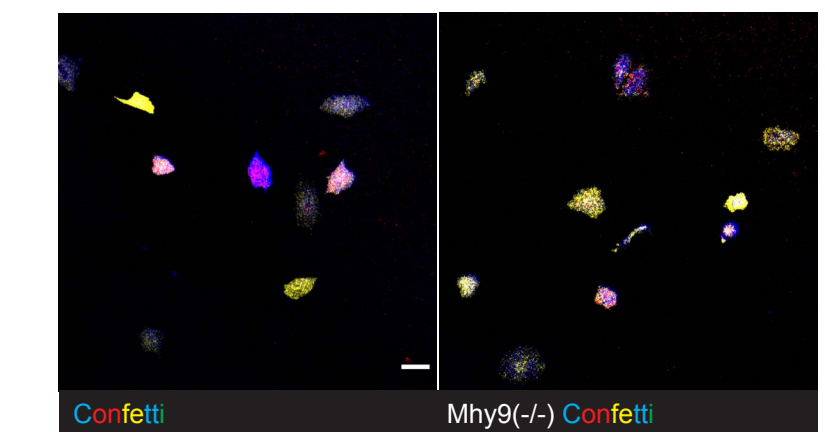

e
Cre-
Cre+
Fibrinogen
Collagen

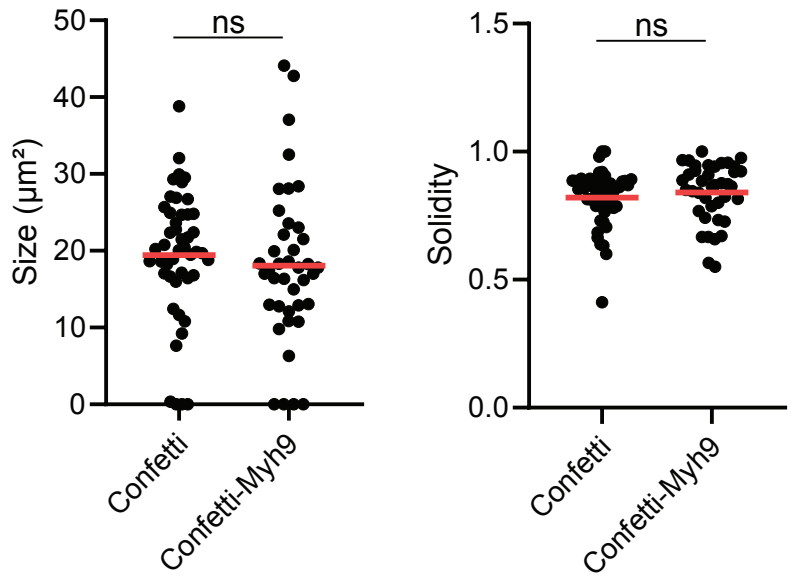

f
Spreading
Spread cells/FOV
40
20
0
ns
Cre-
Cre+
g
size of adherent platelets (µm²)
80
40
0
ns
Cre-
Cre+
h
Spread cells/FOV
30
15
0
ns
Cre-
Cre+

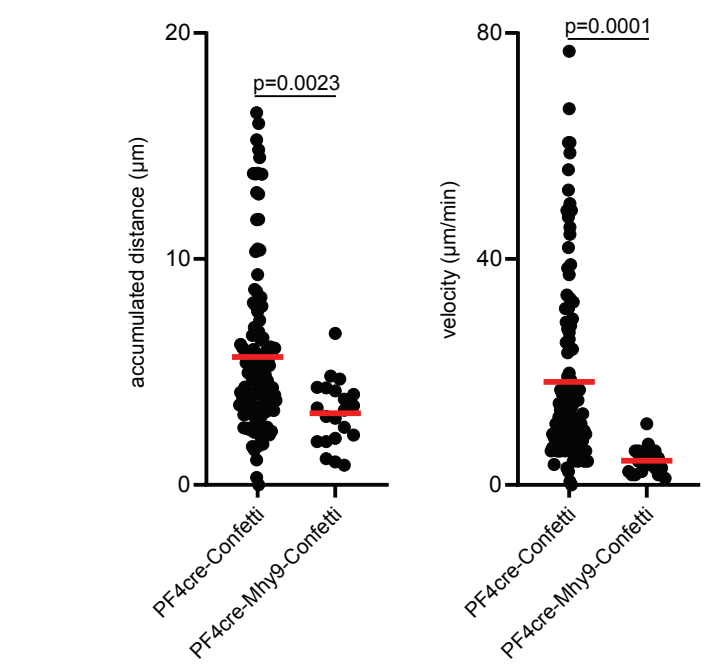
Suppl. Fig. 4

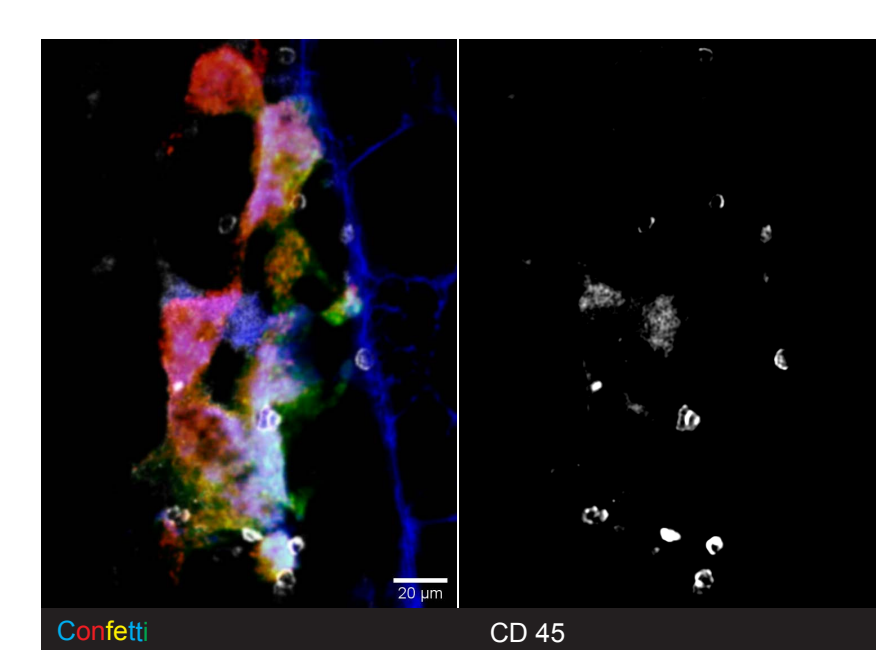

a
area/ cell (µm²)
ns
Area
b
ns
ns
Solidity
AR
YFP
RFP
CFP


c
Confetti
Fbg647
Migration
% of area cleared/cell
ns
GFP
RFP
CFP


d
Confetti
Mhy9(-/-) Confetti


Size (µm²)
ns
Confetti
Confetti-Myh9
Solidity
ns
Confetti
Confetti-Myh9


e
accumulated distance (µm)
p=0.0023
PF4cre-Confetti
PF4cre-Mhy9-Confetti
velocity (µm/min)
p=0.0001
PF4cre-Confetti
PF4cre-Mhy9-Confetti


f
20 µm
Confetti
CD 45