ARTICLE - Platelet Biology & its Disorders

Inter-platelet communication driving thrombus formation is regulated by extracellular calpain-1 cleavage of connexin 62

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Supplementary Material

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Methods:

Immunoblotting

Platelet lysates were prepared using a Triton X-100 lysis buffer supplemented by a protease and phosphatase inhibitor cocktail. Proteins were separated by SDS-PAGE, immunoblotted and stained using primary and secondary antibodies (Sup. Table 1). Blots were visualized using a Typhoon FLA 9500 biomolecular imager (Cytiva, Buckinghamshire, UK) and band intensities were normalized to appropriate loading controls.

Platelet aggregation

Aggregation of washed human platelets ($4x10^8$ mL^{-1}) was measured by optical aggregometry using a Chrono-Log Model 700 Optical-lumi aggregometer (Helena Biosciences, Gateshead, UK). Washed platelets were stimulated with gradient concentrations of collagen or thrombin in the presence or absence of the indicated inhibitors and activators.

Intracellular calcium flux

PRP was stained with Fura-2-AM (2 μM, 1 hour, 30°C) and excess dye was removed by washing and re-suspending cells ($4x10^8$ platelets mL^{-1}) in Ca^{2+}-free Tyrode's-HEPEs buffer. Samples were incubated with the indicated inhibitors or activators prior to stimulation by CRP-XL (10 μg ml^{-1}) or TRAP6 (10 μM). Fluorescence was recorded for three minutes with dual excitation at 340 nm and 380 nm and emission 510 nm, using a NOVOstar plate reader (BMG Labtech, Aylesbury, UK). Intracellular Ca^{2+} mobilisation was calculated using the 340/380 ratio, as described previously.[1]

Temporal assessment of calcein diffusion during thrombus formation

PRP was prepared from an aliquot of whole blood and platelets were stained by calcein-AM dye (0.5 μM, 30 minutes, 30°C) and Alexafluor647-conjugated αCD61 antibodies (1:100 v/v,

10 minutes, 30°C). Stained platelets ($CD61^+calcein^+$) were washed and resuspended in Ca^{2+}-free Tyrode's-HEPEs buffer. Whole blood and stained platelets were independently treated with either ^{Scr}Pept-NT or ^{62}Pept-NT (100 µg mL^{-1}, 5 minutes) and mixed immediately prior to thrombus formation to give 10% (v/v) stained platelets per experiment. Thrombus formation was performed under arteriolar shear (1,000 s^{-1}, 5 minutes), as described previously.[2] Calcein diffusion was calculated using FIJI (Image J, V1.54) by creating a binary mask of $CD61^+$ cells, which was subtracted from the total calcein signal in each frame (0.5 frames per second). CD61 fluorescence was subtracted from the calcein signal in each frame using a binary mask. Resultant calcein signal was considered to arise from calcein diffusion away from $CD61^+Calcein^+$ cells and was quantified as fluorescence increase from baseline (F/F_0, Figure S4).

Thrombus formation assays

Thrombus formation was performed under arteriolar shear (1,000 s^{-1}, 5 minutes), as described previously.[3] Briefly, whole blood was incubated with either ^{62}Pept-NT or ^{scr}Pept-NT (5 minutes, 100µg mL^{-1} each), prior to perfusion through collagen coated Vena 8 Fluoro+ biochips (Cellix, Dublin, Ireland). After 6 minutes thrombi were perfused with paraformaldehyde fixative containing the lipophilic dye 3,3'-Dihexyloxacarbocyanine Iodide (DiOC6; AbCam, Cambridge, UK). Confocal imaging with z stepping at 1.025 µm intervals was performed at predetermined regions for all channels. Image quantification was conducted using FIJI (Image J, V1.54) with Bio-Formats Plugin.

Thromboelastography

Thromboelastography was performed using a TEG5000 (Haemonetics Corp, USA). Calibration, quality control and assay procedures were performed according to the manufacturer's instructions. Briefly, whole blood was incubated with either ^{62}Pept-NT, ^{scr}Pept-NT (5 minutes, 100µg mL-1 each), carbenoxolone (Cbx, 5 minutes, 100µM) before transfer to functional fibrinogen assay kit vials (Haemonetics Corp, USA). Blood was recalcified with $CaCl_2$ and transferred into an assay cup for analysis. Assays were allowed to run for 90 minutes, and assay parameters were recorded.

References

1. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem. 1985;260(6):3440-3450.
2. Bye AP, Hoepel W, Mitchell JL, et al. Aberrant glycosylation of anti-SARS-CoV-2 spike IgG is a prothrombotic stimulus for platelets. Blood. 2021;138(16):1481-1489.
3. Sahli KA, Flora GD, Sasikumar P, et al. Structural, functional, and mechanistic insights uncover the fundamental role of orphan connexin-62 in platelets. Blood. 2021;137(6):830-843.

Table S1: Primary and secondary antibodies

Target	Supplier	Reference
GJA10	Sigma-Aldrich, UK	SAB2105481
Calpain-1	Thermo Fisher, UK	MA535705
β-Actin	AbCam, UK	Ab8226
Phospho-VASP (Ser^{157})	Cell Signalling Technology, UK	#3111S
Talin 1 (TA205)	Sigma-Aldrich, UK	MAB1676
Phospho-PP2A (Tyr^{307})	Thermo Fisher, UK	MA535878
AlexaFluor 647 CD61	Biolegend, UK	#336408
AlexaFlour 647 Donkey anti rabbit	Life Technologies, UK	A31573
AlexaFlour 647 Donkey anti mouse	Life Technologies, UK	A31571

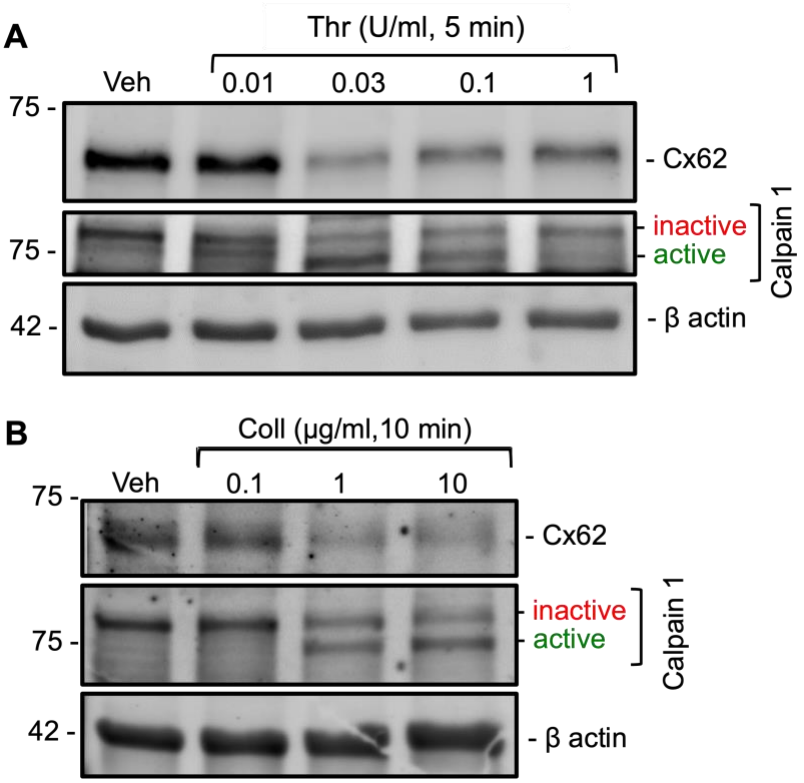


Figure S1: Increasing levels of platelet agonists promote activation of calpain-1 and cleavage of Cx62. (A) Effects of increasing doses of thrombin (0.01-1 U/ml, 5 minutes) on the levels of intact Cx62. **(B)** Effects of increasing doses of collagen (0.1-10 µg/ml, 10 minutes) on the levels of intact Cx62. Calpain 1 levels were used an indicator for calpain activation status. β actin levels were used as a loading control.

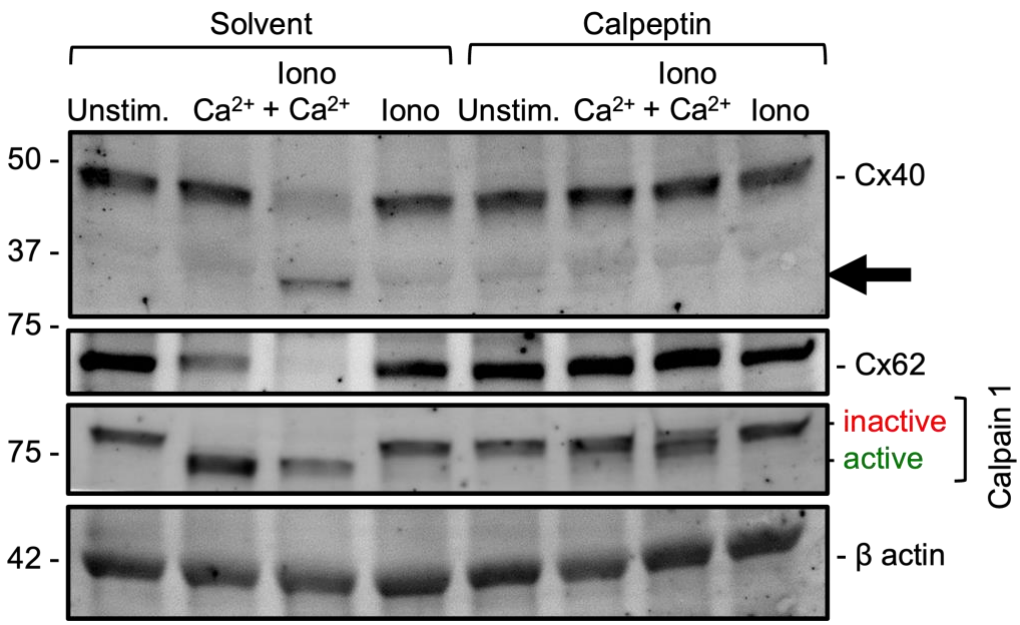


Figure S2: Cx40 is a calpain substrate but requires high levels of Ca^{2+}. Effects of high levels of extracellular calcium (Ca^{2+}; 5mM, 15 minutes) in the presence or absence of ionophore (Iono; 1µM, 5 minutes) on the levels of intact CX40 and CX62 in the presence or absence of calpeptin (50 µM, 30 minutes). Arrow is indicating the cleavage product of intact levels of CX40. Calpain-1 isoforms were used an indicator for calpain activation status. β actin levels were used as a loading control.

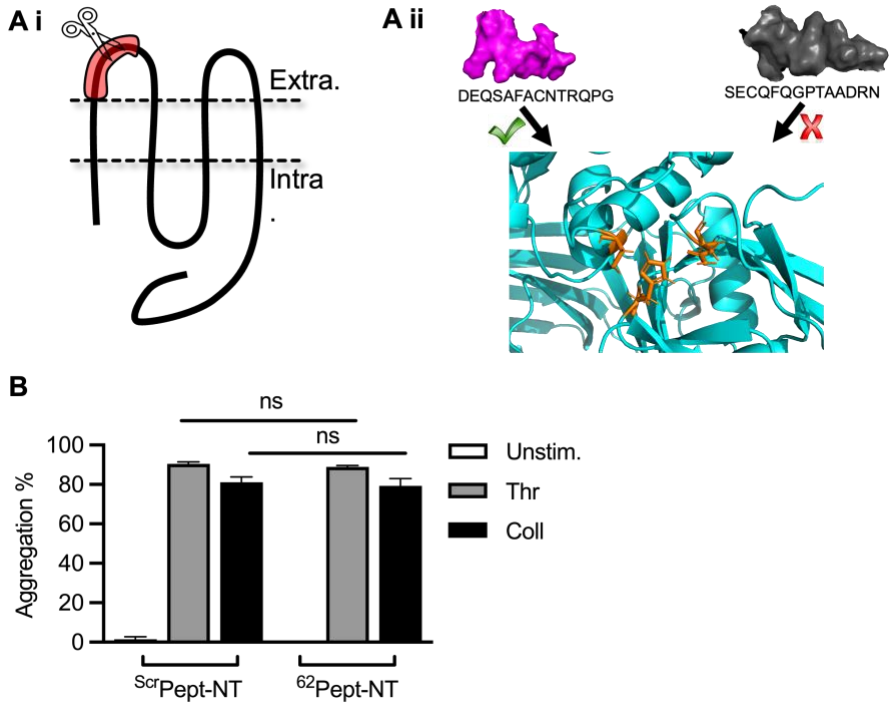


Figure S3: The calpain decoy peptide ^{62}Pept-NT does not alter agonist-evoked platelet aggregation. (A) Topological cartoon showing calpain cleavage site DEQSAFACNT|RQPG. (Aii) Model of the calpain-1 active site (cyan) and the peptides ^{62}Pept-NT (blue) and ^{Scr}Pept-NT (orange). **(B)** Platelet aggregation in response to thrombin (Thr; 0.03 U mL^{-1}, 5 minutes) or collagen (Coll; 1 µg mL^{-1}, 5 minutes) in the presence ^{Scr}Pept-NT or ^{62}Pept-NT (100 µg/ml each, 30 minutes); n=8 per group (One-way Anova+ Bonferroni).

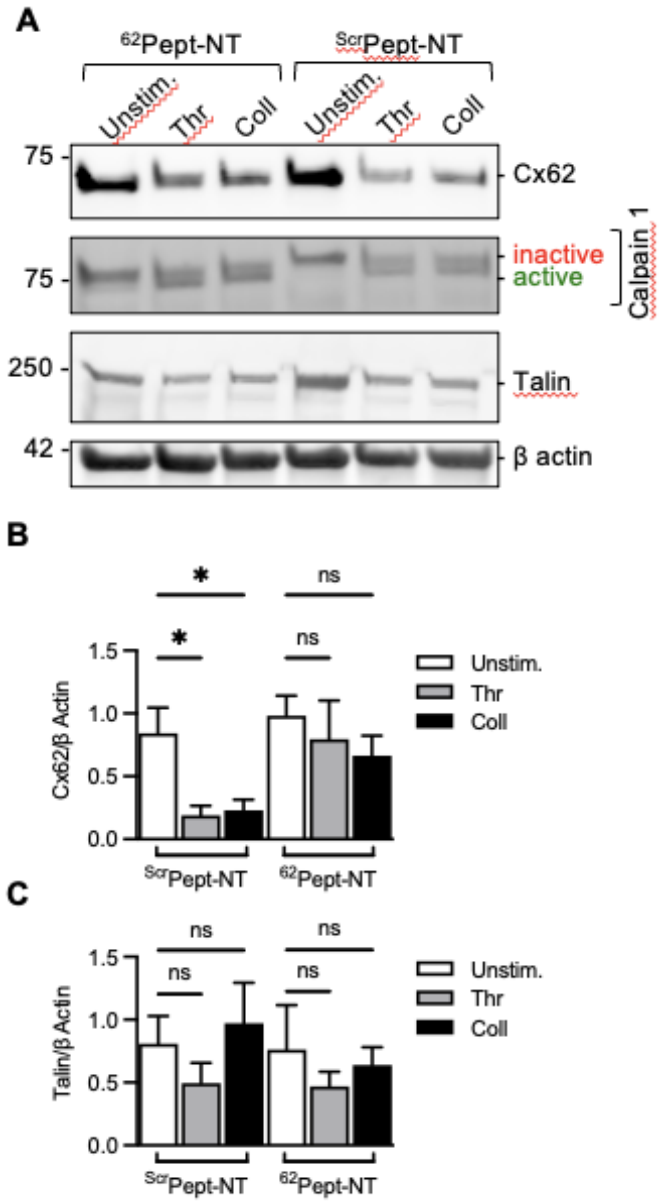


Figure S4: The calpain decoy peptide ^{62}Pept-NT does not affect agonist-evoked talin cleavage. Platelets were stimulated by thrombin (Thr; 0.03 U mL^{-1}, 5 minutes) or collagen (Coll; 1 µg mL^{-1}, 5 minutes) in the presence ^{Scr}Pept-NT or ^{62}Pept-NT (100 µg/ml each, 5 minutes). **(A)** Representative images for immunoblot detection of Cx62, calpain-1, talin and and β actin as loading control. Relative levels of Cx62 **(B)** and talin **(C)** were assessed by densitometry. Data are representative of seven independent experiments. Statistical significance was determined by ANOVA and is denoted as ns = *P>0.05 and * = P<0.05.*

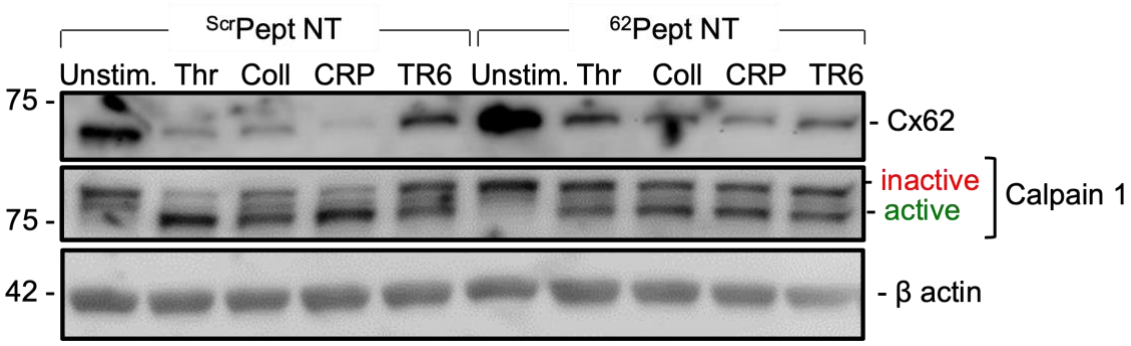


Figure S5: Decoy of active calpain-1 by ^{62}Gap27 protects against cleavage of platelet Cx62. The levels of active calpain and intact Cx62 in response to the platelet agonists thrombin (Thr; 0.03 U/ml; 5 minutes), collagen (Coll; 10 µg/ml; 5 minutes), CRP-XL (CRP; 10 µg/ml, 5 minutes) or TRAP6 (TR6; 10 =µM, 5 minutes) in the presence of the calpain decoy peptide (^{62}Pept-NT; 100 µg/mL, 30 minutes) or scrambled control (^{Scr}Pept-NT; 100 µg/mL, 30 minutes). Calpain-1 isoforms were used an indicator for calpain activation status and β actin levels served as a loading control.

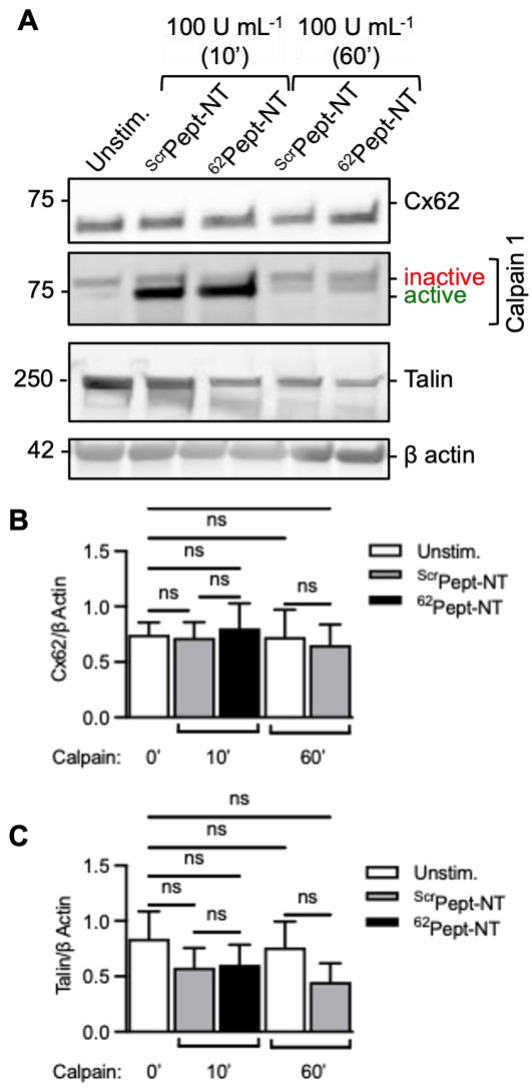


Figure S6: Recombinant calpain does not cleave calpain in resting platelets. (A) Platelets treated with ^{Scr}Pept-NT or ^{62}Pept-NT (100 µg/ml each, 5 minutes) were incubated with 100U/mL recombinant calpain for 10 minutes or 60 minutes. Representative images for immunoblot detection of Cx62, calpain-1, talin and and β actin as loading control. Relative levels of Cx62 **(B)** and talin **(C)** were assessed by densitometry. Data are representative of five independent experiments. Statistical significance was determined by ANOVA and is denoted as ns = *P>0.05.*

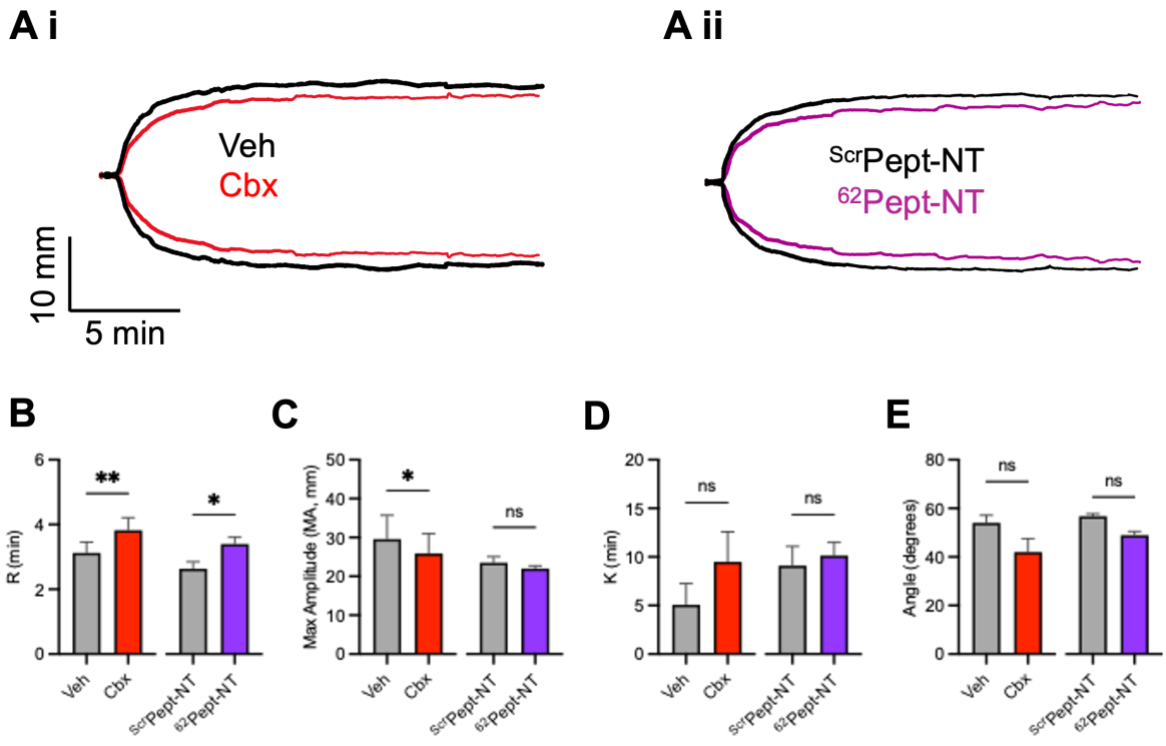


Figure S7: Calpain decoy peptide modestly reduces time to initiation of clotting. The role of connexins in regulating platelet clotting parameters were assessed by thromboelastography (TEG). Platelets were pre-treated with vehicle (Veh; black), a generic gap junction blocker carbenoxolone (Cbx; red; 100 µM), ^{62}Pept-NT (black; 100 µg/mL) or ^{Scr}Pept-NT (magenta; 100 µg/mL). (**A**) representative TEG traces are shown for each condition. Readouts for time to clot initiation (**B**; R, min), maximum amplitude (**C**; MA, mm), time to 20mm amplitude (**D**; K, min) and angle (**E**; degrees). Data are representative of 3-4 independent experiments and statistical significance was determined by paired t test, ** = *P<0.01, * = P<0.05 and ns = P>0.05.*

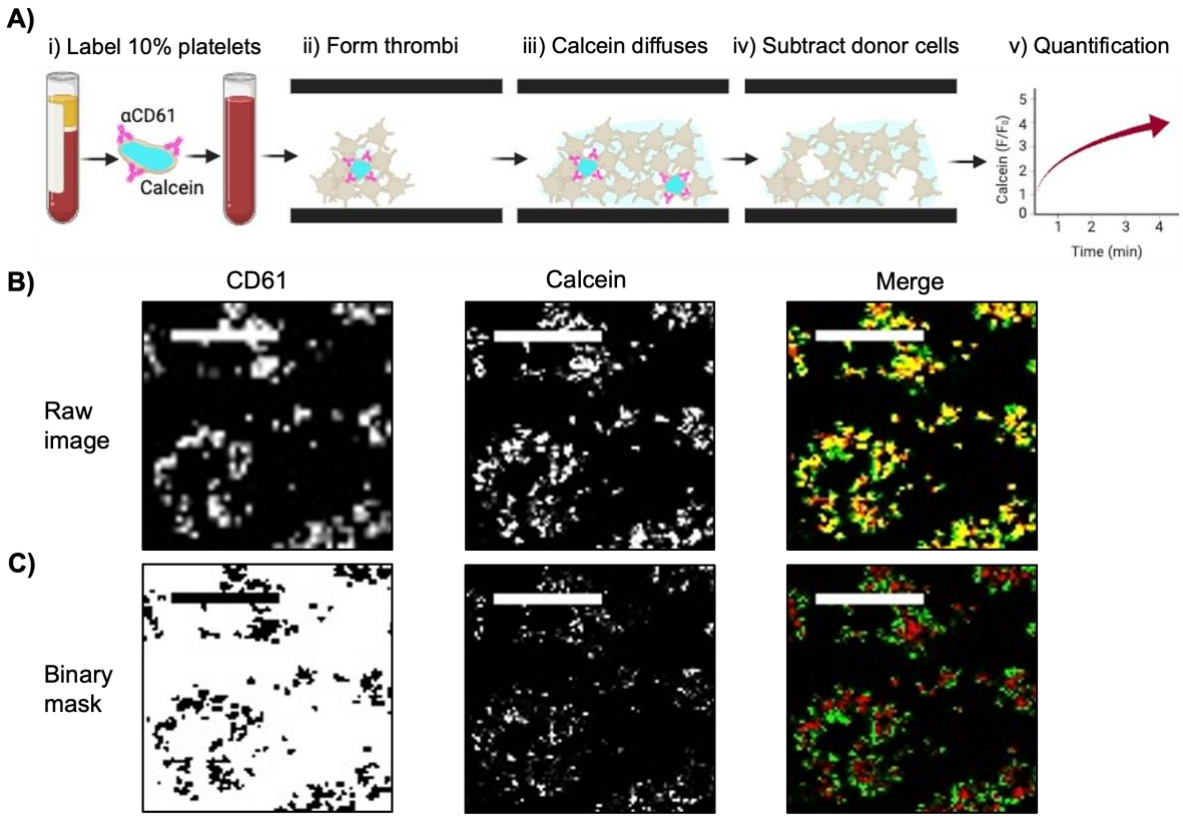
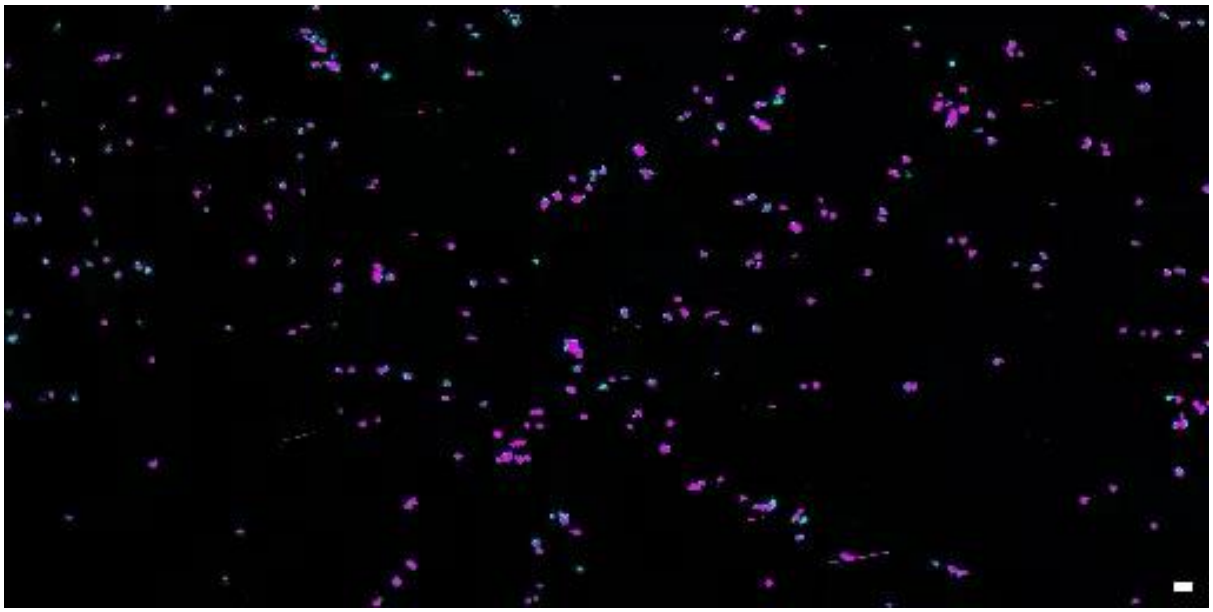


Figure S8: Workflow for real-time assessment of calcein dye diffusion via connexin gap junctions during thrombus formation. A) Cartoon workflow of calcein diffusion assay (www.biorender.com). i) Isolate PRP and stain 10% with cytosolic dye (calcein; cyan) & surface marker (αCD61; magenta). Reconstitute $Calcein^{+}CD61^{+}$ platelets & blood (10% *v/v*). ii) Form thrombi under arteriolar shear ($1000s^{-1}$), iii) monitor calcein diffusion (0.5fps), iv) subtract donor cells and quantify calcein signal (v). B) Raw data showing incorporation of $CD61^{+}Calcein^{+}$ platelets into thrombi. C) Mask generated using $CD61^{+}Calcein^{+}$ signal and subtracted from each frame. Donor $Calcein^{+}CD61^{+}$ platelets are superimposed onto the resultant calcein image for composites and videos. Scale bars = 50μm.

Movie S1: Calcein transfer in the presence of ^{Scr}Pept NT. Representative movie of real-time thrombus formation with 10% (v/v) platelets co-stained by CD61 (magenta) and cytosolic calcein (cyan) dye. Thrombi were formed under arteriolar shear rates (1,000 s^{-1}) for five minutes. As connexins activate and form gap junctions calcein dye is able to diffuse away from the donor cells, generating a cyan 'halo' around the donor cells. Whole blood and donor platelets were pre-treated with 100µg/mL ^{Scr}Pept-NT prior to the start of perfusion. Scale bar is 10µm and movie is representative of four independent experiments.



Movie S2: Calcein transfer in the presence of ^{62}Pept NT. Representative movie of real-time thrombus formation with 10% (v/v) platelets co-stained by CD61 (magenta) and cytosolic calcein (cyan) dye. Thrombi were formed under arteriolar shear rates (1,000 s^{-1}) for five minutes. As connexins activate and form gap junctions calcein dye is able to diffuse away from the donor cells, generating a cyan 'halo' around the donor cells. Whole blood and donor platelets were pre-treated with 100µg/mL ^{62}Pept-NT prior to the start of perfusion. Scale bar is 10µm and movie is representative of four independent experiments.