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

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**Running Head:** Extracellular calpain regulates thrombus signaling

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**Authorship Contributions**

KAT, AE, TLH and SP performed experiments. KAT, AE, and JMG critically analyzed the data. KAT, AE, RA, CEH, LJM and JMG wrote and edited the manuscript. RA and LJM directed the modelling studies. KAT, AE and JMG designed the study.

**Disclosure of Conflicts of Interest**

The authors have no competing interests to declare.

**Data Availability**

Data are available upon request.

**Abstract:**

Connexin (Cx) gap junction proteins are expressed by a multitude of cells and function as plasma membrane hemichannels or dock to form intercellular communication tunnels. Whilst Cx43 has garnered considerable attention, less is known about the structure and function of Cx62 channels. Platelets and megakaryocytes express Cx37, Cx40 and Cx62, which contribute to hemostatic and thrombotic responses. Our study explores an unexpected finding that following platelet activation, an extracellular region of Cx62 undergoes proteolytic cleavage by calpain-1. We adopted an interdisciplinary approach to evaluate structural and functional consequences of calpain-mediated cleavage of Cx62. Cellular signaling was assayed by immunoblotting, aggregation and calcium flux assays. Gap junction function and thrombus formation were assessed under arteriolar flow. *In silico* modelling was used to predict calpain-mediated changes to the pore diameter and design a decoy peptide ($^{62}$Pept-NT). Mechanistically, Cx62 cleavage is $Ca^{2+}$-dependent and requires calpain-1 externalization. Modelling a predicted calpain-1 cleavage site on the first extracellular loop, shows that calpain can dock to Cx62 monomers, promoting stepwise channel cleavage. Consequently, we predict a significant pore dilation enhancing diffusion of signaling molecules between cells and into the extracellular milieu. We designed a decoy peptide that abrogated calpain-1-mediated cleavage, reduced intercellular communication and restricted thrombus growth. Cx62 cleavage was dependent upon sequential action of protein kinase A, protein phosphatase 2A and $Ca^{2+}$ release from intracellular stores. Extracellular calpain cleavage represents a fundamentally new regulatory mechanism for connexin 62, culminating in an irreversible open state.

**Key Words:** Gap junctions, calpain-1, thrombosis, structural biology, signaling

## Introduction

Platelet activation and occlusive thrombus formation underpins the etiology of myocardial infarction and ischemic stroke. These cells store and release over 300 bioactive molecules and that regulate angiogenesis, proliferative responses, necrosis, proteolysis and inflammation.[1-3] The importance of platelets beyond cardiovascular disease is garnering increasing attention within the arenas of cancer biology and inflammatory responses.[4-6] Connexin gap junctions and pannexin-1 are critically important in health and disease.[7] Aberrant activation of connexins and channel-specific mutations are associated with cancers, cardiovascular disease and neurological complications.[7-9] Given the global burden of these conditions on patients and healthcare systems, there is mounting interest in developing therapeutic approaches and drugs to target connexins, which have centered on connexin 43 (Cx43), due to its ubiquitous expression profile. Strategies have included mimetic peptides, channel modulators and repurposing of existing drugs (e.g. carbenoxolone).[10, 11] However, given that connexins are broadly distributed, it is important to look beyond Cx43 and further our understanding of the structure and function of other family members. Thus, providing critical groundwork for the rational design of connexin-specific therapies using gene editing and small molecule approaches.

Connexins are synthesized and assemble into hexameric hemichannels that are trafficked to the plasma membrane, where they function as hemichannels or can dock with opposing channels to form gap junctions.[12] This dual role enables connexins to serve as conduits for the exchange of small molecules between the cytosol and extracellular milieu to directly convey intercellular signals.[13] Connexins are considered to be non-selective channels that facilitate passage of molecules ≤1 kDa in size. Therefore, enabling the release or transfer of bioactive molecules, such as ATP, $Ca^{2+}$, inositol-1,4,5-trisphosphate and micro RNAs.[13, 14]

Connexins are sub-divided into five families (□, β, γ, δ and ε) and three members of the □-connexin family have been identified in platelets: Cx37, Cx40 and Cx62.[15, 16] Targeted inhibition of Cx37, Cx40 or Cx62 reduces thrombotic responses in an *in vivo* murine model. Gap junction blockers also reduce $Ca^{2+}$ responses, platelet aggregation and thrombus formation *in vitro,* highlighting their importance in coordinating platelet responses.[17, 18] While research into the roles and regulation of Cx37 and Cx40 have been studied in a range of cell types, we were first to report functional expression of *GJA10* (Cx62 in human and Cx57 in mouse).[18] The platelet therefore presents itself as a useful tool for the assessment of Cx62 function providing insights into the role of this channel in relevant cell types and disease models. Mechanisms underpinning the regulation of gap junction function have been explored with roles for phosphorylation, proteolytic cleavage, ubiquitination and

sumoylation.[19] We recently demonstrated that platelet Cx62 is regulated by protein kinase A (PKA) in a cAMP-independent manner[18].

In the present study, we sought to further explore the regulation and function of Cx62 in human platelets. We made the surprising discovery that upon platelet stimulation, Cx62 channels are subject to $Ca^{2+}$-dependent proteolytic cleavage, likely via calpain-1. *In silico* modelling reveals that calpain-1-mediated cleavage substantially increases the Cx62 pore diameter, and our functional data report increased diffusion of the cytosolic dye calcein. This process was regulated by the sequential action of protein kinase A (PKA), protein phosphatase 2A (PP2A) and elevation of intracellular $Ca^{2+}$.

## Methods

*Additional methods are provided in the Supplementary Material.*

*Reagents*

$PGI_2$, thrombin, calpeptin and EDTA (Ethylenediaminetetraacetic acid) were purchased from Sigma–Aldrich (Poole, UK). TRAP6, collagen and cross-linked collagen-related peptide (CRP) were supplied by Bachem (Bubendorf, Switzerland), Takeda (Linz, Austria) and CambColl (Cambridge, UK), respectively. Custom peptides listed in Table 1 were synthesized by Sigma-Aldrich. Fura2-AM and calcein-AM were from Thermo Fisher (Loughborough, UK). 5,5'-dimethyl-Bapta, AM ester was from Sartorius (Beit HaEmek, Israel) and LB-100 was from SelleckChem (Cambridge, UK). Recombinant calpain was purchased from Scientific Laboratory Supplies (Nottingham, UK). Unless specified, all other reagents were from Sigma-Aldrich.

*Platelet isolation*

*This study was approved by the University of Reading Research Ethics Committee.* Blood was collected from healthy human donors, who had abstained from anti-platelet medication for a minimum of two weeks, after informed consent in accordance with the declaration of Helsinki. Blood was collected into Na-citrate vacutainers (3.8%) and centrifuged to obtain platelet-rich plasma (PRP, 100 xg, 20 min).[20] Washed platelets were collected by centrifugation (900 xg, 7 min) and re-suspended into $Ca^{2+}$-free Tyrode's-HEPES buffer (134mM NaCl, 0.34mM $Na_2HPO_4$, 2.9mM KCl, 12mM $NaHCO_3$, 20mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5mM glucose and 1mM $MgCl_2$, pH 7.3) at a concentration of $4x10^8$ platelets $mL^{-1}$ and rested for 30 minutes at 30°C before use.

*In silico modelling of interactions between Cx62 and calpain-1*

In our previous study[18] we modelled Cx62 and its interaction with the mimetic peptide, we initially predicted the Cx62 monomer using the IntFOLD server.[21] Subsequently, we built the Cx62 hemichannel by utilizing the crystal structure of Cx26 as a template. Then, the Swisdock webserver was also employed to dock the mimetic peptide to Cx62 monomer, generating alternative conformations to predict the peptide binding site and their interactions.[22]

With the development of AlphaFold2, advanced neural network-based tools can predict protein-protein, and protein-peptide at high accuracy without the need for additional docking tools and the explicit usage of crystal structures.[23] Consequently, our *in silico* pipeline underwent a significant upgrade with the development of MultiFOLD, specifically designed to predict protein-protein, protein-peptide interactions, and multimeric states.[24]

Firstly, we excluded from the model the long-disordered region within C-terminus based on DISOPRED3 server prediction to focus on the functional sites.[25] Then, the Cx62 monomer and its interacting partners, including the decoy peptide and calpain-1, were modelled using MultiFOLD.[24] Additionally, various combinations of Cx62 hemichannel and its cleavage were also modelled without the need for explicit crystal structures, utilizing MultiFOLD's capability to accurately predict the multimeric state.

Statistics

Data are expressed as mean ± SEM. Statistical evaluation was performed using Student's t test for paired data or one-way ANOVA with Bonferroni post-test for repeated measures, as indicated in the figure legends. Statistical significance is denoted as ns, *, ** or *** for *P>0.05*, *P<0.05*, *P<0.01* or *P<0.001*, respectively.

**Results**

*Cx62 is a calpain substrate in activated platelets.*

In our previous study of platelet Cx62 we noticed that there was a loss of immunoreactivity following stimulation by platelet agonists. We explored this phenomenon further by performing immunoblotting experiments. Stimulation of platelets by thrombin or collagen led to a significant reduction of intact Cx62 (Figure 1A). Interestingly, decreased immunoreactivity was accompanied by the appearance of a second smaller band below 37kDa (Figure 1B). Cx62 cleavage also took place following exposure of platelets with supraphysiological levels of $Ca^{2+}$ (5mM), indicating that this was a $Ca^{2+}$-mediated process (Figure 1 B-C). Cleavage of Cx62 was concentration-dependent with thrombin concentrations ≥0.03 Units $mL^{-1}$ or collagen concentrations ≥ 1µg $mL^{-1}$, sufficient to induce platelet activation (Figure S1). We also explored whether other platelet connexins are subject to $Ca^{2+}$-dependent proteolytic modification. Our experiments revealed a similar

phenomenon for Cx40, which underwent proteolytic cleavage following stimulation by the $Ca^{2+}$ ionophore ionomycin (Figure S2).

Stimulation of platelets by thrombin or collagen leads to a rapid increase of intracellular $Ca^{2+}$ mediated by release from intracellular stores and activation of plasma membrane ion channels.[26-28] We studied the $Ca^{2+}$-dependence of Cx62 cleavage through chelation of extracellular or intracellular $Ca^{2+}$. Chelation of extracellular $Ca^{2+}$ by EGTA (Figure 2A) or intracellular $Ca^{2+}$ by BAPTA-AM (Figure 2B) protected against proteolytic cleavage of Cx62. $Ca^{2+}$-dependent proteolysis of Cx32 and Cx50 has been shown to be mediated by calpain in hepatocytes and lens fiber cells.[29, 30] Calpain activation can be detected by immunoblot with an electrophoretic shift from its inactive (78 kDa) to active (76 kDa) conformation.[31] Probing our blots for calpain-1 isoforms, we noted that activation of calpain-1 coincided with the cleavage of Cx62 (Figure 2). We therefore examined whether calpain-1 activity was required to achieve cleavage of Cx62. The calpain-1 inhibitor calpeptin protected against agonist-evoked Cx62 cleavage, pointing to a role for this protease (Figure 2C). These data point to a mechanism for post-activation regulation of Cx62 by a $Ca^{2+}$-dependent protease and the involvement of calpain-1.

*Cx62 has an extracellular calpain-1 cleavage site that regulates intercellular communication.*

We next adopted an *in silico* approach to search for potential calpain-1 cleavage sites within the Cx62 sequence. We utilized an open-source calpain-1 cleavage site prediction program (GPS-CCD) to identify and score calpain-1 cleavage sites. This approach predicted a calpain-1 cleavage site within the first extracellular loop (DEQSAFACNTRQPG), with a score of 0.693, which exceeded the threshold of 0.654.

We previously developed a model of Cx62 within IntFOLD based on the crystal structure of Cx26.[18, 21] To enhance understanding, an advanced computational pipeline was utilized to model the interaction between Cx62, calpain-1, the decoy peptide, and Cx62 hemichannel. Using our model, we highlighted the predicted calpain cleavage site on a Cx62 monomer (Figure 3A, yellow). Models of calpain-1 docking onto a Cx62 monomer show interactions between the catalytic region of calpain-1 and the predicted cleavage site (Figure 3B). We further tested our hypothesis by modelling interactions between calpain-1 and a hexameric Cx62 hemichannel (Figure 3C). These data show that whilst calpain-1 can dock onto a Cx62 hemichannel, it is unlikely that multiple calpain-1 enzymes can interact with the connexin simultaneously. We therefore predict that calpain-1 cleaves Cx62 channels in a stepwise manner. Finally, we modelled the structural consequence of calpain cleavage upon the pore structure of Cx62. Prior to calpain cleavage the Cx62 channel has a minimum pore diameter of 12.5 Å (reference molecules glucose, ATP and albumin are predicted to have diameters of

9 Å, 14 Å and 30 Å, respectively; Figure 3D). Removal of the cleaved region of Cx62 results in a substantial increase of minimum pore diameter to 29.5 Å (Figure 3E).

Our model predicts that calpain acts on an extracellular site. Notably, calpain release from endothelial cells, lymphocytes, osteoblasts and chondrocytes has been reported.[32, 33] Calpain release may occur through active secretion or leak from apoptotic cells.[34] Released calpain modulates endothelial cell protease-activated receptor function.[35] Thus, we investigated whether calpain-1 can be detected in platelet releasate and whether we could interrupt calpain-1-mediated cleavage. We prepared supernatants from platelets stimulated by thrombin or collagen and ran immunoblots for calpain-1 and Cx62. Consistent with previous studies, we detected active calpain-1 isoforms in the releasate of platelets stimulated by thrombin or collagen (Figure 4A).

To evaluate the functional role of calpain-1 cleavage upon Cx62, we designed a calpain-1 decoy peptide ($^{62}$Pept-NT; DEQSAFACNTRQPG) based upon the predicted cleavage sequence within Cx62. We also designed a control peptide from a scrambled sequence of the same residues ($^{Scr}$Pept-NT; SECQFQGPTAADRN). Modelling these peptides suggests that the decoy, but not scrambled control, peptide is able to bind to the catalytic region of calpain-1 (Figure S3). This peptide protected against Cx62 cleavage by calpain-1, despite clear activation of the protease (Figure 4B). The decoy peptide did not affect cleavage of the intracellular calpain target talin-1 (Figure S4). Similar findings were observed when platelets were stimulated by TRAP6 (PAR1 agonist) or collagen-related peptide (CRP; GPVI agonist; Figure S5). To assess whether extracellular calpain was sufficient to induce Cx62 cleavage, we evaluated the effect of exogenous calpain applied to unstimulated platelets. Application of active calpain in the presence of extracellular $Ca^{2+}$ for up to one hour failed to induce cleavage of Cx62 (Figure S6). These data suggest that either calpain-mediated cleavage of Cx62 requires platelet activation and conformational change of the channel, or that there is not sufficient protein at the surface in resting platelets.

It is unclear whether calpain-1-mediated cleavage of Cx62 represents a primary activation pathway for the channel, or if it may be a mechanism to prevent channel closure. We have shown that selective inhibition of Cx62 leads to a reduction of thrombin- and collagen-evoked platelet aggregation.[18] However, pre-incubation of platelets with our decoy peptide did not lead to a reduction of platelet aggregation responses (Figure S3B), indicating that calpain-1 cleavage is not essential for primary activation of Cx62. We next studied the impact of calpain cleavage upon thrombus formation under arteriolar shear (1,000 $s^{-1}$) in an endpoint *ex vivo* assay. Imaging of fixed thrombi revealed that inhibition of calpain cleavage did not affect platelet adhesion to collagen as surface coverage values were comparable between groups (Figure 4C). Importantly, decoy of calpain cleavage by $^{62}$Pept-NT reduced

thrombus height and volume (Figure 4D-F), consistent with a role for calpain-mediated cleavage in regulating thrombus formation. Given that calpain cleavage influenced thrombus formation but not aggregation responses, we explored whether Cx62 influences clot structure by thromboelastography. In our assay, whole blood was pre-incubated with a generic gap junction blocker (carbenoxolone, Cbx) or the calpain decoy peptide $^{62}$Pept-NT prior to clot formation. Inhibition of connexins delayed the onset of clot formation and reduced the maximum amplitude, indicating reduced clot strength (Figure S7). A smaller impact on the time to initiation of clot formation was observed in the presence of the calpain decoy peptide (Figure S7). Overall, there was a trend towards reduced clot strength, but it is not likely to be the main function of Cx62 cleavage.

To further assess the functional consequences of calpain cleavage, we directly assessed hemichannel and gap junction using a calcein diffusion assay. Previous studies have used calcein diffusion within pre-formed thrombi or single cells using fluorescence recovery after photobleaching (FRAP) technology or flow cytometry, respectively.[16, 18, 36] We developed an alternative approach to measure temporal dynamics of calcein dye diffusion during thrombus formation (Figure S4). This approach utilizes donor platelets that are co-stained with calcein dye and fluorescent CD61 antibodies to enable tracking of calcein diffusion over time. Thrombi were formed under arterial flow conditions (1,000$s^{-1}$) and calcein diffusion was found to initiate soon after platelets began to adhere to the collagen-coated surface. Under control conditions, calcein diffusion occurred at a steady rate across the five-minute time course (Figure 4G-I and Movie S1). Pre-incubation of platelets with our decoy peptide significantly blunted calcein diffusion (Figure 4G-I, Movie S2). These data indicate that calpain-1 influences the permeability through modification of the Cx62 pore diameter.

*Calpain-1 regulation of Cx62 is dependent on intracellular $Ca^{2+}$, cAMP and PP2A activity*.

We have demonstrated that PKA regulates Cx62 in a cAMP-independent manner.[18] We therefore investigated other pathways that regulate PKA activity and their influence upon Cx62 cleavage. Elevation of cAMP leads to activation of PKA and downstream phosphorylation of the IP3 receptor, abrogating $Ca^{2+}$ release.[37, 38] In the presence of $PGI_2$ immunoblot analysis shows increased levels of VASP phosphorylation at $Ser^{157}$, indicative of PKA activation (Figure 5A). PKA activation was associated reduced intracellular $Ca^{2+}$ mobilization (Figure 5B), coinciding with a reduction of calpain-1 activation and protected against Cx62 cleavage (Fig 5A).

Protein phosphatase 2A (PP2A) is a negative regulator of PKA and is activated following platelet stimulation.[39] PP2A has been shown to disrupt PKA-dependent phosphorylation of neuronal Cx36 channels.[40] Platelet stimulation by collagen or thrombin increased PP2A

phosphorylation, reduced VASP phosphorylation and was accompanied by cleavage of Cx62 (Fig 5C). Pre-incubating platelets with LB-100, a PP2A inhibitor, abrogated PKA activation, prevented calpain-1 activation and Cx62 remained intact (Figure 5C). Inhibition of PP2A also led to a significant reduction of intracellular $Ca^{2+}$ mobilization, presumably through phosphorylation of the IP3 receptor (Figure 5D-E).

Finally, we determined whether direct inhibition of Cx62 by the mimetic peptide $^{62}$Gap27 influenced calpain-mediated cleavage. Binding of $^{62}$Gap27 to its target site on the second extracellular loop of Cx62 inhibits channel activity and downregulates platelet function.[18] Incubation with the inhibitory peptide reduced levels of calpain activation and protected against calpain-1-mediated cleavage of Cx62 (Figure 6). The mechanism by which $^{62}$Gap27 protects against cleavage is unclear but may be related to reduced $Ca^{2+}$ flux, or by locking the channel in a closed conformation, rendering the calpain-1 cleavage site inaccessible.

**Discussion**

Connexins represent a universal mechanism for the regulation of intercellular communication between adjacent cells and tissues.[11, 41] The nature by which they are regulated is of fundamental importance and provides insights for the development of novel therapeutics. Calpain-mediated regulation of the intracellular domain of specific connexins has been highlighted previously.[30, 32] We report for the first time that the orphan connexin, Cx62, possesses a calpain-1 cleavage site that is situated within the first extracellular loop. Cleavage at this site is predicted to increase the size of the pore promoting intercellular communication and thrombus growth. Using platelets as a Cx62 expressing cellular model, we noted that proteolytic cleavage was mediated by calpain-1 and critically dependent upon elevation of intracellular $Ca^{2+}$. Efforts to reduce $Ca^{2+}$ flux through induction or modulation of the PKA signaling pathway abrogated calpain-1 activation and protected against cleavage of Cx62. Furthermore, selective inhibition of Cx62 by the peptide $^{62}$Gap27 or decoy of active calpain by $^{62}$Pept-NT prevented channel cleavage (Figure7).

Studies in other cell types have identified connexins as substrates for calpain cleavage. Mouse liver Cx32, but not Cx26, is cleaved by both μ-calpain and m-calpain.[29] Cleavage occurs at an intracellular site within the C-terminus and phosphorylation of the channel by PKC, but not PKA, was shown to protect against protease activity.[29] Cx50 in lens fiber cells was also shown to undergo calpain cleavage of a c-terminal region, resulting in the removal of a 32kDa portion of the channel.[30] We were therefore surprised to identify an extracellular calpain-1 cleavage site within the sequence of Cx62. Cleavage resulted in detection of a smaller fragment that was rapidly degraded following stimulation with collagen or thrombin. It is important to note that the 37kDa band that was detected following stimulation (Figure 1)

cannot be fully explained by the predicted extracellular calpain cleavage site, as the N-terminal anti-Cx62 antibody would be expected to detect a fragment of approximately 5kDa, although we cannot exclude potential alternative cleavage sites. While none were detected using a bioinformatic approach, this may reflect a limitation of such experimental tools. It is therefore possible that the larger Cx62 fragment detected (Figure 1) represents intermediate products of protein degradation, mediated by calpain or other proteases, following initiation by an extracellular calpain-dependent cleavage event. We are therefore unable to confirm that the DEQSAFACNT|RQPG sequence is the definitive site that mediates or initiates Cx62 cleavage, pore dilation and protein degradation. Our modelling data (Figure 3) highlight that Cx62 is likely to be cleaved in step-wise manner, but we are unable to determine the functional consequences of this. Surprisingly, incubation with recombinant calpain did not result in Cx62 cleavage of resting platelets. This may be explained by low levels of Cx62 on the surface of resting platelets, which we have previously shown.[18] Alternatively, this may be explained by the calpain site being inaccessible in its closed conformation. Given that the calpain decoy peptide $^{62}$Pept-NT is not cell permeable, our data support calpain-1-mediated cleavage at an extracellular site.

Calpain-1 release from cells has been reported from a variety of cells, including platelets.[32-35] For example, extracellular calpain-1 has been implicated in vascular repair in a model of glomerulonephritis.[32] The mechanism by which calpain-1 is released from cells is debated. Whilst release from apoptotic cells is a likely source, there is evidence also for active secretion from intact cells, such as lymphocytes, chondrocytes and endothelial cells.[33, 42] Calpain-1 has been detected within platelet-derived extracellular vesicles.[35] Calpain-1 released through this mechanism was shown to cleave PAR1 on vascular endothelial cells, stimulating synthesis of pro-inflammatory TNF-α. An alternative route for calpain-1 release may be through ATP-binding cassette transporters (i.e. ABCA1), which have been reported in human and mouse lymphocytes.[43] Platelets express ABCA1 and deletion of these transporters has been shown to reduce platelet reactivity to collagen and thrombin and downregulated thromboxane A2 synthesis.[44] Whilst the authors state that this is likely due to altered lipid conversion in the absence of ABCA1, it is also possible that this deletion impacts calpain-1 release. Further studies are required to evaluate the precise mechanism(s) for calpain-1 release from activated platelets.

The functional role of calpain-1-dependent cleavage of connexin proteins remains unclear. Given that other connexin channels have a relatively short membrane half-life of a few hours, it is possible that calpain cleavage serves as a way to regulate Cx62 channel turnover.[29, 41] An alternative prospect is that cleavage of Cx62 by calpain-1 is required to facilitate irreversible transition to an additional open-state. In the absence of single channel

recordings for Cx62, we do not know the open probability and to what extent calpain-1 influences permeability of specific ions. Pannexin-1 is structurally-related to connexins and has been shown to possess a caspase cleavage site within its intracellular C-terminus.[45, 46] Cryo-EM modelling shows that caspase cleavage removes an auto-inhibitory domain, leading to enhanced channel permeability and release of ATP.[47] Our modelling of Cx62 cleavage by calpain-1 suggests that similar regulatory mechanisms may be at play. Cleavage increases the minimum pore diameter from 12.5 Å to 29.5 Å, which would increase permeability to ATP and other molecules (e.g. microRNA, glucose and $IP_3$). Cx62 cleavage by calpain may also serve to stabilize the channel in an open state, which facilitates recruitment of platelets into a thrombus and/or coordination of signaling events between platelets and the endothelium.

Our study highlights differences between platelet aggregation responses and the properties of thrombi formed *in vitro*. We show that aggregation in response to collagen or thrombin is not reliant upon Cx62 cleavage. However, the decoy peptide $^{62}$Gap27 had mixed effects on thrombus formation, whereby surface coverage was unchanged but overall height and volume decreased. This difference likely relates to a static vs aqueous platelet agonists and the increased shear forces experienced by thrombi in whole blood assays. Calpain-deficient mice have reduced laser-induced thrombus formation[48] and attenuated procoagulant activity[49]. We therefore conclude that these differences may relate to altered stability of platelet-platelet interaction in the more physiologically relevant thrombus formation assay.

We previously demonstrated that selective inhibition of Cx62 by $^{62}$Gap27 attenuates platelet activation responses through reduction hemichannel and gap junction function.[18] Binding of this peptide to Cx62 led to cAMP-independent activation of PKA, which is inhibitory in platelets.[18] Platelets incubated with $^{62}$Gap27 were protected from calpain-1-mediated cleavage. Notably, the binding sites for calpain-1 and $^{62}$Gap27 reside within the first and second extracellular loops, respectively. The protective mechanism for the $^{62}$Gap27 peptide is unclear but may relate to $^{62}$Gap27 locking the channel in a closed conformation or disruption of calpain-1 binding by the peptide. Whilst the precise mechanisms by which Gap27 peptides inhibit connexins remain unclear, there is strong evidence that these peptides attach to, and are retained, at the extracellular face of the channel and their binding rapidly reduces the pore diameter.[11] We hypothesize that Cx62 channel activation is a pre-requisite to calpain-1-mediated cleavage and that this serves as a regulatory mechanism to protect against the action of calpains that have been released from other cells.

In conclusion, we propose that agonist-evoked elevation of intraplatelet $Ca^{2+}$ leads to the activation of Cx62 and release of calpain-1 from the cytosol, where it will be exposed to $Ca^{2+}$ (2 mM) in the plasma. Subsequent interactions between calpain-1 and its substrate

promotes further conformational change of Cx62. This presumably irreversible activation state facilitates increased intercellular communication through hemichannels and gap junctions. Targeting calpain-1 cleavage of Cx62 may represent a mechanism to downregulate the response to injury in the context of arterial thrombosis. Our findings suggest a novel extracellular role for calpain-1 in the regulation of connexin 62 structure and function. Further studies are therefore justified to explore the impact of this mechanism on other cell types and tissues in the context of health and disease.

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## Tables:

Table 1: Custom peptides for the study of Cx62 function

| **Peptide name** | **Sequence ($NH_2$-COOH)** |
|---|---|
| $^{62}$Gap27 | SRPTEKTIFML |
| $^{Scr}$Gap27 | REPTISFMKLT |
| $^{62}$Pept-NT | DEQSAFACNTRQPG |
| $^{Scr}$Pept-NT | SECQFQGPTAADRN |

## Figure Legends:

**Figure 1: Cx62 is post-translationally modified in activated human platelets. (A)** Representative immunoblot showing a reduction of full-length Cx62 expression by resting platelets (Veh) or following stimulation by thrombin (Thr; 0.03 U $mL^{-1}$, 5 minutes) or collagen (Coll; 1 µg $mL^{-1}$, 5 minutes) n=7 per group. **(B)** Stimulation of platelets by thrombin, collagen or extracellular $Ca^{2+}$ (5mM, 15 minutes) resulted in the appearance of a smaller cleavage product close to 37kDa. **(C)** Cx62 occurs at early timepoints with cleavage products detected at 50s onwards. Arrows indicate the cleavage fragment of Cx62 and β actin was used as a loading control. Data were analyzed by one-way ANOVA and ns, ** or *** denote *P>0.05*, *P<0.01, P<0.001*, respectively.

**Figure 2: Cx62 is a calpain substrate in activated human platelets.** Effect of EGTA (A: 5 mM, 5 minutes) BAPTA-AM (B; 20 µM, 30 minutes) or calpeptin (C; 50 µM, 30 minutes) upon detection of full-length Cx62 and calpain-1 activation. Comparisons were drawn between unstimulated (Unstim.) platelets and those stimulated by thrombin (Thr; 0.03 U $mL^{-1}$, 5 minutes) or collagen (Coll; 1 µg $mL^{-1}$, 5 minutes). Data are representative of three independent experiments and were analyzed by one-way ANOVA and ns or * denotes *P>0.05* or *P<0.05,* respectively.

**Figure 3: Structural consequences of calpain-mediated cleavage of Cx62 channels. (A)** Cx62 monomer shown in green with the predicted calpain-1 cleavage site (DEQSAFACNTRQPG) mapped in yellow. Side (i) and $90^{o}$ rotation showing extracellular aspect (ii) are shown. **(B)** Docking of calpain-1 (cyan) to a Cx62 monomer. Side (i) and $90^{o}$ rotation showing extracellular aspect (ii) are shown. **(C)** Docking of calpain-1 to a Cx62 hemichannel (green). Side (i) and $90^{o}$ rotation showing extracellular aspect (ii) are shown. **(D)** View through intact Cx62 channel pore from intracellular aspect with a minimum internal diameter of 12.5 Å (ii). **(E)** View through cleaved Cx62 channel pore from intracellular aspect. Cleavage increases the minimum pore diameter from 12.5 Å to 29.5 Å (ii). All images were rendered using PyMOL (http://www.pymol.org/pymol)

**Figure 4: The calpain decoy peptide $^{62}$Pept-NT protects against cleavage of Cx62, restricts thrombus growth and reduces gap junction activity. (A)** The detected levels of active calpain in platelet releasate in the presence or absence of thrombin (Thr; 0.03 U $mL^{-1}$, 5 minutes) or collagen (Coll; 1 µg $mL^{-1}$, 5 minutes). **(B)** Effects of $^{62}$Pept-NT on calpain active form levels and Cx62 modifications in platelets stimulated by Thr or Coll; n=6 per group, ****P<0.0001 vs $^{Scr}$Pept-NT. Calpain-1 isoforms were used an indicator for calpain activation status. β actin levels were used as a loading control. **(C)** Representative images of thrombi formed on collagen coated surfaces under arteriolar shear (1,000 $s^{-1}$). Whole blood was pre-

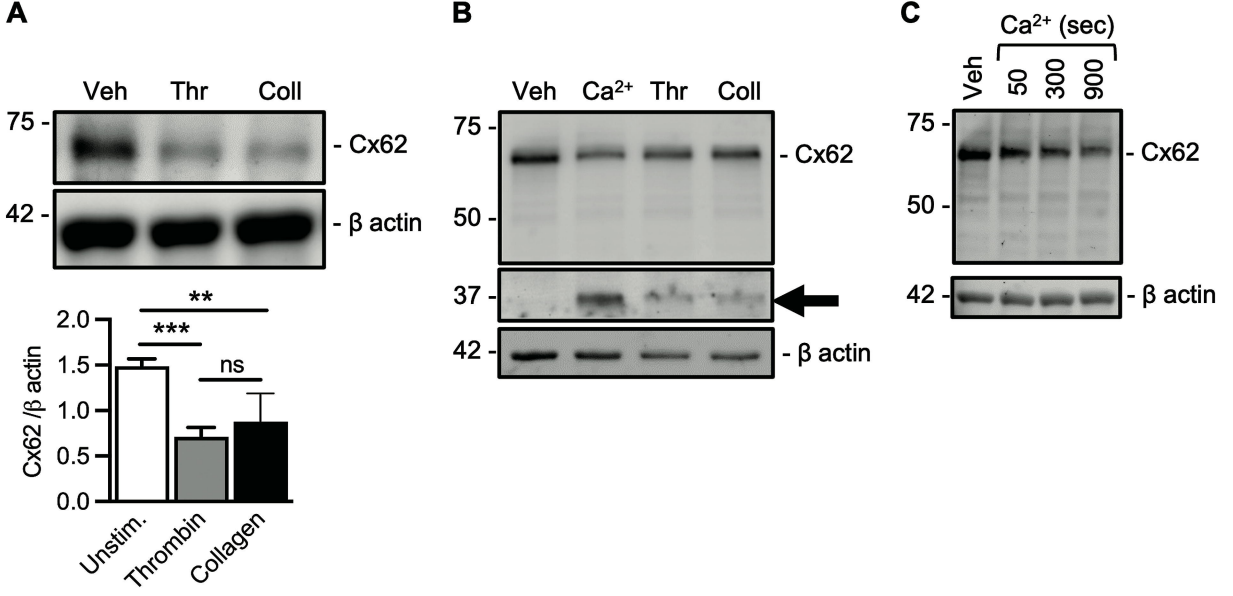
incubated with either $^{Scr}$Pept-NT or $^{62}$Pept-NT (each 100 µg mL$^{-1}$). Percentage surface coverage **(D)**, thrombus height **(E)** and thrombus volume **(F)** were calculated for whole blood treated with either $^{Scr}$Pept-NT (gray) or $^{62}$Pept-NT (white; n=6 per group). **(G)** Representative images of thrombi at 5 minutes with CD61 stained donor platelets (magenta) and diffused calcein (cyan). **(H)** LOWESS curves of calcein diffusion over time with SEM error bars for $^{Scr}$Pept-NT (black) or $^{62}$Pept-NT (red). **(I)** Summary data for calcein diffusion at 5 minutes. Data are representative of 4 independent experiments and scale bars = 10µm. Data were analyzed by paired-t-test, *= P<0.05.

**Figure 5: Stimulation of PKA or inhibition of PP2A protects against calpain-1 mediated cleavage of Cx62. (A)** The impact of PKA activation by prostacyclin ($PGI_2$; 280 nm., 5 minutes) on levels of full-length Cx62, calpain-1 activation and VASP phosphorylation. Platelets that were resting (Unstim.) or stimulated by thrombin (Thr; 0.03 U mL$^{-1}$, 5 minutes) or collagen (Coll; 1 µg mL$^{-1}$, 5 minutes). β-actin was included as loading control and data are representative of 6 independent experiments. **(B,D,E)** Peak $Ca^{2+}$ responses were monitored in response to stimulation by TRAP-6 (10 µM) or cross-linked collagen-related peptide (CRP-XL; 10 µg mL$^{-1}$). Where indicated platelets were pre-incubated with $PGI_2$ (280 nM, 5 minutes) or LB-100 (2.5 µM, 20 minutes) to establish contributions by PKA or PP2A, respectively. **(C)** The impact of PP2A inhibition by LB-100 (2.5 µM, 20 minutes) on levels of full-length Cx62, calpain-1 activation, VASP phosphorylation and PP2A phosphorylation. Platelets that were resting (Unstim.) or stimulated by thrombin (Thr; 0.03 U mL$^{-1}$, 5 minutes) or collagen (Coll; 1 µg mL$^{-1}$, 5 minutes). β-actin was included as loading control and data are representative of 7 independent experiments. Data are representative of 4 independent experiments. Statistical significance was determined by ANOVA and is denoted as ns, *, ** or *** for *P>0.05*, *P<0.05*, *P<0.01* or *P<0.001*, respectively.

**Figure 6: $^{62}$Gap27 limits calpain-1 activation and Cx62 cleavage.** Effect of the selective Cx62 mimetic peptide inhibitor $^{62}$Gap27 upon channel cleavage. Platelets were pre-incubated with either $^{Scr}$Gap27 or $^{62}$Gap27 (25 µg mL$^{-1}$, 5 minutes) and stimulated by thrombin (Thr; 0.03 U mL$^{-1}$, 5 minutes) or collagen (Coll; 1 µg mL$^{-1}$, 5 minutes). Data are representative of 3 independent experiments and were analyzed by one-way ANOVA; ns and *** denote *P>0.05* and *P<0.001*, respectively.

**Figure 7: Schematic for the proposed regulation of calpain-1-dependent cleavage of platelet Cx62.** The binding of platelet agonists thrombin or collagen to their receptors activate phospholipase C (PLC) β and γ isoforms, respectively. PLC triggers $Ca^{2+}$ mobilization pathways, increasing cytosolic $Ca^{2+}$, inactivation of PKA (protein kinase A) by PP2A (protein phosphatase 2A) and release of granular contents. Calpain-1 may be released or secreted from the cytosol where it will be activated by plasma $Ca^{2+}$ (2mM).

Calpain-1 is then able to dock to its cleavage site on the first extracellular loop of Cx62, facilitating transition to an irreversible open state. Chelation of $Ca^{2+}$ by EGTA or BAPTA-AM prevented calpain-1 activation and protect against Cx62 cleavage. Furthermore, activation of PKA by incubation with $PGI_2$ (prostaglandin $I_2$) or inhibition of PP2A by LB-100, prevented $Ca^{2+}$ mobilization and cleavage of Cx62. Cartoon created using Biorender (www.biorender.com).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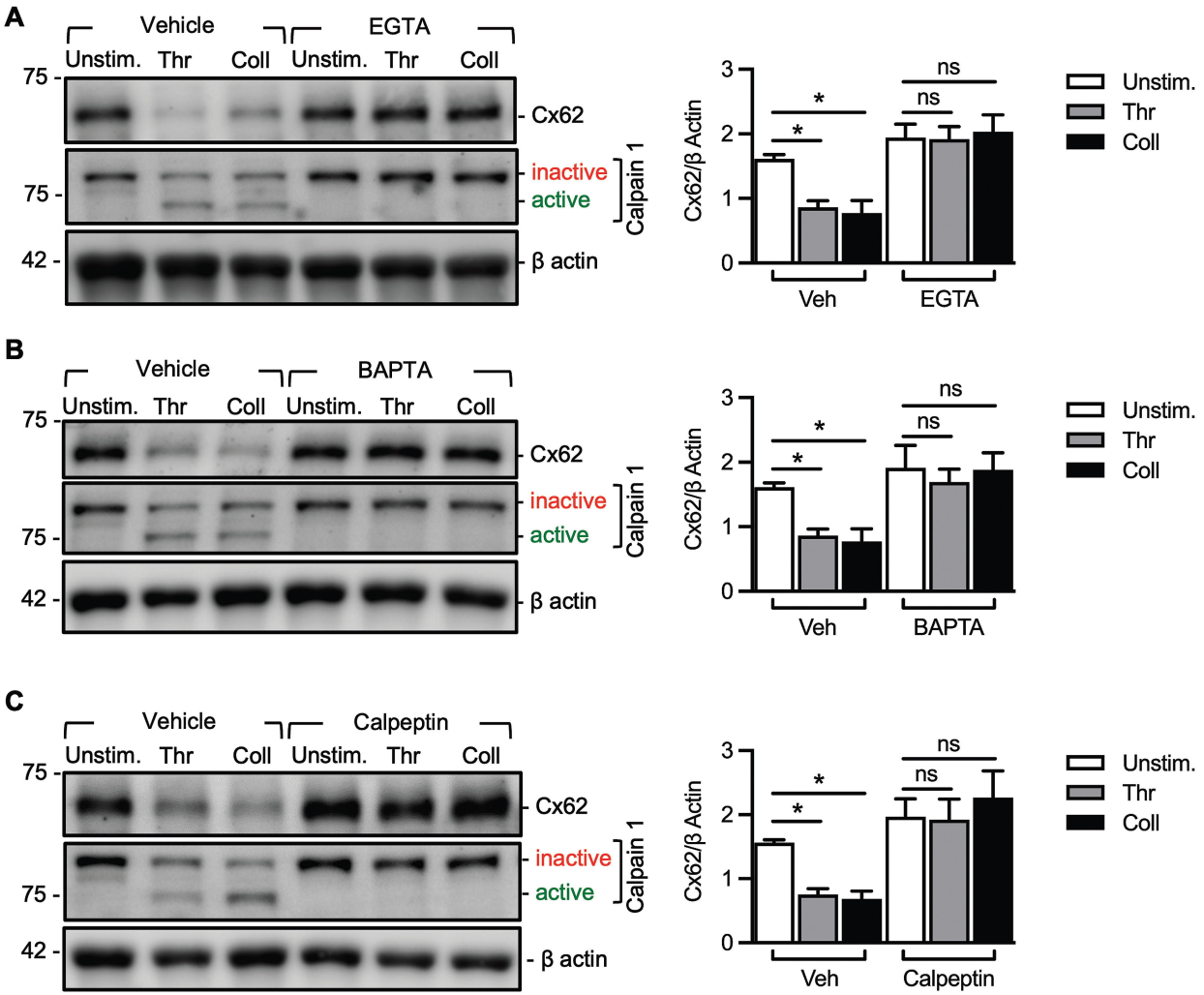
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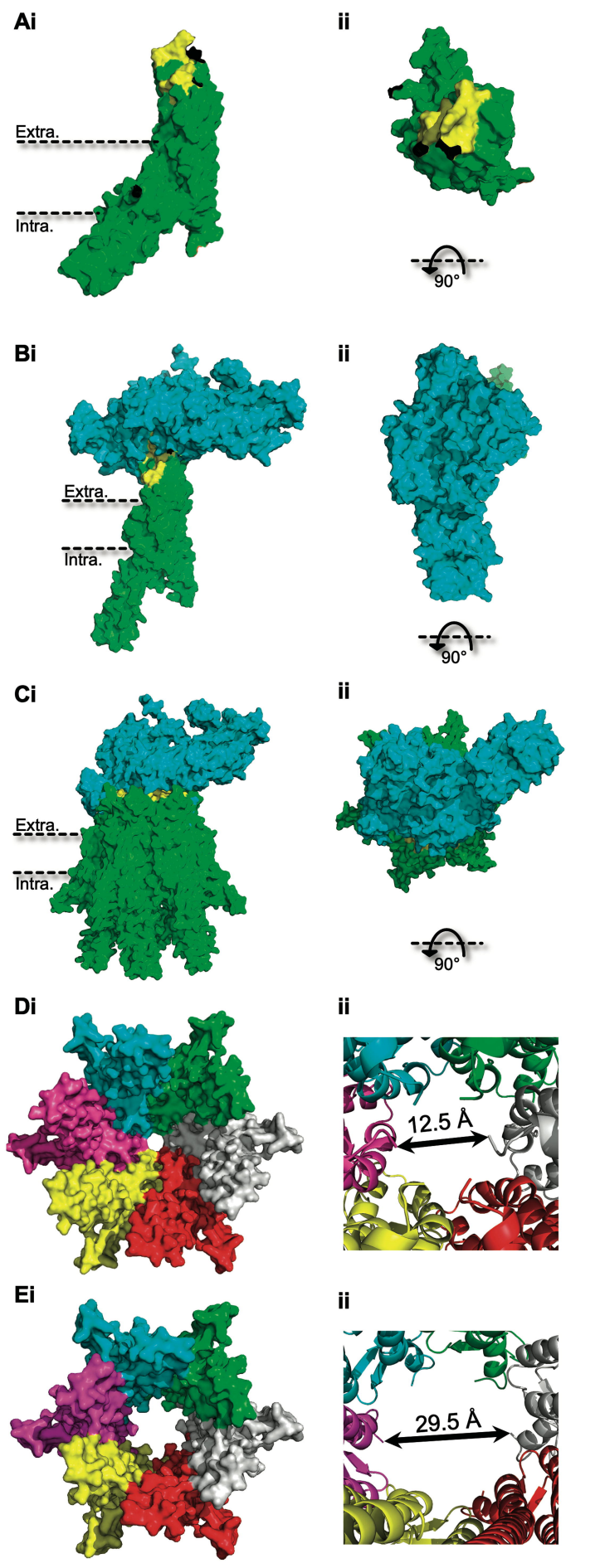
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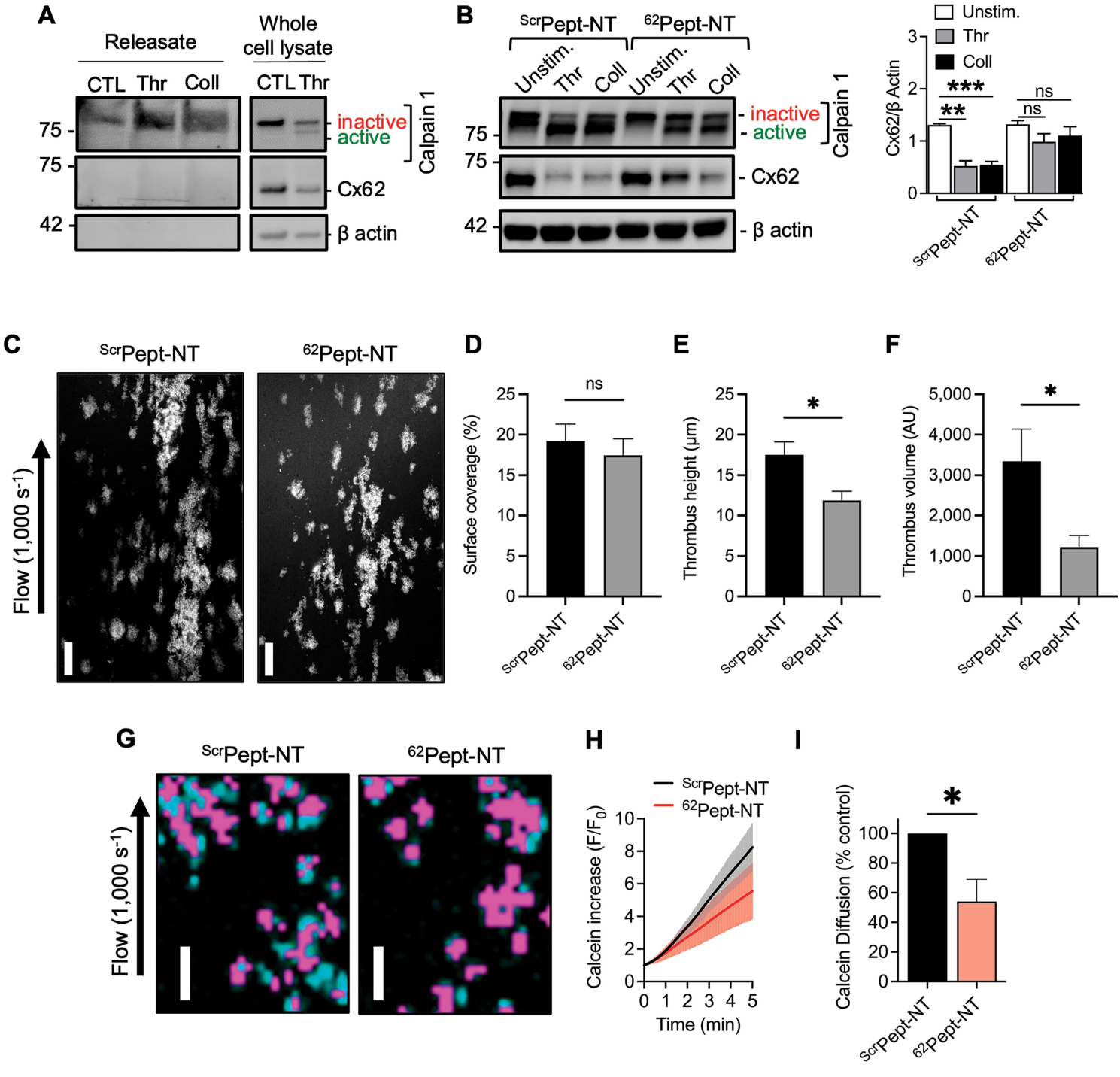
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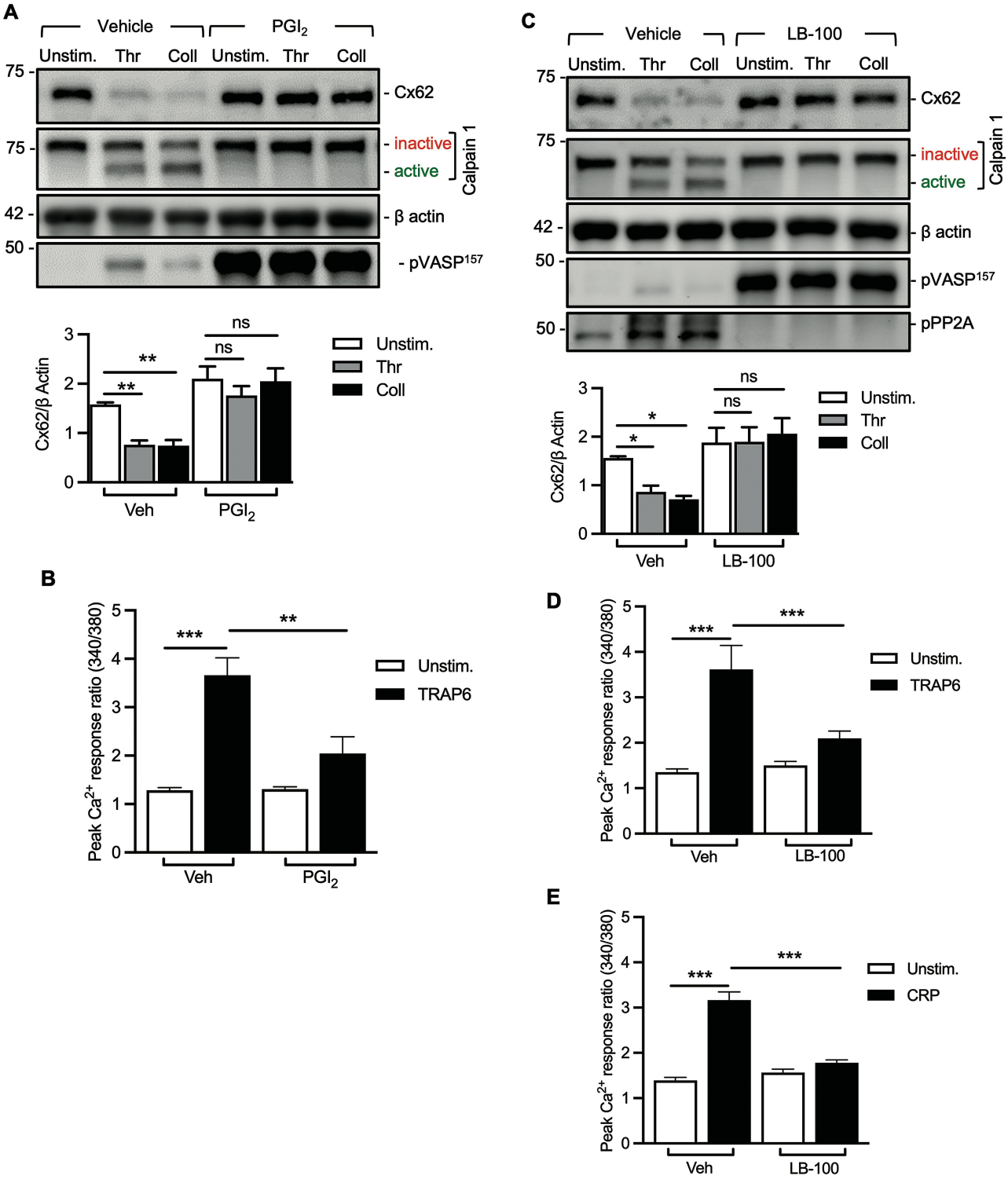
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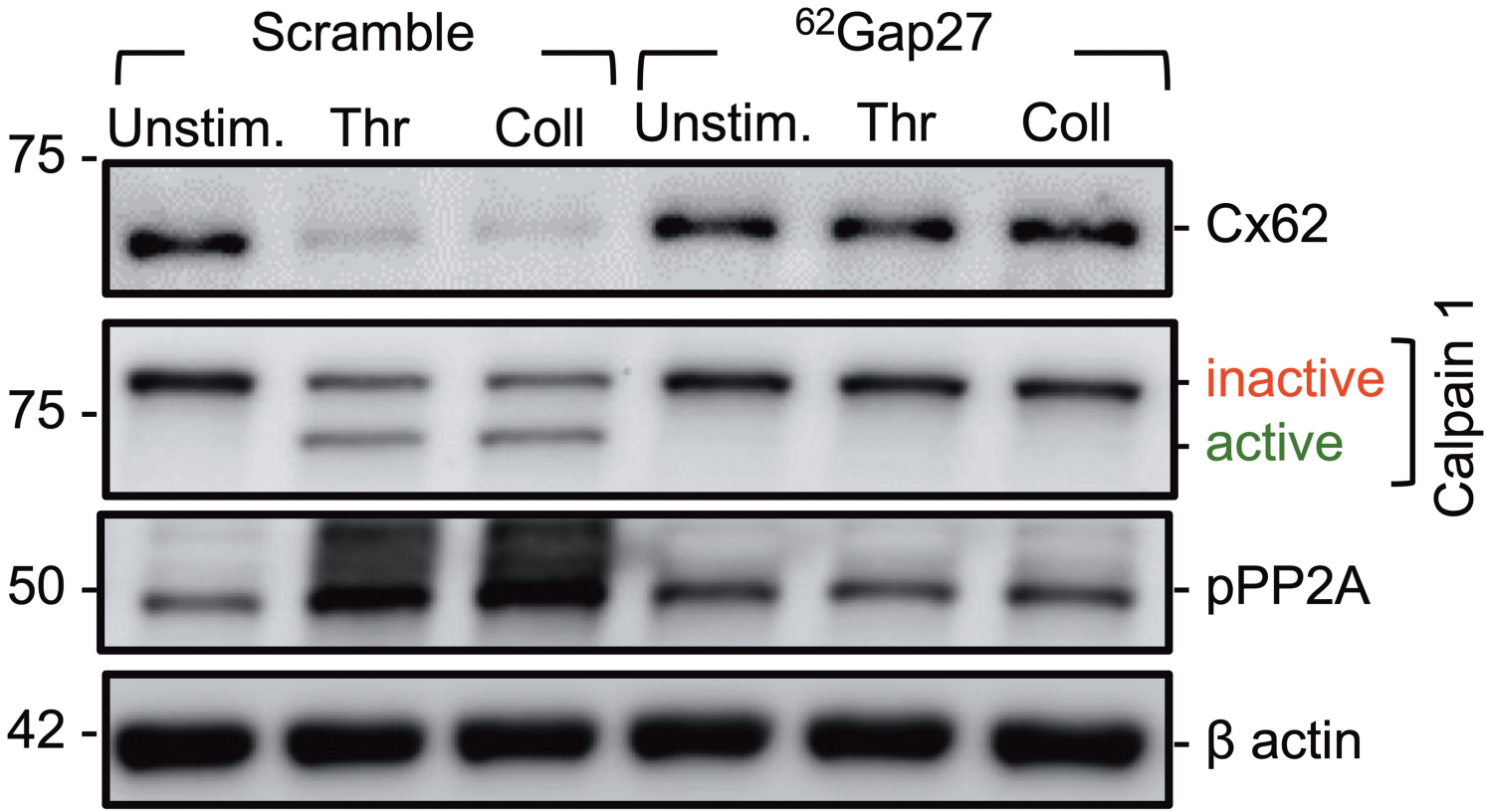
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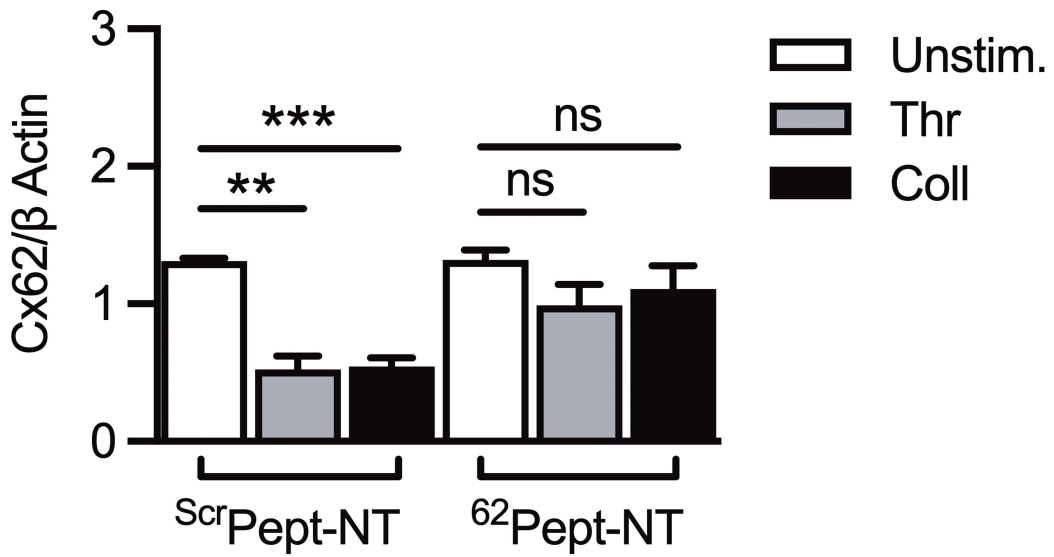
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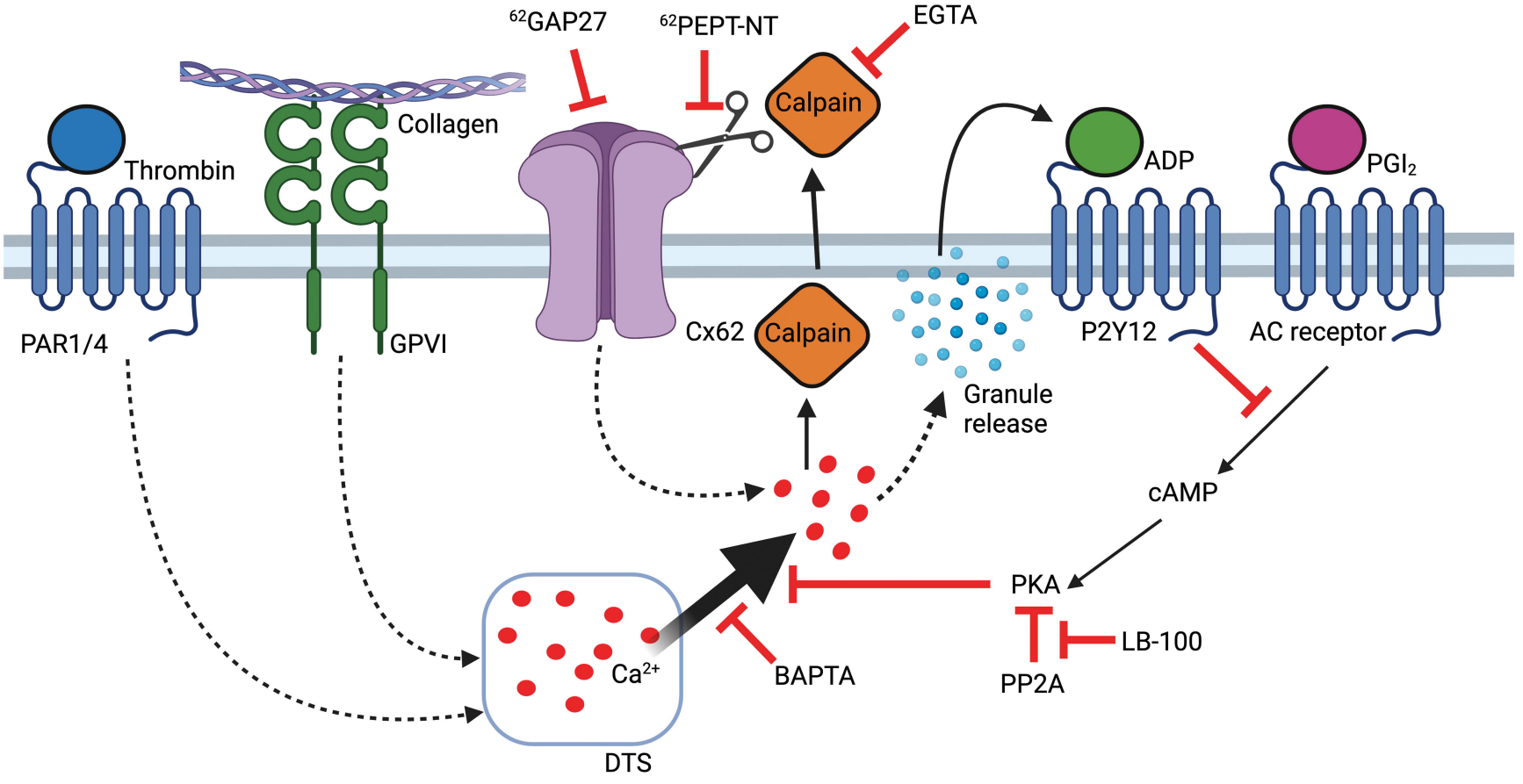
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PKA
LB-100
PP2A
Ca2+
BAPTA
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# Supplementary Material

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

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*KAT and AE contributed equally as co-first authors.

**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


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**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 |

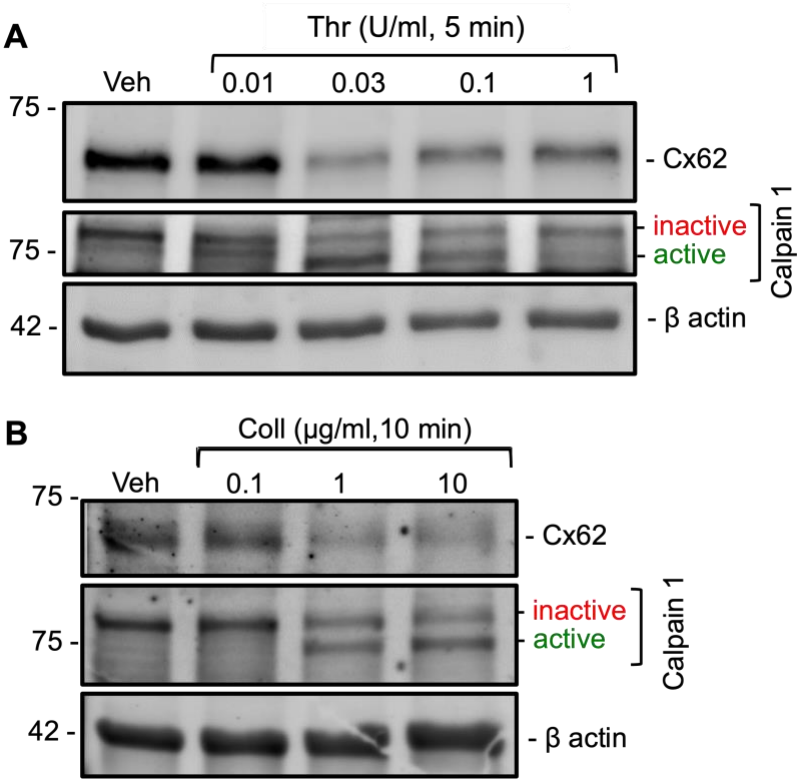



**Figure S1: Increasing levels of platelet agonists promote activation of calpain-1 and cleavage of Cx62. (A)** Effects of increasing doses of thrombin (0.01-1 U/ml, 5 minutes) on the levels of intact Cx62. **(B)** Effects of increasing doses of collagen (0.1-10 µg/ml, 10 minutes) on the levels of intact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

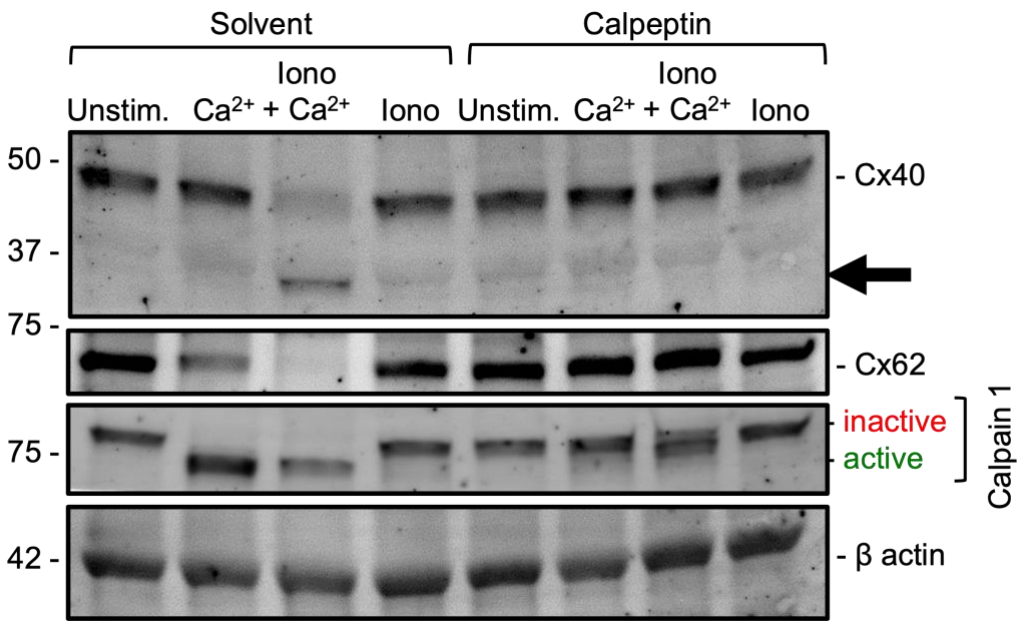



**Figure S2: Cx40 is a calpain substrate but requires high levels of $Ca^{2+}$.** Effects of high levels of extracellular calcium ($Ca^{2+}$; 5mM, 15 minutes) in the presence or absence of ionophore (Iono; 1µM, 5 minutes) on the levels of intact CX40 and CX62 in the presence or absence of calpeptin (50 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.

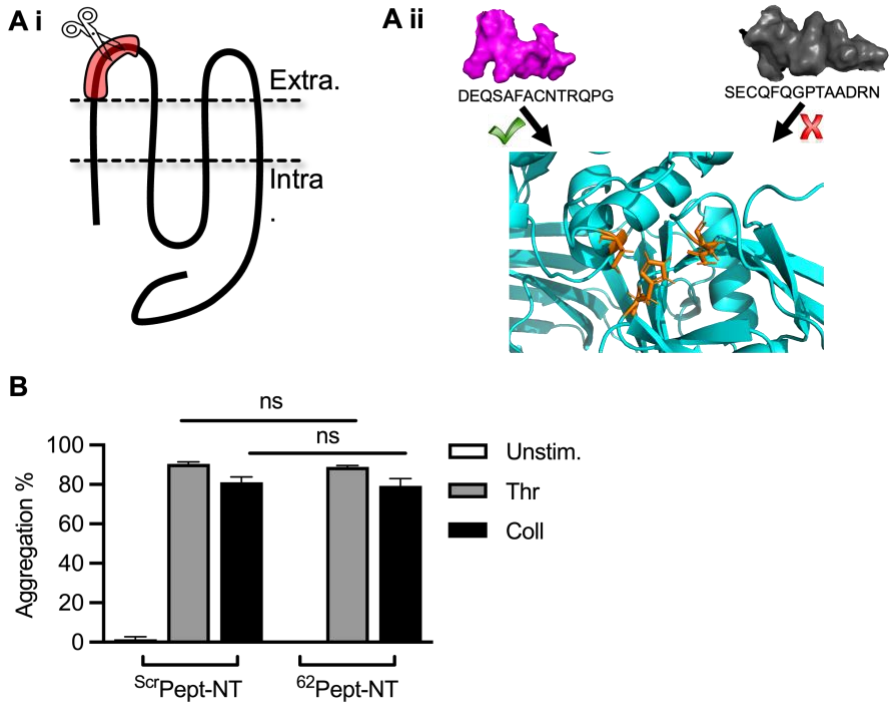



**Figure S3: The calpain decoy peptide $^{62}$Pept-NT does not alter agonist-evoked platelet aggregation. (A)** Topological cartoon showing calpain cleavage site DEQSAFACNT|RQPG. (Aii) Model of the calpain-1 active site (cyan) and the peptides $^{62}$Pept-NT (blue) and $^{Scr}$Pept-NT (orange). **(B)** 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).

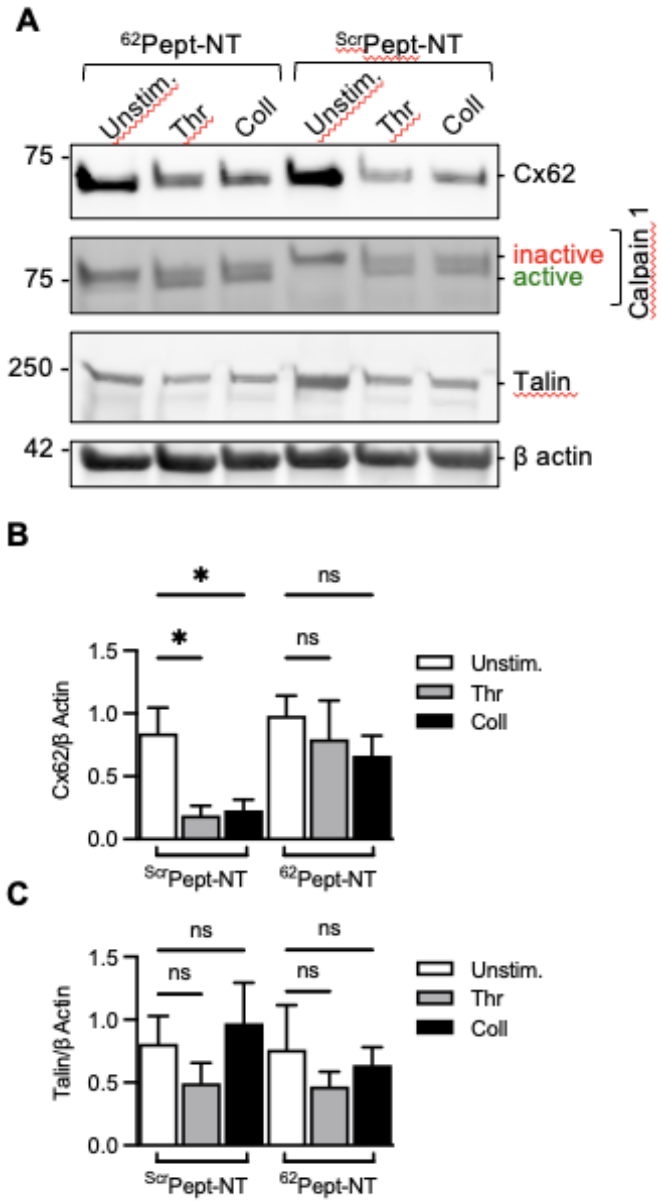



**Figure S4: The calpain decoy peptide $^{62}$Pept-NT does not affect agonist-evoked talin cleavage.** Platelets were stimulated by thrombin (Thr; 0.03 U mL$^{-1}$, 5 minutes) or collagen (Coll; 1 µg mL$^{-1}$, 5 minutes) in the presence $^{Scr}$Pept-NT or $^{62}$Pept-NT (100 µg/ml each, 5 minutes). **(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.*

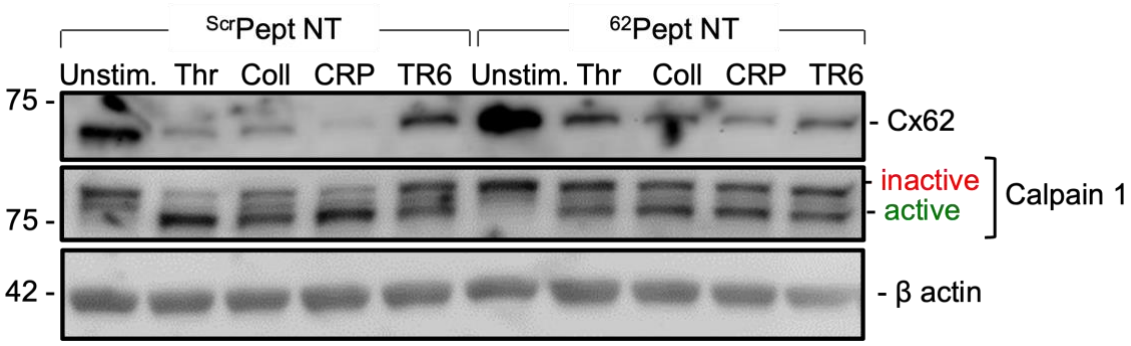



**Figure S5: Decoy of active calpain-1 by $^{62}$Gap27 protects against cleavage of platelet Cx62.** The levels of active calpain and intact Cx62 in response to the platelet agonists thrombin (Thr; 0.03 U/ml; 5 minutes), collagen (Coll; 10 µg/ml; 5 minutes), CRP-XL (CRP; 10 µg/ml, 5 minutes) or TRAP6 (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.

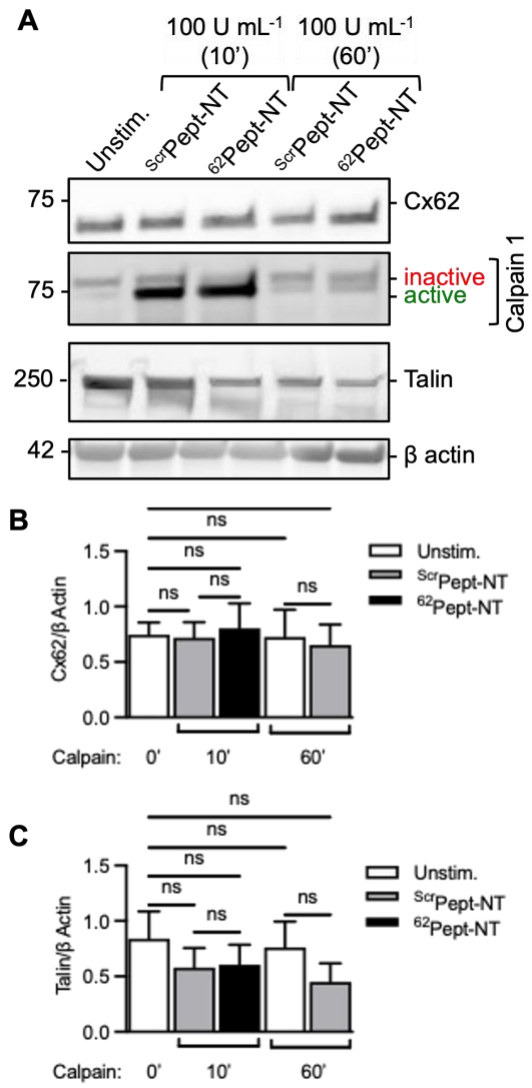



**Figure S6: Recombinant calpain does not cleave calpain in resting platelets. (A)** Platelets treated with $^{Scr}$Pept-NT or $^{62}$Pept-NT (100 µg/ml each, 5 minutes) were incubated with 100U/mL recombinant calpain for 10 minutes or 60 minutes. Representative images for immunoblot detection of Cx62, 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.*

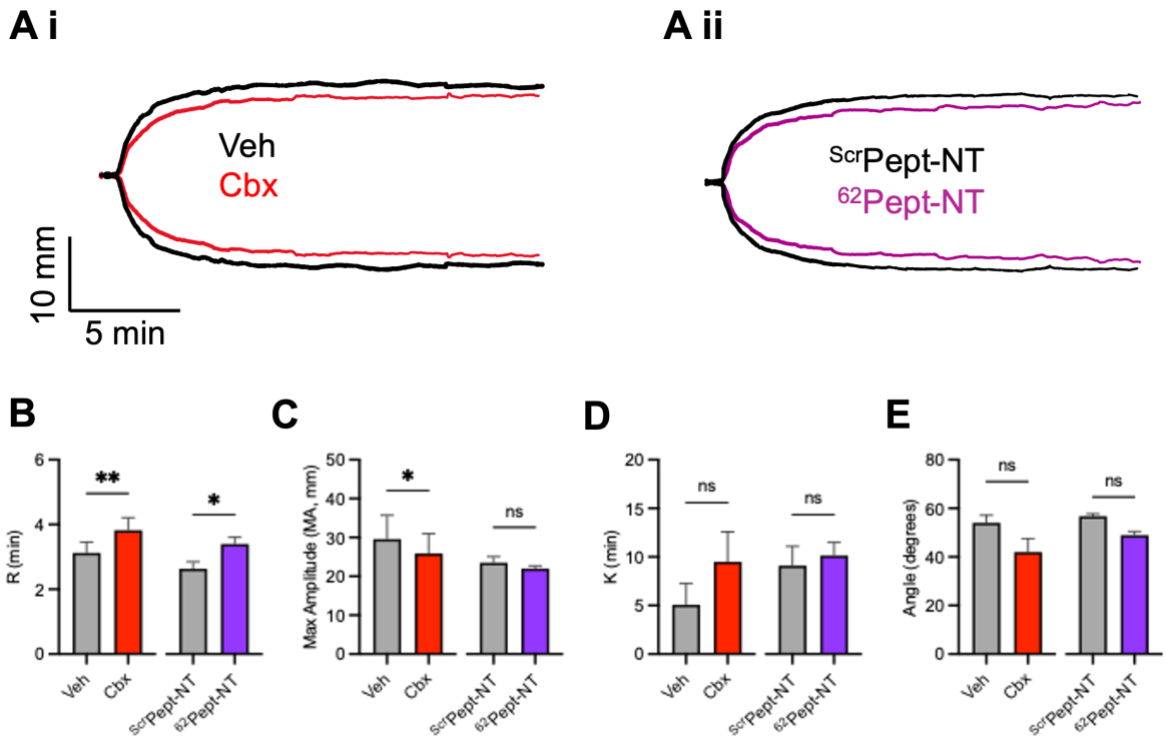



**Figure S7: Calpain decoy peptide modestly reduces time to initiation of clotting.** The role of connexins in regulating platelet clotting parameters were assessed by thromboelastography (TEG). Platelets were pre-treated with vehicle (Veh; black), a generic gap junction blocker carbenoxolone (Cbx; red; 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.*

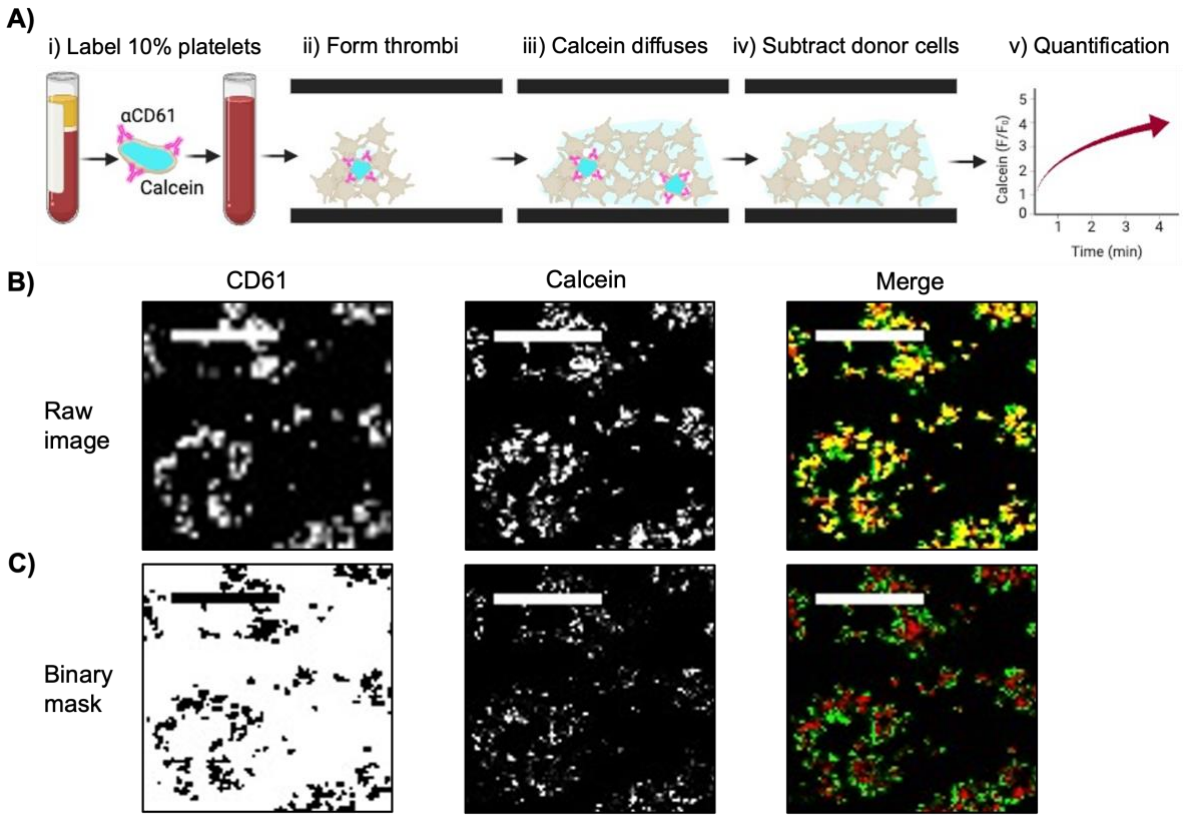
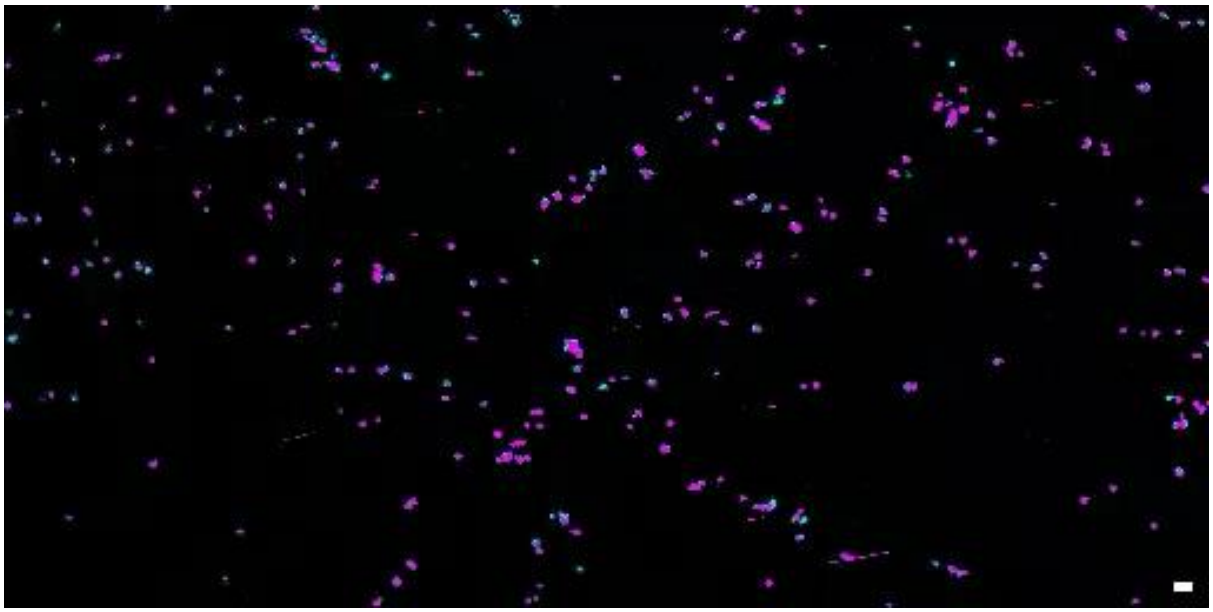



**Figure S8: Workflow for real-time assessment of calcein dye diffusion via connexin gap junctions during thrombus formation.** A) Cartoon workflow of calcein diffusion assay (www.biorender.com). i) Isolate PRP and stain 10% with cytosolic dye (calcein; cyan) & surface marker (αCD61; magenta). Reconstitute $Calcein^{+}CD61^{+}$ 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.