Supplemental information

Structural, Functional and Mechanistic Insights Uncover the Fundamental Role of Orphan Connexin 62 in Platelets

Authors

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Materials

Cx62 antibody was obtained from Sigma-Aldrich. The anti-phosphotyrosine antibody 4G10 was obtained from Millipore, USA and the phospho-specific antibodies - PLCγ2 (Y759), AKT (S473), myosin light chain (S19) and vasodilator-stimulated phosphoprotein (VASP) phospho-Ser157 and Ser239 were obtained from cell signaling technologies, USA. Syk (pY525/526) and LAT (Y200) antibodies were from Abcam. Anti-Phospho–PKC substrate antibody was purchased from New England BioLabs, USA. Mouse anti-human 14-3-3ζ (Santa Cruz Biotechnology, USA) was used to detect 14-3-3ζ to ensure equivalent levels of protein loading in immunoblots. The secondary antibodies used for immunoblotting; Cy5 goat anti-rabbit IgG, AlexaFluor488 goat anti-rabbit and AlexaFluor 488 goat anti-mouse IgG antibodies were obtained from Life Technologies, UK. All other reagents were from previously described sources[1-3].

Methods

Preparation of human Platelets

Human blood was taken from consenting, drug-free volunteers on the day of the experiment according to the methodology approved by the University of Reading Research Ethics Committee. Blood was taken using 3.8% (w/v) sodium citrate and Acid Citrate Dextrose (ACD; 110 mmol/L glucose, 80 mmol/L citric acid, 120 mmol/L sodium citrate) as an anticoagulant. Whole blood was centrifuged at 102*g* for 20 minutes at 20°C to yield platelet-rich plasma (PRP). Where washed platelets were required, they were isolated from the PRP by further centrifugation at 1413*g* for 10 minutes at 20°C in the presence of 0.1 μg/ml prostacyclin to prevent activation. The supernatant was discarded in Klorsept disinfectant (Medentech, Wexford, Ireland) and the platelet pellet was resuspended in 25ml of modified Tyrodes-HEPES buffer (134 mmol/L NaCl, 0.34 mmol/L Na_2HPO_4, 2.9 mmol/L KCl, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, 5 mmol/L glucose, 1 mmol/L $MgCl_2$, pH 7.3) and 3 ml of ACD in the presence of 0.1 μg/ml prostacyclin. Platelets were centrifuged at 1413*g* for 10 minutes at 20°C and resuspended to a density of $4x10^8$ cells/ml in modified Tyrodes-HEPES buffer using a platelet count obtained with a Z Series Coulter Counter (Beckman Coulter, CA, USA). Washed platelets were rested for at least 30 minutes at 30°C prior to the experiment to allow responses to recover. Platelet preparations typically contained fewer than 1 contaminating erythrocyte or leukocyte per 6500 platelets.

ADP-sensitive washed platelets were prepared by collecting blood into 3.8% (w/v) sodium citrate and centrifugation at 102*g* for 20 minutes at 20°C to yield PRP (without the addition of ACD). Platelets were isolated from the PRP by further centrifugation at 350*g* for 20 minutes. The supernatant was discarded, and the platelet pellet was resuspended to a density of $4x10^8$ cells/ml in the modified Tyrodes-HEPES buffer.

Preparation of Mouse Platelets

Mouse blood was collected through cardiac puncture after termination by rising CO_2 concentration and cervical dislocation as per Schedule 1 of the Animals (Scientific Procedures) Act 1986. After the mice were euthanized, their blood was drawn through the cardiac puncture into a syringe containing 4% sodium citrate (1 part sodium citrate to 9 parts blood). Red blood cells and leukocytes were eliminated by reducing the concentration of blood with Tyrode's-HEPES buffer followed by centrifugation at 203 *g* for 8 minutes. The upper layer comprising PRP was gently aspirated with a pipette. After the addition of PGI_2 (final concentration, 12.5 ng/mL) to the PRP, the platelets were subjected to centrifugation at 1,028 *g* for 5 minutes. The resulting platelet pellet was resuspended in modified Tyrode's-HEPES buffer (4×10^8 cells/mL) and left to rest at 30°C for 30 minutes.

Sucrose gradient sub-cellular fractionation

Platelet fractionation was performed as previously described with minor modifications[4]. Platelets were transferred into a cell-disruption bomb (Parr 4639, Parr Instrument Co.) and homogenized by nitrogen cavitation. A pressure of 1200 psi was applied with N_2 to the platelet suspension and after 15 min the pressure was quickly released. This procedure was repeated three times and the final platelet homogenate was cleared from the cell debris and partially disrupted cells by centrifugation at 500g for 10 min. The platelet homogenate was fractionated over a linear sucrose gradient (from 60 to 30%, w/v in 5mM EDTA) by centrifugation at 284,061 x g for 2 hours at 4°C. Fractions were collected from the top of the tube and aliquots analyzed by immunoblotting.

Immunofluorescence microscopy

Human blood was collected in vacutainers containing sodium citrate as described previously. The blood was centrifuged at 100g for 20 minutes to collect PRP. Resting or activated platelets

(stimulated with 5 μM U46619; in the presence of 4 μM integrillin) in PRP were fixed with an equal volume of 8% paraformaldehyde-PBS (PFA-PBS) to make a final concentration of 4% (v/v) and incubated for 15 min. Thereafter, platelets were centrifuged at 950g for 10 minutes. The supernatant was removed, and the platelet pellet was resuspended in 2 ml of PBS-ACD (pH 6.1) for washing. Platelets were centrifuged for 10 minutes at 950g and resuspended in 1 ml of PBS-ACD to concentrate platelets. Platelets were centrifuged again at the same speed for 10 minutes and then resuspended in 500μl of 1% (w/v) BSA-PBS, to concentrate platelets even more. Poly-L-lysine coated-12mm coverslips (VWR micro cover glass No.1.5) were put in a 6x6 culture plate and 90μl of platelets were added on each coverslip. Culture plates were placed at 37^{o}C for 90 minutes. After 2-3 washes with PBS, samples were blocked with 0.2% (v/v) Triton-X-100, 2% (v/v) serum from same species as secondary antibody and 1% (w/v) protease-free BSA for 1h. Thereafter, primary antibodies diluted (1:100) in 0.2% (v/v) Triton-X-100, 2% (v/v) serum from the same species as secondary antibody and 1% (w/v) protease-free BSA were added and left overnight. The following day, samples were washed with PBS (2-3 times) and secondary antibodies (1:200) were added for 1 hour at room temperature. The unbound antibodies were washed off with PBS (2-3 times) and samples were fixed using 4% (v/v) PFA-PBS for 5 minutes. The coverslips were washed again with PBS (2-3 times). Coverslips were placed on glass slides after adding ProLong Gold Antifade mounting media (Life technologies). The slides were kept at room temperature until mounting media dried and then kept in the fridge until they were imaged using a Nikon A1-R confocal microscope (100x oil immersion).

Stochastic optical reconstruction microscopy (STORM)

Tyrode's-HEPES buffer was used to dilute the PRP (1:20). The polymerization of fibrin was prevented by treatment with GPRP (0.5 mg/mL). The samples were activated with thrombin (1 U/mL) for 5 minutes, then the unstimulated and stimulated samples were fixed with 2% (v/v) formyl saline and subjected to centrifugation for 15 minutes at 500 *g*. After removal of the supernatants, the pellets containing the platelets were resuspended in Perm Buffer III (100 μL; BD Biosciences, Oxford, UK) and incubated on ice for 30 minutes. Platelets were then washed with Tyrode's-HEPES buffer (2 × 20 minutes) and subjected to centrifugation at 500 *g*. The supernatant was discarded, and the resultant pellet was resuspended in Tyrode's-HEPES buffer (50 μL). The samples were incubated with the primary antibodies (diluted 1:50;

mouse monoclonal IgG against integrin β_3 and rabbit polyclonal IgG against Cx62) at 4 °C overnight. Platelets were then washed twice with Tyrode's-HEPES buffer (2 mL) followed by centrifugation for 20 minutes at 550 *g*. The samples were incubated with secondary antibodies (diluted 1:50 in Tyrode's-HEPES buffer; Alexa Fluor® 647-labeled donkey anti-rabbit to detect Cx62 and Alexa Fluor® 555-labeled goat anti-mouse to detect β_3 integrin) at 37 °C for 30 minutes. Platelets were then washed with Tyrode's-HEPES buffer (2 mL) and subjected to centrifugation for 20 minutes at 550 *g*. The resulting pellet was suspended in Tyrode's-HEPES buffer (100 µL). Finally, platelets (100 µL) were applied to the ibidi® slides coated with poly-L-lysine. The slides were incubated at 4 °C overnight to allow the platelets to adhere. The next day, the unbound platelets were removed and blinking buffer was added (Stock A: 0.90 g/mL catalase [Sigma–Aldrich], 0.182 mM Tris [2-carboxyelthyl] phosphine hydrochloride [Sigma–Aldrich], 2.27% [v/v] glycerine, 1.14 mM KCl, 0.91 mM Tris-HCl [pH 7.5], 0.045 mg/mL glucose oxidase [Sigma–Aldrich] and 5 mL diH_2O; stock B: 36 mg/mL glucose, 3.6% [v/v] glycerine and 36 mL H_2O; and stock C: 0.09 M mercaptoethylamine-HCl [Sigma–Aldrich] and 1 mL diH_2O). For 3D STORM imaging of the platelets, the 100× oil immersion lens of the microscope was used.

Coordinate-based colocalization (CBC) analysis[5] was performed to assess changes in the colocalization of Cx62 and β_3 integrin upon platelet stimulation using ImageJ and the open-source Thunderstorm plugin.[6]

Calcein Dye Efflux

Platelets (in PRP) were loaded with calcein-AM (0.5 µM; Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at 37 °C as described previously [7]. The platelets were then treated with scrambled peptide or ^{62}Gap27 for 5 minutes. Next, the platelets were stimulated with thrombin (0.1 U/mL). In order to prevent fibrin polymerization, the thrombin-treated samples were also treated with GPRP (25 µg/mL). Stimulation was carried out with gentle mixing for different time periods over 5 minutes. Finally, the reaction was stopped with 0.2% (v/v) formyl saline. Flow cytometry (488 nm excitation, 530 ± 30 nm emission) was performed with a BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK). For each sample, 10,000 events, gated on platelets by forward scatter and side scatter, were collected. Data were analyzed with the built-in BD Accuri™ C6 Plus software (version 1.0.264.21).

Fluorescence Recovery after Photobleaching (FRAP)

FRAP analysis was performed as previously described with minor modifications[8,9]. Each of eight wells of each ibidi® slide was coated with fibrinogen (100 μg/mL) and collagen (10 μg/mL) in modified PBS for 1 hour. 1% (w/v) BSA was added to the wells followed by a 1-hour incubation to prevent the binding of platelets to the glass. The wells were washed three times with PBS. Calcein-loaded PRP was added to the coated coverslips and incubated for 45 minutes. Unbound platelets were washed from the wells with PBS (three washes). The samples were then treated with the scrambled peptide or ^{62}Gap27 (100 μg/mL) for 5 minutes. A high-intensity laser (488 nm) was trained on the central circular area (8-μm-diameter region of interest [ROI]) of the monolayer of cells thrombus for 300 milliseconds, resulting in an 85% loss of fluorescence. Then, a 488-nm wavelength laser was used to excite the samples and the fluorescence emission was detected at 500–520 nm. Finally, fluorescence recovery was recorded for 500 seconds. The 100× oil immersion objective of an A1R confocal microscope was used to capture images of single sections every second for 500 seconds. Five thrombi from each of seven donor samples treated with scrambled peptide or ^{62}Gap27 were analyzed. NIS-Elements software (Nikon, Tokyo, Japan) was used to compute the mean fluorescence intensities. For each time point, the average fluorescence intensities were computed for the background, non-bleached (reference) and bleached areas.

Protein Bioinformatics

The complete sequence of Cx62 was obtained from GenBank[10], and for the physicochemical analysis, ProtParam[11] was utilized. In the absence of an experimental structure, state-of-art structure prediction tools were employed to obtain 3D models for the Cx62 protomer. The IntFOLD4-TS method[12] from the IntFOLD server[13] was used to predict tertiary structure models for the Cx62 protomer (monomeric subunit). Additionally, the quality estimation method, ModFOLD6[14], was employed to provide both global and local (per-residue) scores for estimating 3D model quality. The calculated local (or per-residue) errors from ModFOLD6 were mapped onto the model using the temperature coloring scheme ranging from blue (indicating residues modeled with high quality) to red (indicating residues with lower model quality, which are often more flexible or disordered).

Multiple sequence alignment of human connexin sequences was performed using ClustalW to ensure the selectivity of 62Gap27. A scrambled peptide control was designed using Mimotopes, (http://www.mimotopes.com/peptideLibraryScreening.asp?id=97) and BLAST searches performed to ensure that designed sequences were not present in any other proteins.

To predict the most likely interactions occurring between Cx62 and the ^{62}Gap27 inhibitor, protein-ligand docking was performed using the SwissDock server[15]. The FullFitness and Gibbs free energy (ΔG) score of each run of the docking was evaluated and the final ranking of each cluster was based on the FullFitness scores.

The quaternary structures of the Cx62 hemichannels (2x 6-mers) were successfully modeled using the PDB entry 2zw3 (crystal structure of Cx26 gap junction) as a template. The docked hemichannel assembly (12-mer) template for PDB ID 2zw3 was downloaded from PISA [16] service at the EBI (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html). For each hemichannel, the template was used to orientate six of the modeled protomers by a six-fold symmetry axis perpendicular to the membrane plane and build the complete model of the docked hemichannel (12-mer) complex. Residues in the modeled protein-protein and protein-ligand complexes were considered to be interacting if the distance between the closest heavy atoms (i.e. non-hydrogen) in the residues belonging to different chains was <= 5Å

Platelet aggregometry

Light transmission aggregometry (LTA) was performed in an optical platelet aggregometer (Chrono-Log, PA, USA, and Helena Biosciences Europe, Gateshead, UK). Washed platelets ($4x10^8$ cells/ml) treated with ^{62}Gap27 or scrambled peptide were stimulated in the presence of agonist (collagen, CRP-XL, thrombin, U46619 or ADP) with continuous stirring (1200 rpm at 37^{o}C) for 3 minutes and aggregation was measured as an increase in light transmittance. The data were quantified by considering scrambled peptide-treated samples as 100% aggregation and the level of aggregation obtained in scrambled peptide-treated samples was normalized to it.

Fibrinogen binding and alpha granule secretion

Fibrinogen binding and P-selectin exposure to the platelet surface were detected by flow cytometry as measures of integrin αIIbβ3 activation and the secretion of α-granules respectively. Fluorescein isothiocyanate (FITC)-labelled rabbit anti-human fibrinogen antibody (Dako, Ely, UK) was used to measure fibrinogen binding. PE-Cy™ 5-labeled mouse anti-human CD62P antibody (BD Biosciences, Oxford, UK) was used to measure the exposure of P-selectin. The assay volume comprising human or mouse PRP, an inhibitor of Cx function or appropriate scrambled peptide control and each of the antibodies in modified Tyrode's-HEPES buffer, was incubated for 5 minutes at room temperature in the dark. The platelet agonists thrombin (in the presence of GPRP to prevent fibrin polymerization) or CRP-XL were added and incubated for an additional 20 minutes. The reaction was stopped by the addition of 0.2% (v/v) formyl saline. A BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK) and BD Accuri™ C6 software were used for the acquisition of the flow cytometry data. The median fluorescence intensity was calculated for 10,000 gated events. Fluorescence in FL1-A and FL3-A channels were used to analyze fibrinogen binding and P-selectin exposure, respectively.

Dense granule secretion

Dense granule secretion was determined by measuring changes in the extracellular ATP concentration. These changes were observed concurrently with aggregation in a Model 700 Whole Blood/Optical Lumi-Aggregometer with the use of a luciferase kit (Chrono-Log, Havertown, PA, USA). ATP release from dense granules was monitored with a bioluminescence system comprising D-luciferin, firefly luciferase and magnesium. ATP interactions with these reagents produce light, in direct proportion to the ATP concentration, which is observed and quantified using a Lumi-aggregometer. Platelets (4×10^8 cells/mL) were pre-treated with ^{62}Gap27 or scrambled peptide at 37°C for 5 minutes. Luciferase was added under stirring conditions during the last 2 minutes of the incubation. The platelets were stimulated with the indicated concentrations of thrombin or CRP-XL under stirring conditions (1,200 rpm at 37°C). ATP release and aggregation at 37°C were recorded for 3 minutes following the addition of agonist using the AggroLink8 software, which calculates ATP secretion levels from the 2nM ATP standard.

TxB_2 Assay

The TxB_2 measurements were performed with a TxB_2 immunoassay kit based on a competitive ELISA (Cayman Chemical, Cambridge, UK), according to the manufacturer's instructions. Washed platelets (4×10^8 cell/mL) were treated with ^{62}Gap27 or scrambled peptide for 5 minutes in glass cuvettes. The samples were then activated with CRP-XL or thrombin. After 5 minutes, stop solution (1 mM EGTA and 10 μM indomethacin) was added to terminate the reaction. The samples were then immediately subjected to centrifugation for 2 minutes at 12,000 rpm and the supernatants were frozen at −80°C. Later, the supernatants were thawed and diluted 1:40 in ELISA buffer (0.01% [w/v] sodium azide, 1 mM EDTA, 400 mM NaCl, 0.1% [w/v] BSA and 100 mM phosphate). The dilutions were plated in wells coated with polyclonal goat anti-mouse IgG antibodies. To determine the relationship between the TxB_2 concentration and absorbance, TxB_2 standards were prepared. TxB_2-acetylcholinesterase and anti-TxB_2 monoclonal antibody were added to each well, then the plate was incubated at room temperature for 2 hours. After incubation, the plate was washed 4 times with washing buffer. Next, Ellman's reagent was added to each well and the plate was incubated in the dark. A NOVOstar plate reader (BMG Labtech, Aylesbury, UK) was used to determine the absorbances of the wells at 405 nm. A standard curve was plotted using the absorbance readings for the TxB_2 standards. The inverse function was used to compute the TxB_2 concentrations from the test sample readings.

Measurement of intracellular calcium mobilization

The mobilization of Ca^{2+} from intracellular stores into the platelet cytosol was measured in a fluorescence-based 96-well plate assay. PRP was incubated with 2 μM Fura-2 AM for 60 minutes at 30°C. The PRP was washed and subjected to centrifugation at 350 *g* for 20 minutes, then resuspended in modified Tyrode's-HEPES buffer containing $CaCl_2$ (1mM) at 4×10^8 cell/mL. Fura-2-loaded platelets were incubated with ^{62}Gap27 or scrambled peptide for 5 minutes at 37°C, then stimulated with the agonists, CRP-XL or thrombin. A NOVOstar plate reader (BMG Labtech, Aylesbury, UK) was used to measure the fluorescence (excitation at 340 and 380 nm and emission at 510 nm). The ratio of the excitation signals at 340 and 380 nm was used to estimate the concentration of Ca^{2+}. To measure the mobilisation of calcium from intracellular stores, the above-mentioned steps were performed using Fura-2 loaded washed

platelets (in the absence of 1 mM $CaCl_2$) in the presence of saturating concentration of EGTA (2mM).

The cells were lysed with digitonin (5 μM) to release the Fura-2 into the assay buffer (which contained 1 mM $CaCl_2$) and facilitate the measurement of the maximum fluorescence ratio.

The minimum fluorescence ratio was measured by chelating Ca^{2+} ions with EGTA (10 mM) and Tris base (10 mM; added to ensure that the pH remained alkaline for optimum Ca^{2+} buffering by EGTA). Non-Fura-2-loaded cells at the same final density were used to measure the autofluorescence levels. Using the calibration values from above, experimental $[Ca^{2+}]_i$ concentrations were calculated using the following equation:

$$[Ca^{2+}]_i = K_d \times \frac{S_f}{S_b} \times \frac{R - R_{min}}{R_{max} - R}$$

Where K_d is the dissociation constant of Fura-2AM (~224 nM). S_f and S_b are the values of the fluorescence at 380nm excitation (corrected to background auto-fluorescence), with zero or saturating $[Ca]^{2+}$ respectively. R is the 340/380nm fluorescence ratio, corrected for background fluorescence. R_{min} and R_{max} are the ratio limits at zero or saturating $[Ca]^{2+}$, respectively, adjusted using a viscosity constant of 0.85. This corrects for the effects of the cellular environment on the fluorescence of Fura-2.

Platelet adhesion and spreading

To study platelet spreading, glass coverslips coated with fibrinogen (100 μg/mL in modified PBS) were placed in 6-well plates. After coating for 1 hour, 1% (w/v) BSA was added to the coverslips followed by 60 minutes incubation to prevent platelets from binding the glass. The coverslips were then washed three times with PBS. The washed platelet suspensions (2×10^7 cells/mL) that had been incubated for 5 minutes with ^{62}Gap27 or scrambled peptide were then added to the coverslips and incubated at 37°C for 45 minutes. Unbound platelets were removed, and the coverslips were washed three times with PBS. Then, the coverslips were fixed in 0.2% (v/v) formyl saline for 10 minutes. The coverslips were again washed three times with PBS. Next, the platelets were permeabilized with 0.2% (v/v) Triton™ X-100 for 5 minutes, then washed three times with PBS. The coverslips were incubated with Alexa Fluor® 488-conjugated phalloidin for 1 hour in the dark to label filamentous actin. The supernatants were removed, the coverslips were washed with PBS and placed on glass slides and fluorescence was preserved by adding ProLong™ Gold Antifade Mountant. The 100× oil

immersion lens of the Nikon A1R confocal microscope (Nikon, Tokyo, Japan) was used to image samples (excitation at 488 nm from an argon laser, emission between 500 and 520 nm). Images were taken in a single focal plane. In order to determine platelet adhesion, the numbers of platelets in five random images of each coverslip were counted. Platelets were categorized as spread fully (lamellipodia formed), partially spread (defined as filopodia) or adhered (not spreading). Finally, the relative frequencies of these groups were computed.

Clot retraction

Human PRP was prepared and rested at 30°C for 30 minutes. Red blood cells and ^{62}Gap27 or scrambled peptide were mixed with the PRP. The mixture was adjusted to a final volume of 1 mL with modified Tyrode's-HEPES buffer and incubated for 5 minutes at room temperature. Thrombin (final concentration, 1 U/mL) was added to initiate clot generation. A glass pipette was added to the center of each test tube, around which the clot would form, and samples were placed in an incubator chamber at 37^{o}C. Photographs were taken every 10 minutes and the assay was terminated after 60 minutes at which time the clot in the scrambled peptide-treated samples was seen to have retracted completely. Clot weight was measured as a marker for clot retraction. Clots were removed from the glass pipettes and transferred into the pre-weighed microfuge tubes. Clot mass was determined by subtracting the weight of pre-weighed microfuge tubes from the weight of microfuge tubes containing clot.

***In vitro* thrombus formation under flow**

Whole human blood was incubated with the lipophilic dye DiOC6 (5 μM) at 30 °C for 1 hour. Vena8 BioChip microfluidic channels were coated with type I collagen (100 μg/mL) for 1 hour. Channels were washed with modified Tyrode's-HEPES buffer to remove excess collagen. Whole blood was incubated with ^{62}Gap27 or scrambled peptide for 5 minutes. Then, the blood samples were perfused through the collagen-coated channels at an arteriolar shear rate of 20 dyne/cm^2. An argon laser was used to excite fluorescence (488 nm) and emission was recorded at 500–520 nm. Thrombus formation on the microfluidic chip was observed through the 20x objective of the Nikon A1R confocal microscope. Images of single sections were obtained every second for 600 seconds. Finally, NIS-Elements software (Nikon, Tokyo, Japan) was used to compute the mean thrombus fluorescence intensity.

In vivo thrombus formation

C57BL/6 mice were anaesthetised with intraperitoneally administered atropine (0.25 mg/kg), xylazine (12.5 mg/kg) and ketamine (125 mg/kg). When needed, pentobarbital (5 mg/kg) was used to sustain anesthesia. After the exteriorization of the cremaster muscle and removal of the connective tissue, an incision was made in the muscle, resulting in its adherence as a single layer to the glass slide. A buffer (135 mM NaCl, 4.7 mM KCl, 2.7 mM CaCl2 and 18 mM NaHCO3; pH 7.4) was used to hydrate the muscle.

Before the injury (made with a MicroPoint Ablation Laser Unit; Andor Technology, Belfast, UK), ^{62}Gap27 or scrambled peptide and DyLight® 649-conjugated anti-GPIbα antibody (to label platelets; 0.2 μg/g mouse weight), were introduced into the circulation through a cannula in the carotid artery. After 5 minutes of administration of ^{62}Gap27 or scrambled peptide, the formation of thrombi was observed with an Olympus BX61W1 microscope (Olympus, Tokyo, Japan). A Hamamatsu digital camera (C9300; Hamamatsu Photonics, Welwyn Garden City, UK) with charge-coupled device camera in 640×480 format was used to obtain images before and after injury. The images were analyzed with SlideBook 6 software (Intelligent Imaging Innovations, Denver, CO, USA). The protocol from the Home Office license was followed for the sacrifice of the mice. The protocol was also approved by the Animal Welfare and Ethics Research Board and the University of Reading local ethics review panel.

Tail Bleeding Assay

C57BL/6 mice were anesthetized by intraperitoneal administration of xylazine (12.5 mg/kg) and ketamine (125 mg/kg). ^{62}Gap27 or scrambled peptide was administered through injection via the femoral vein. After 5 minutes of infusion, the tips of the tails (0.3 cm) were cut with a scalpel and immediately placed in tubes with saline in a manner that prevented the cut ends of the tails from touching the walls of the tubes. The bleeding time was recorded until blood flow stopped or for up to 20 minutes. The mice were sacrificed according to the protocol that was approved by the University of Reading local ethics review panel, the Animal Welfare and Ethics Research Board and the Home Office.

cAMP ELISA

A cAMP immunoassay kit based on a competitive ELISA (Cell Signaling Technology, Hitchin, UK) was used to assess cAMP levels, according to the protocol provided by the manufacturer. Washed platelets (4×10^8 cell/mL) were added to a glass cuvette and treated with ^{62}Gap27 or scrambled peptide for 5 minutes. After 5 minutes of stimulation with CRP-XL or thrombin, lysis buffer (Triton™ X-100: 1% polyethylene glycol octylphenol ether) was added to the samples. The samples were immediately frozen at −20°C. The samples were later thawed and added to microwells coated with cAMP XP® rabbit monoclonal antibody. The association between cAMP concentration and absorbance was determined using cAMP standards. The assay plate was covered and incubated on a horizontal orbital plate shaker for 3 hours at room temperature. After incubation, the contents of the wells were removed and the wells were washed with 1x washing buffer three times. Then, 3,3',5,5'-tetramethylbenzidine substrate was added to the wells and the plate was incubated for 30 minutes. Stop solution was added to terminate the reaction. The absorbance at 450 nm was periodically determined with a NOVOstar plate reader (BMG Labtech, Aylesbury, UK). A standard curve was plotted from the absorbance readings of the cAMP standards. cAMP concentrations were computed for the test sample readings via the inverse function.

Western blotting

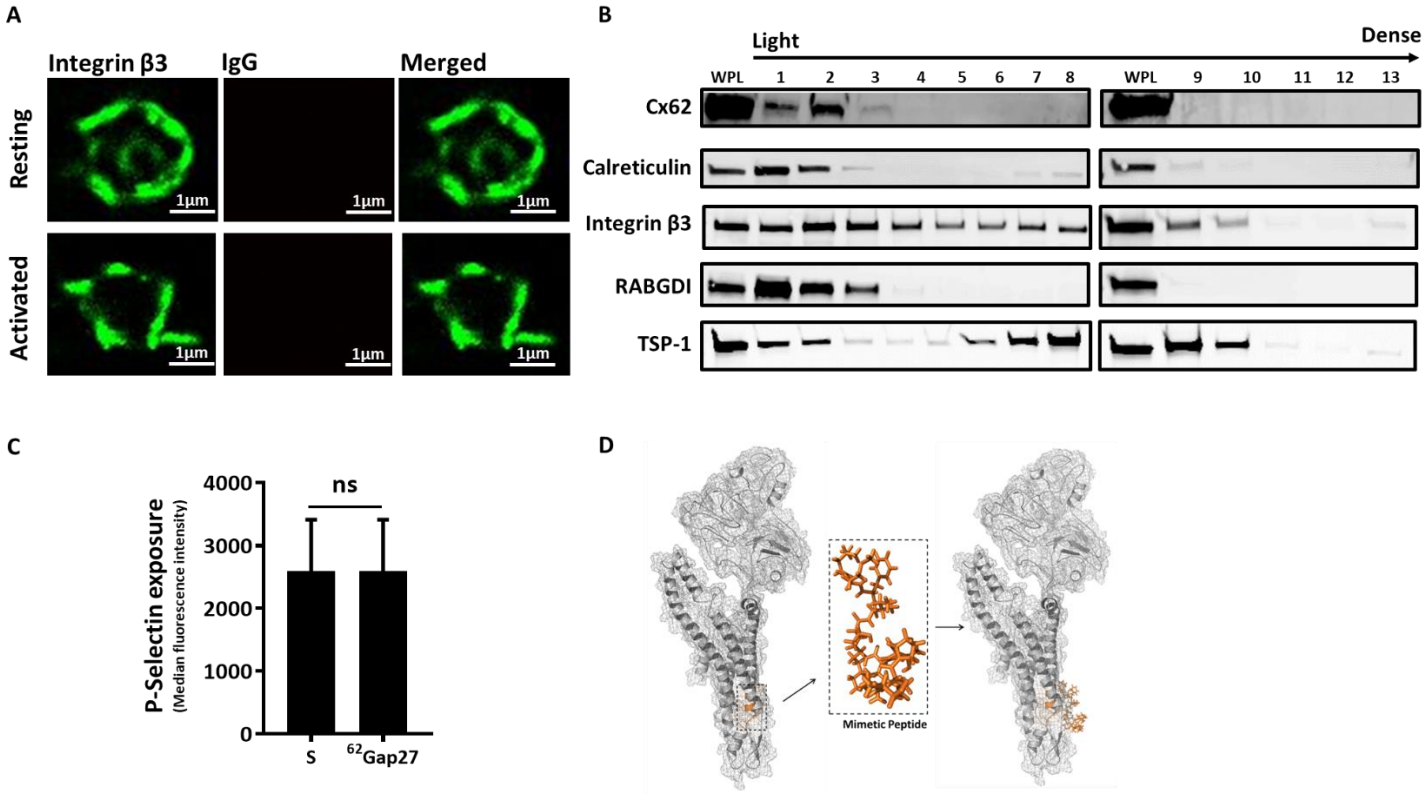
To study cell signaling, human or mouse washed platelets were prepared at a density of $4x10^8$ cells/ml under non-aggregation conditions [indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). These platelets were treated with an inhibitor of Cx function or scrambled peptide control for 5 minutes and then stimulated with platelet agonists in the aggregometer. Unstimulated or stimulated samples were lysed with 6X Laemmli sample reducing buffer and heated to 95^{o}C for 5 minutes before storing at -20^{o}C until use.

The proteins in the extracts of the platelet lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) after heating to 95°C for 10 minutes in 6x Laemmli reducing buffer. The samples and molecular weight standards were loaded onto 4%–20% acrylamide gradient gels (Bio-Rad precast gels; Bio-Rad, Watford, UK). The gels were run at a constant voltage (100 V) for 90 minutes in a Mini-PROTEAN® II apparatus (Bio-Rad, Watford, UK) with Tris-glycine buffer in the running reservoir. The separated proteins were transferred to PVDF membranes (Bio-Rad, Watford, UK) by semi-dry transfer. PVDF membranes, soaked in methanol, were

placed under the resolving gels. Four sheets of 3-mm filter paper soaked in anode buffer (300 mM Tris base and 20% [v/v] methanol; pH 10.4) were placed below the membranes and 4 sheets of 3-mm filter paper soaked in cathode buffer (25 mM Tris base and 40 mM 6-amino-n-hexanoic acid; pH 9.4) were placed above the resolving gels. The proteins were transferred from the gels to the membranes by applying a constant voltage (15V) for 2 hours.

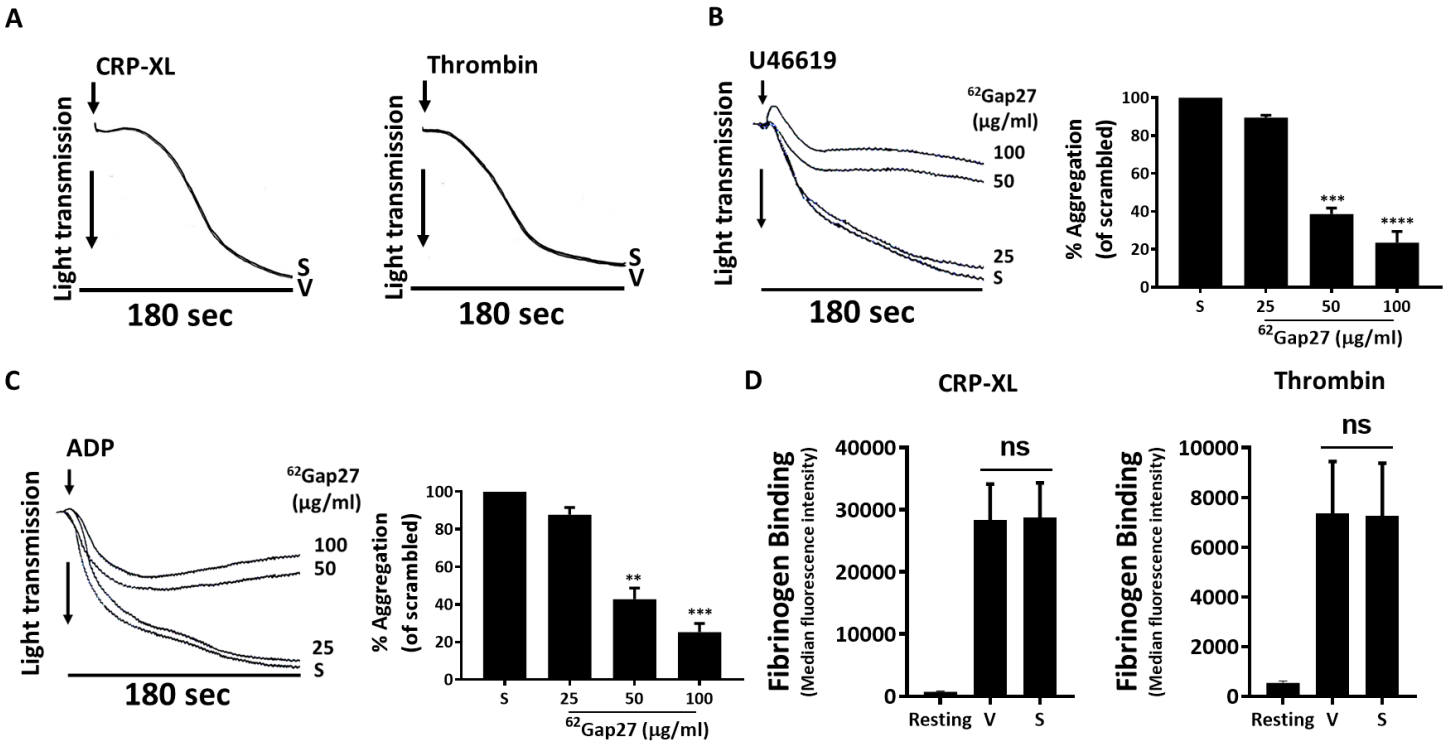
The PVDF membranes were blocked with 5% (w/v) BSA dissolved in 1% (v/v) Tris-buffered saline–TWEEN® 20 for 1 hour at room temperature. The membranes were incubated with the primary antibodies, which were diluted in 1% (v/v) TBST with 2% (w/v) BSA, overnight at 4°C. After overnight incubation, the membranes were washed with TBST (3 × 5 minutes) to remove unbound antibodies. Fluorescently labeled secondary antibodies diluted in 1% (v/v) TBST containing 2% (w/v) BSA were then applied to the membranes, which were incubated for 1 hour at room temperature in the dark. The membranes were then washed with TBST (3 × 5 minutes) and their fluorescence visualized using a Typhoon FLA 9500 fluroimager (Amersham Biosciences, Buckinghamshire, UK). Image Quant software version 8.1 (GE Healthcare) was used to quantify the fluorescence intensities of the individual bands.

Supplemental Figure 1



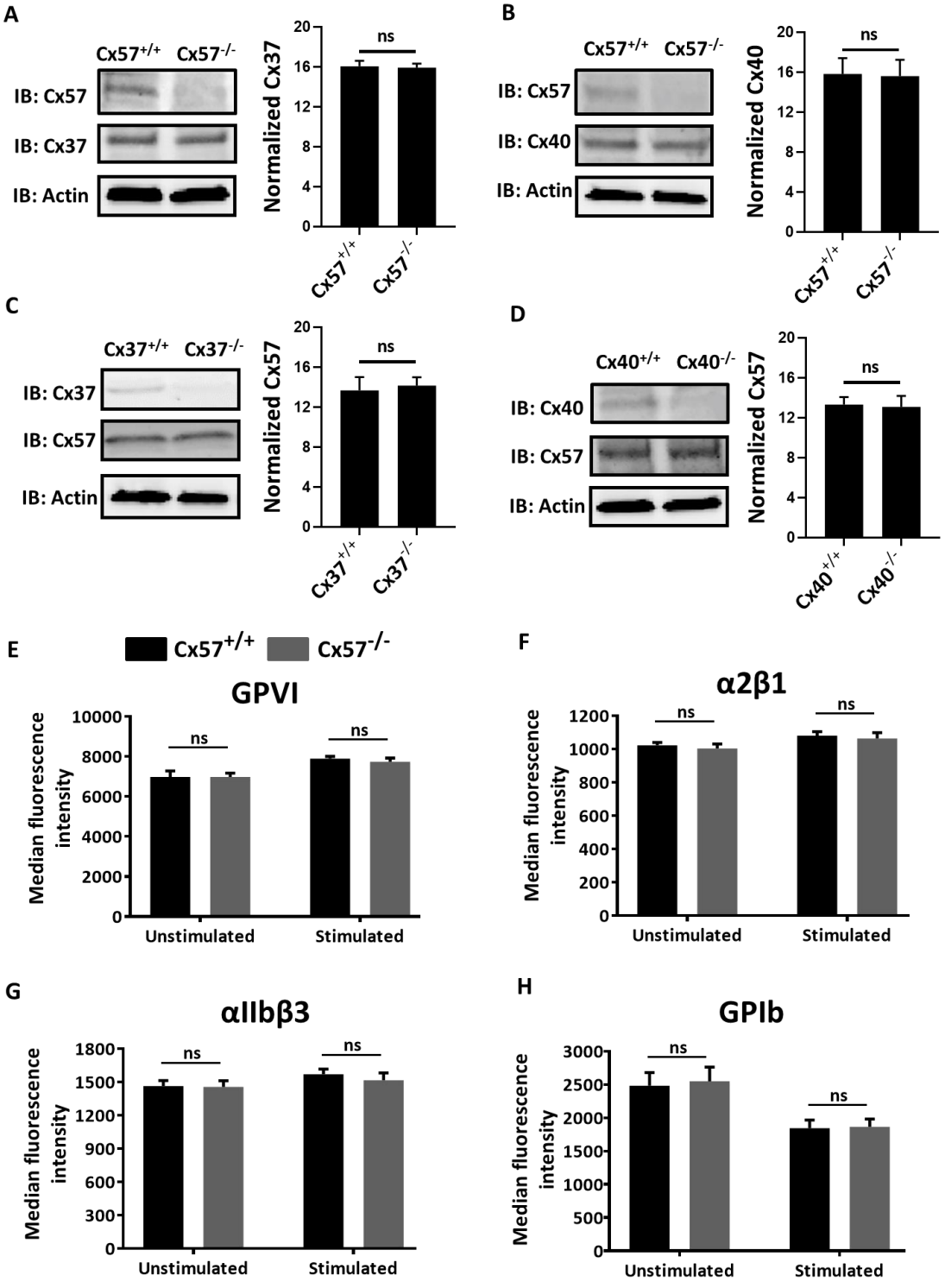
Supplemental Figure 1. Localization of Cx62 in platelets. (A) Treatment of platelets with secondary antibody alone (in the absence of anti-GJA10 primary antibody) was performed during STORM microscopy to exclude the possibility of non-specific staining **(B)** Cx62 is distributed in low-density subcellular platelet fractions. Ultracentrifugation was utilized to separate platelet homogenates on a sucrose density gradient. The fractions were separated by SDS-PAGE and immunoblotted for Cx62, β3 integrin, calreticulin, RabGDIb and TSP-1. The lower-density platelet fractions (lanes 1–6) are identified by the DTS protein calreticulin, surface marker integrin β3, and the cytosolic marker RabGDIb. The α-granule protein TSP-1 was used to identify the heavier fractions. A Typhoon™ FLA 9500 fluorimager was utilized to examine the immunoblots (GE Healthcare, UK). The results are representative of 3 individual experiments. WPL: whole platelet lysate. **(C)** Effects of ^{62}Gap27 (100 µg/ml) on thrombin (0.1 U/ml) mediated P-selectin exposure, in comparison to the scrambled peptide (S; 100 µg/ml) was evaluated using flow cytometry. **(D)** Structural representation of the target region to which the ^{62}Gap27 mimetic peptide was designed, and the putative binding site of the inhibitor on Cx62.Statistical analysis was performed using the student t-test.

Supplemental Figure 2



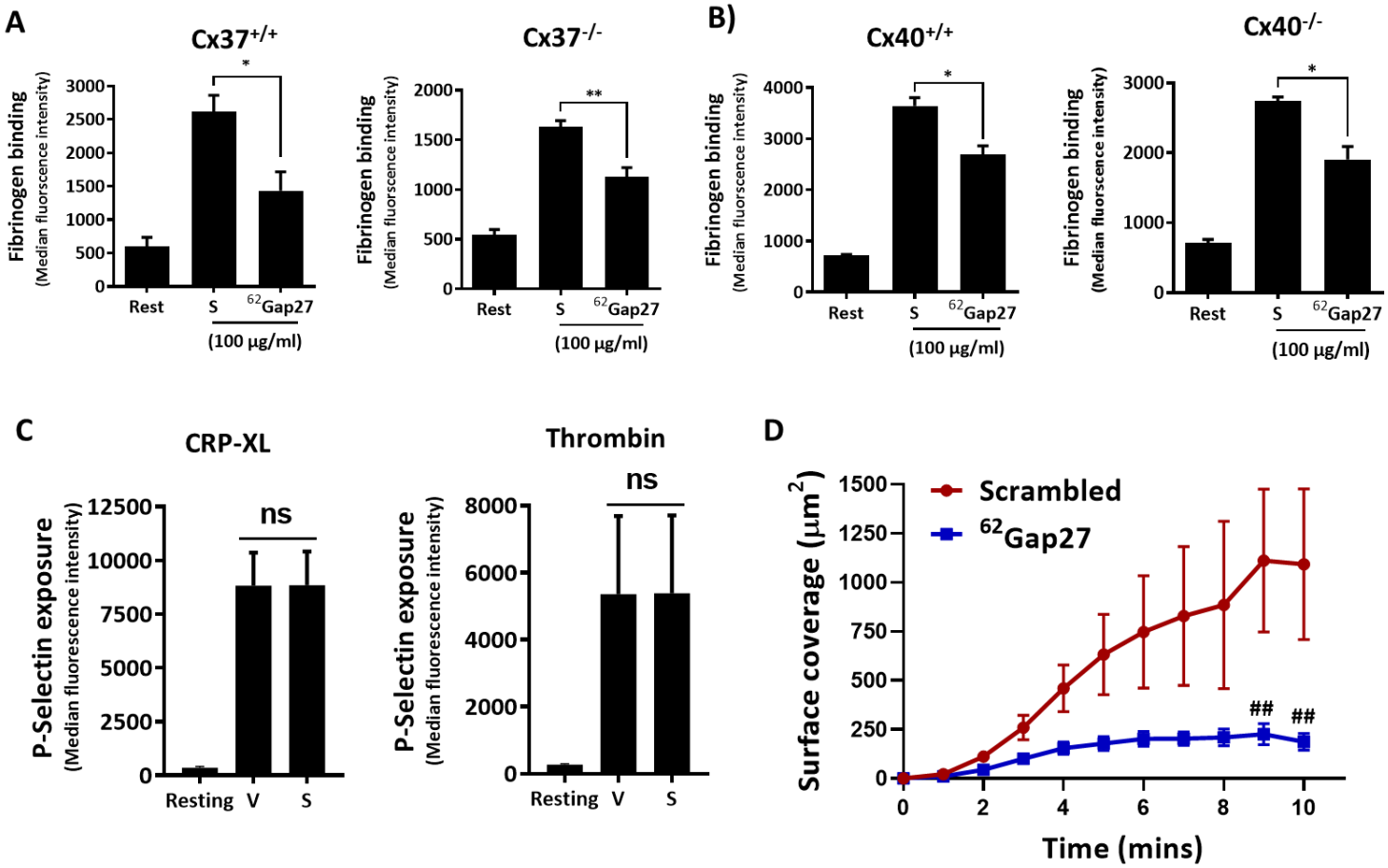
Supplemental Figure 2. ^{62}Gap27 inhibits platelet aggregation and fibrinogen binding to integrin αIIbβ3. **(A)** Washed human platelets (4×10^8 cells/mL) were treated with vehicle (V; ddH_2O) or scrambled peptide (S; 100 µg/ml) and stimulated with CRP-XL or thrombin. Aggregation was measured using optical light transmission aggregometry for 180 seconds. Representative aggregation traces are shown **(B, C)** Washed human platelets (4×10^8 cells/mL) were treated with ^{62}Gap27 or scrambled peptide (100 µg/ml) for 5 minutes prior to their stimulation with **(B)** U46619 (EC_{50}: 0.25–0.4 µM) or **(C)** ADP (EC_{50}: 5–10 µM). Aggregation was measured optical light transmission aggregometry for 180 seconds. Representative aggregation traces and quantified data shown (Scrambled-treated samples represent 100% aggregation) **(D)** Effects of the vehicle (V; ddH_2O) and scrambled peptide (S; 100 µg/ml) on CRP-XL (0.25 µg/ml) and thrombin (0.05 U/ml) mediated fibrinogen binding was evaluated in platelets (in PRP) using flow cytometry. Data represent mean ± SEM (n≥3), **P<0.01, ***P<0.001 and ****P<0.0001 was calculated by one-way ANOVA.

Supplemental Figure 3



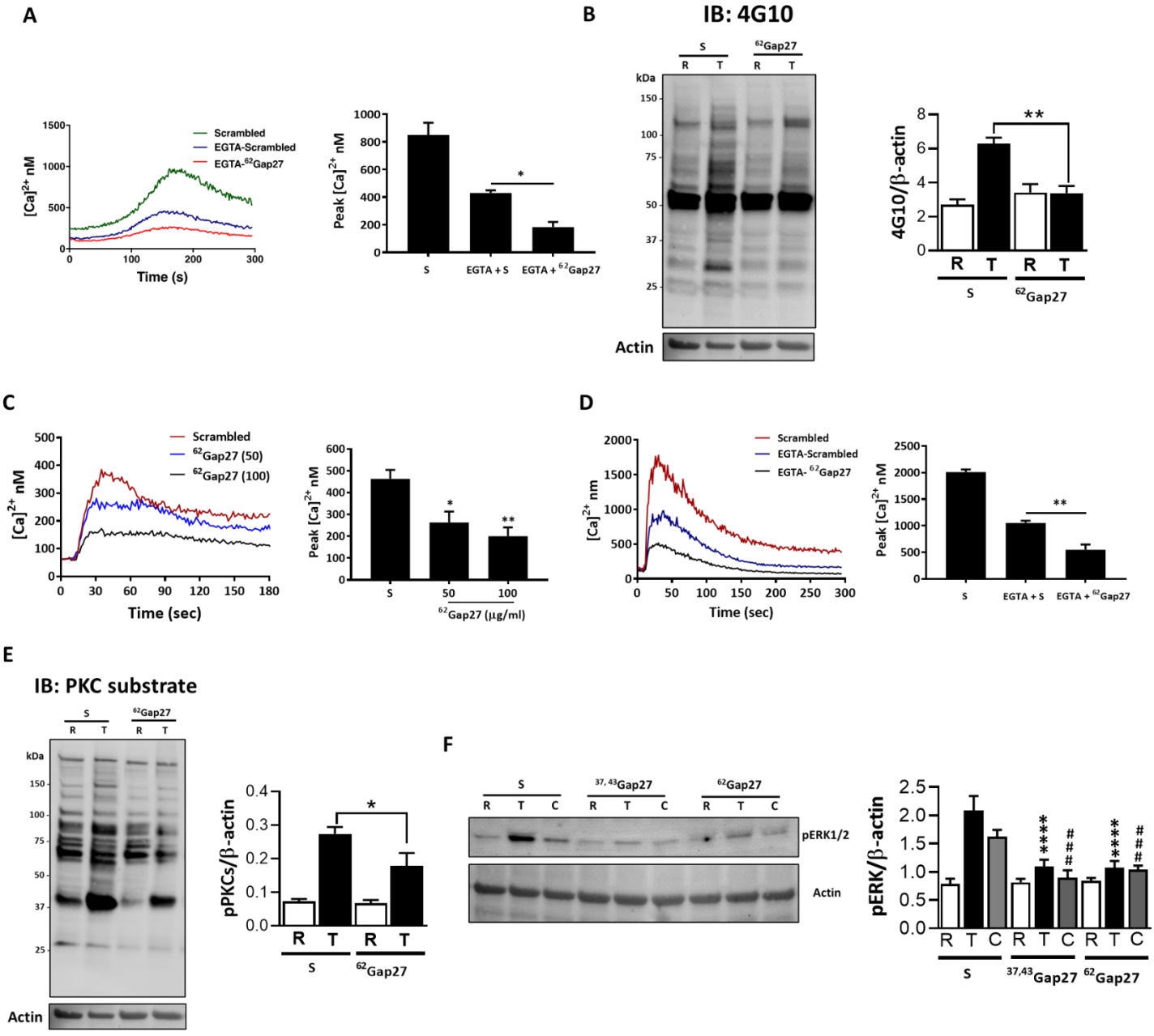
Supplemental Figure 3. Characterization of Cx57$^{-/-}$ platelets. Cx57$^{+/+}$ and Cx57$^{-/-}$ platelets were used to evaluate the expression of **(A)** Cx37 and **(B)** Cx40 by immunoblotting. **(C)** Cx37$^{+/+}$, Cx37$^{-/-}$ and **(D)** Cx40$^{+/+}$, Cx40$^{-/-}$ platelets were used to analyze the expression of Cx57 by immunoblotting. Actin was used as a loading control. Quantified data shown. The expression levels of **(E)** GPVI **(F)** α2β1, **(G)** αIIbβ3 and **(H)** GPIb were analyzed in resting and CRP-XL-activated (1 μg/ml) platelets from Cx57$^{+/+}$ and Cx57$^{-/-}$ mice by flow cytometry. The student t-test was used for statistical analysis.

Supplemental Figure 4



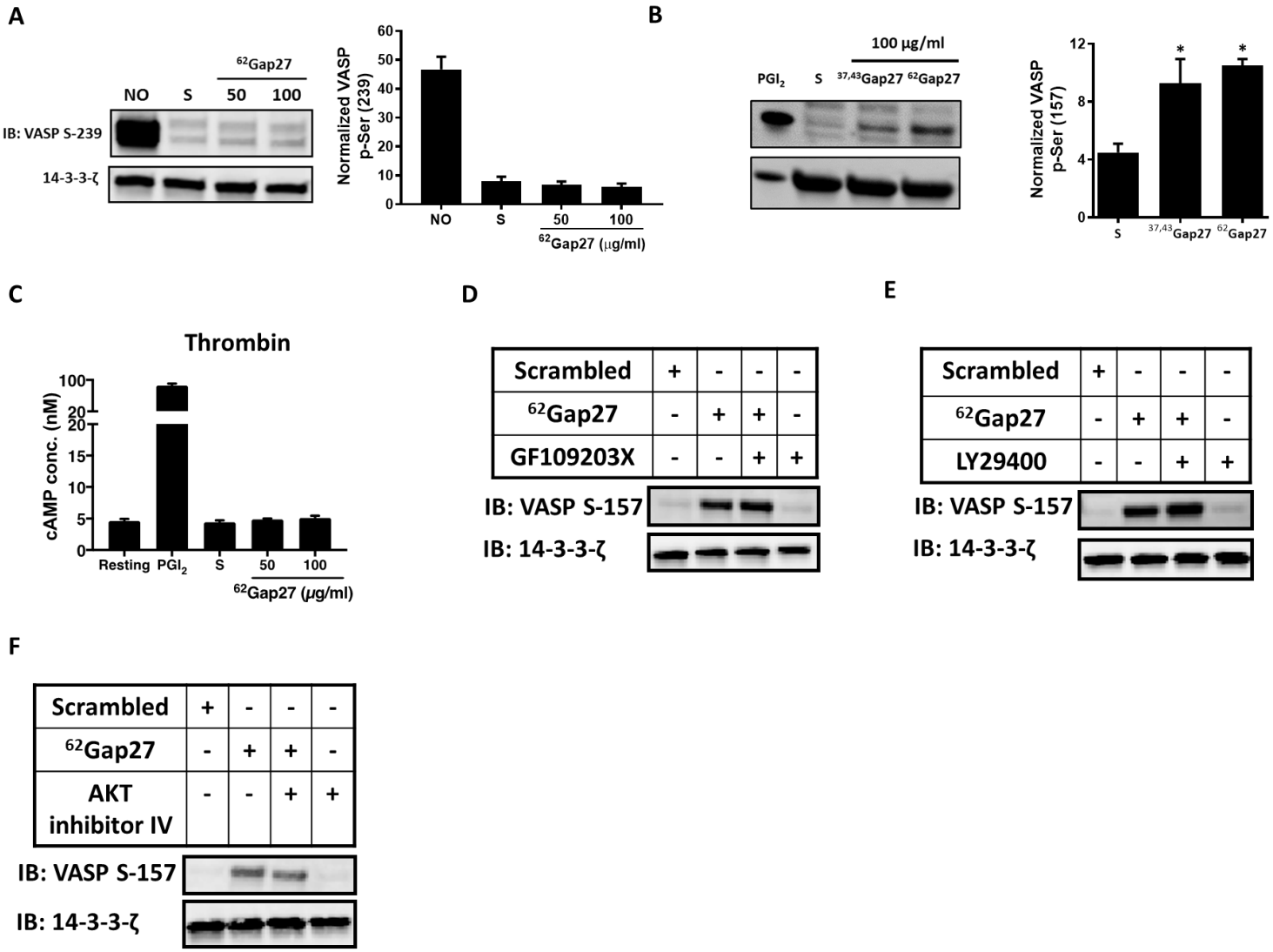
Supplemental Figure 4. Cx57 functions independently of Cx37 and Cx40 in platelets. PRP from **(A)** $Cx37^{+/+}$ and $Cx37^{-/-}$ and **(B)** $Cx40^{+/+}$ and $Cx40^{-/-}$ mice was treated with ^{62}Gap27 (100 µg/ml) or scrambled peptide (S; 100 µg/ml) for 5 minutes. Fibrinogen binding levels were evaluated after stimulation with CRP-XL (1 µg/ml). **(C)** Effects of scrambled peptide (S; 100 µg/ml) and vehicle (V: ddH_20) on CRP-XL (0.25 µg/ml) and thrombin (0.05 U/ml) mediated P-selectin exposure was evaluated in platelets (in PRP) using flow cytometry. **(D)** DiOC6-loaded human whole blood was treated with scrambled peptide or ^{62}Gap27 (100 µg/ml) for 5 min before perfusion through collagen-coated (100 µg/mL) Vena8Biochips at a shear rate of 500 s^{-1} (20 dyne/cm^2). Quantified data display surface coverage of thrombus over a period of 10 minutes. Data represent mean ± SEM (n≥3). *P<0.05 and **P<0.01 was calculated by the Student t-test. ##P<0.01 was calculated by two-way ANOVA.

Supplemental Figure 5



Supplemental Figure 5. ^{62}Gap27 inhibits thrombin-mediated signaling in human platelets. (A) Fura-2AM-loaded washed platelets (4×10^8 cells/mL) were incubated with ^{62}Gap27 (50 or 100 μg/mL) or scrambled peptide (S, 100 μg/mL) for 5 minutes in the presence of EGTA and stimulated with CRP-XL (0.5 μg/mL) for 5 minutes. Spectrofluorimetry was used to measure the release of calcium from intracellular stores. Representative traces of calcium mobilization over a period of 5 minutes and quantified data (peak calcium levels) are shown. **(B)** Representative blot and quantified data indicate the levels of phosphorylated total tyrosine in washed human platelets (4×10^8 cells/mL). Resting human platelets (R) were pre-incubated with scrambled peptide (S) or ^{62}Gap27 (100 μg/mL) for 5 min then were stimulated with thrombin (T; 0.05 U/ml). **(C)** Calcium mobilization and **(D)** release of calcium from intracellular stores was measured in Fura-2AM-loaded washed platelets (4×10^8 cells/mL) treated with scrambled peptide (S; 100 μg/mL) or ^{62}Gap27 and stimulated with thrombin (0.05 U/ml). Representative traces of calcium mobilization over a period of 5 minutes and quantified data (peak calcium levels) are shown. **(E)** Representative blot and quantified data indicate the levels of phosphorylated PKC substrate in washed human platelets (4×10^8 cells/mL). Resting human platelets (R) were pre-incubated with scrambled peptide (S) or ^{62}Gap27 (100 μg/mL) for 5 min then were stimulated with thrombin (T; 0.05 U/ml). **(F)** Representative blot and quantified data indicate the levels of pERK1/2 in washed human platelets (4×10^8 cells/mL). Resting human platelets (R) were pre-incubated for 5 minutes with Scrambled peptide (S) or ^{37,43}Gap27 or ^{62}Gap27 (100 μg/mL) and stimulated with thrombin (T; 0.05 U/ml) or CRP-XL (C; 1 μg/ml). Actin was used as a loading control. Data represent the mean ± SEM (n≥3). *P<0.05, **P<0.01, ****P<0.0001 and ####P<0.0001 was calculated by one-way ANOVA.

Supplemental Figure 6



Supplemental Figure 6. ^{62}Gap27 modulates PKA activity. (A) Resting washed human platelets (4×10^8 cells/mL) treated with scrambled peptide (S; 100 μg/mL) or ^{62}Gap27 (50 and 100 μg/mL) for 5 minutes were tested for VASP S239 phosphorylation (a marker of PKG activity). Platelets treated with PAPA-Nonoate (NO; 100 μM) for the stimulation of PKG-mediated phosphorylation were used as positive controls. **(C)** Resting washed human platelets (4×10^8 cells/mL) treated with scrambled peptide (S) or ^{37,43}Gap27 or ^{62}Gap27 (100 μg/mL) for 5 minutes were tested for VASP S157 phosphorylation. Platelets treated with PGI_2 (1 μg/mL) were used as a positive control. **(D)** Resting washed human platelets (4×10^8 cells/mL) were treated with GF109203X (10 μM), **(E)** LY29400 (100 μM) or **(F)** AKT inhibitor IV (5 μM) for 5 minutes before incubation with the scrambled peptide or ^{62}Gap27 (100 μg/mL) for 5 minutes. Samples were assayed for VASP-S157 phosphorylation. 14-3-3-ζ was detected by immunoblotting as a loading control. Representative blots are shown. Data represent the mean ± SEM (n≥3). *P<0.05 was calculated by one-way.

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