SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Platelet isolation – Healthy, drug-free human volunteers were recruited for the study under approval of the Royal Veterinary College Ethics Committee. Human blood was drawn on the day of experiment and washed platelets were prepared. Acid Citrate Dextrose (ACD: 120mM sodium citrate, 110mM glucose, 80mM citric acid, used at 1:7 vol/vol) was used as anticoagulant. Mouse blood was obtained by intracardiac puncture on carbon dioxide euthanized animals. Platelet rich plasma (PRP) was prepared by centrifugation (200g, 20min) and platelets were then isolated by centrifugation of PRP (400g, 10 min), in the presence of prostaglandin E1 (40ng/ml, Sigma, cat. no. P5515) and indomethacin (10µM, Sigma, cat. no. I7378). The pellet was resuspended to a density of $4x10^8$ platelets/ml in a modified Tyrode's-HEPES buffer (145mM NaCl, 2.9mM KCl, 10mM HEPES, 1mM MgCl2, 5mM glucose, pH 7.3).

Platelet stimulation – In order to stimulate platelet activation and aggregation, thrombin from human plasma (Sigma, cat. no. T6884) was added to washed human or mouse platelets (0.1unit/ml and 1unit/ml, respectively). A Chrono-Log 490D aggregometer was used to maintain the temperature at 37ºC and stir the platelets (700rpm) during stimulation, which lasted 5 minutes. Where indicated, platelets were treated for 30 min with 100µM 6-(2-aminoethyl)amino-5-chlorouracil (AEAC), 50µM 5-phenylthiocyclouridine (PTAU), or 50µM 8-aminoguanosine (AG) (Carbosynth Limited, cat. no. NA004479).

Mass spectrometry sample preparation – Platelet supernatants were collected after stimulation and intact platelets were removed by rapid centrifugation (20 seconds, 1000g). Where indicated, the supernatants from platelet suspensions were filtered using Vivaspin

15R Hydrosart (2,000 MWCO) columns to eliminate molecules with sizes above 2kDa (Sartorius Stedim Biotech GmbH, cat. no. VS02H92). For platelet extract preparation, intact platelets were separated from their supernatant by rapid centrifugation (20 seconds, 1000g) and cells permeabilised by addition of extraction buffer (methanol:water 70%:30%) with 10 freeze-thaw-vortex cycles. Cell debris was eliminated by centrifugation (2500g, 10 min).

Detection of dRP by gas chromatography-mass spectrometry (GC-MS) – Platelet extracts and supernatants were lyophilised for 12 hours at 25°C (HETO VR MAXI vacuum centrifuge attached to a Thermo Savant RVT 4104 refrigerated vapour trap; Thermo Life Sciences, Basingstoke, UK). All samples were chemically derivatised to induce higher volatility by addition of 50µl of 20mg/m^1 O-methoxylamine hydrochloride in pyridine, vortexed, and incubated at 40°C for 75 minutes in a dri-block heater. 50µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was then added and after vortexing the extracts were incubated at 40°C for a further 75 minutes. On completion, 20µl of retention index marker solution was added (0.3 mg/ml docosane, nonadecane, decane, dodecane and pentadecane in pyridine) prior to centrifugation at 15,800*g* for 15 minutes. The resulting supernatant (90µl) was transferred to GC-MS vials for analysis.

Samples were analysed on a 6890 gas chromatograph (Agilent, Cheadle, UK) coupled to a LECO Pegasus III mass spectrometer (LECO, Stockport, UK). A 30mm x 0.25mm i.d. x 0.25µm column thickness DB17 GC column (Supelco, Gillingham, UK) was employed with a constant flow rate of 1.0 ml/min. Samples were injected into a split injector (1:5) at a temperature of 230°C. Chromatographic separation was performed with a temperature program; 70°C – 4 minutes, temperature ramp of 20°C.min-1 to 290°C, hold at 290°C for 5 minutes. The transfer line and source temperatures were 230 and 250°C, respectively. Data were

acquired in electron-impact mode in the m/z range 50-600. Raw data were processed using ChromaTof software v2.12. All samples were randomly analysed in a single analytical batch. An authentic chemical standard of deoxyribose-1-phosphate (dRP) was prepared and analysed under identical conditions to provide accurate identification of dRP in all samples applying a retention index (+/- 10) and mass spectral match (>700).

Detection of dRP by direct infusion mass spectrometry (DIMS) – Platelet extracts were analysed by direct infusion into an electrospray hybrid LTQ-Orbitrap mass spectrometer operating in negative ion mode (ThermoFisher Scientific, Bremen, Germany). Samples were infused for one minute at a flow rate of 5µl/min with data acquired in the mass range 100-1000Da. The mass spectrometer was tuned for detection of dRP and operated with a mass resolution of 100 000 (FWHM at mass 400Da). All scans were averaged and the ion count was employed for semi-quantification of the dRP concentration. Accurate method descriptions for GC-MS and DIMS have been published previously [1].

Endothelial cell culture – Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords collected under ethical approval, as previously described [2]. Cells were grown in complete growth medium (medium M199 supplemented with 20% (v/v) FCS (FCS), 0.02mg/ml Endothelial Cell Growth Supplement (ECGS), 4mM glutamine, 100units/ml penicillin, 100units/ml streptomycin, and 20mM $NaHCO_3$) and cultured at 37 °C in a 5%CO_2, 95% air atmosphere in 25-mm^2 tissue culture flasks (BD Biosciences) precoated with 1% (w/v) gelatin. Cells were passaged into 75-mm^2 tissue culture flasks and cultured to confluence in full medium containing endothelial cell growth supplement (20µg/ml). Human microvascular endothelial cells (HMVECs) from lung were purchased from Lonza (cat. no. cc-2527) and cultured at 37°C in a 5% CO_2, 95% air atmosphere in 25-mm^2 tissue culture flasks

(BD Biosciences) without pre-coating using complete EGM-2-MV medium (Lonza, cat. no. cc-3202) with 2% (v/v) FCS.

Endothelial cell transmigration assay – 10^5 HUVECs/well were cultured in minimal growth medium (medium M199/10% foetal calf serum) diluted 1:1 with Tyrode's-HEPES buffer in 24well-transmigration inserts of Boyden's microchambers (pore size 8µm, Corning Life Sciences, cat. no. 3422), precoated with 1% (w/v) gelatin. The lower chamber contained minimal growth medium plus platelet releasate or Tyrode's HEPES buffer (ratio 1:1). Where indicated, 200µM dRP (Sigma, cat. no. D6539) was added to the lower chamber. Transmigration was quantified 5 hours after assembly of the chemotaxis microchamber by hematoxylin staining, physical removal of the cells on the inner side of the insert and phase-contrast microscopy of the bottom side of the membrane (Leica DM IRB microscope, HiRes Camera DC500, N Plan objective 40X/0.55 Ph2). Transmigration experiments were quantified by analysing 8 images/well per condition. The transmigration coefficient was calculated as the ratio between pores occupied by transmigrated cells and unoccupied pores.

Endothelial wound repair assay – HUVECs were cultured in 12 well-plates to confluence in complete culture medium (medium M199/20% foetal calf serum). A wound of uniform width was created in the monolayers by scratching with a sterile microtip, cell debris was removed by gentle washing with phosphate buffer saline (PBS: 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4), and minimal growth medium plus platelet releasate or Tyrode's HEPES buffer (ratio 2:1) was added. Where indicated, 200µM dRP (Sigma, cat. no. D6539) was added to the well. Cells were incubated for 0, 12, and 24 hours at 37°C in a 5% CO_2, 95% air atmosphere. Scratch wound closure was monitored by phase contrast

microscopy (Zeiss Axiovert 40C connected to a Canon Powershot A640 camera, A Plan objective 10X/0.25 Ph1). Wound size was measured using ImageJ 1.40g software (Wayne Rasband, National Institute of Health, US) and the surface area of the wound was expressed in pixels.

Platelet protein extraction and immunoblotting – Platelets and HUVECs treated with protein-free platelet supernatants were lysed with radioimmuno-precipitation assay buffer (RIPA) (1%Triton X-100, 1%sodium deoxycholate, 0.1%SDS, 150 mM NaCl, 5mM EDTA, 50mM Tris pH 7.4) containing protease inhibitors at the concentration advised by the supplier (Complete mini, Roche). Proteins were quantified using the Bradford assay and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10-12% gels. Recombinant thymidine phosphorylase (R&D Systems, cat. no 229-PE), T7-tagged uridine phosphorylase (Genway Biotech, cat. no. 10-288-22042F), and GST-tagged purine nucleoside phosphorylase (Abnova Corporation, cat. no. H00004860-P01) were used as positive controls (20ng each). Proteins were transferred to ProTran nitrocellulose transfer membrane (Whatman) and blocked with 5% w/v dry milk in TBS-T (10mM Tris pH 7.5, 150mM NaCl, 0.1% v/v Tween20). Primary antibodies used for immunostaining were: 1) for TP, anti-human PD-ECGF antibody (R&D Systems, cat. no. AF1143, 650ng/ml); 2) for UP, anti-UPP1 (Abnova, cat. no. H0007378-M01, 1µg/ml); 3) for human PNP, anti-NP (Atlas Antibodies, HPA001625, 250ng/ml); 4) for mouse PNP, anti-PNP E-12 (Santa Cruz Biotechnology, cat. no. sc-47334, 1µg/ml); 5) for actin, monoclonal anti-actin AC-40 (Sigma-Aldrich, cat. no. A3853, 1.5µg/ml); 6) for integrin β3, anti-human integrin β3/CD61 antibody (R&D Systems, cat. no. AF2266, 200ng/ml); 7) for integrin αv, anti-integrin αv antibody (BD Transduction Laboratories, cat. no. 611013, 0.1% vol/vol); 8) for integrin α5, anti-integrin α5

antibody (BD Transduction Laboratories, cat. no. 610634, 500ng/ml); 9) for integrin β1, anti-integrin β1 (Cell Signaling Technology, cat. no. #4706, 0.1% vol/vol); 10) for HO-1, anti-HO-1 (Hsp 32) polyclonal antibody (Stressgen, cat. no. SPA-896, 1μg/ml). Depending on the primary antibody, the secondary horseradish peroxidise (HRP)-linked antibodies used were: 1) anti-rabbit IgG, HRP-linked whole Ab (GE Healthcare, cat. no. NA934); 2) anti-mouse IgG, HRP-linked whole antibody (GE Healthcare, cat. no. NXA931); 3) donkey anti-goat IgG, HRP-linked (Santa Cruz Biotechnology, cat. no. sc-2020).

ROS production measurements – HUVECs were cultured on 22mm glass coverslips to 50% confluence. Coverslips were mounted on live cell imaging chambers immediately before microscopy. Cells were imaged while bathing in a modified HBSS solution containing 156mM NaCl, 3mM KCl, 2mM $MgSO_4$, 1.25mM KH_2PO_4, 2mM $CaCl_2$, 10mM glucose, and 10mM HEPES, pH adjusted to 7.35 with NaOH. Dihydroethidium (DHE; 10μM) was added immediately before the start of an experiment and remained in the solution for the duration. Images were obtained using a Zeiss 510 LSM confocal microscope equipped with a 40X oil-immersion lens. Excitation was provided by the 543 line of the helium-neon laser line and emitted fluorescence collected >560 nm. In all experiments using DHE, fluorescence intensity data were collected every 10 sec for all cells in a field view. The rate of DHE oxidation following HUVEC challenge with dRP (100μM-400μM) or glucose (100μM-1mM) was measured as fluorescence increase (relative unit/second) and compared with the rate of DHE oxidation in cells treated with vehicle.

Chick chorioallantoic membrane assay – To assess the effects of platelet supernatants on angiogenesis *in vivo* we used the chick chorioallantoic membrane CAM assay. Fertilised white Leghorn eggs were incubated at 37°C in a humidified incubator and windowed. On

day 7 of development, sterile filters soaked with vehicle, VEGF (100ng/filter) or protein-free platelet supernatants (2kDa cut off, 10^9 platelets/ml) were applied to relatively avascular regions of the CAM. CAMs were fixed (4% paraformaldehyde in PBS) *in ovo* on day 9 and photographed by phase contrast imaging. Vascularisation of the regions in contact with each filter was quantified as angiogenic index by measuring the surface area occupied by the vascular network using Leica QWin Lite software (15 eggs/treatment).

Data analysis and presentation – Data were analysed and presented using Prism software (version 5.00, GraphPad Software, La Jolla, US). The results were analysed by either t-test (for comparisons of two groups), or one-way ANOVA with Bonferroni post-test (for multiple comparisons), or two-way ANOVA with Bonferroni post-test (for multiple comparisons of time courses). $P < 0.05$ was considered significant.

REFERENCES

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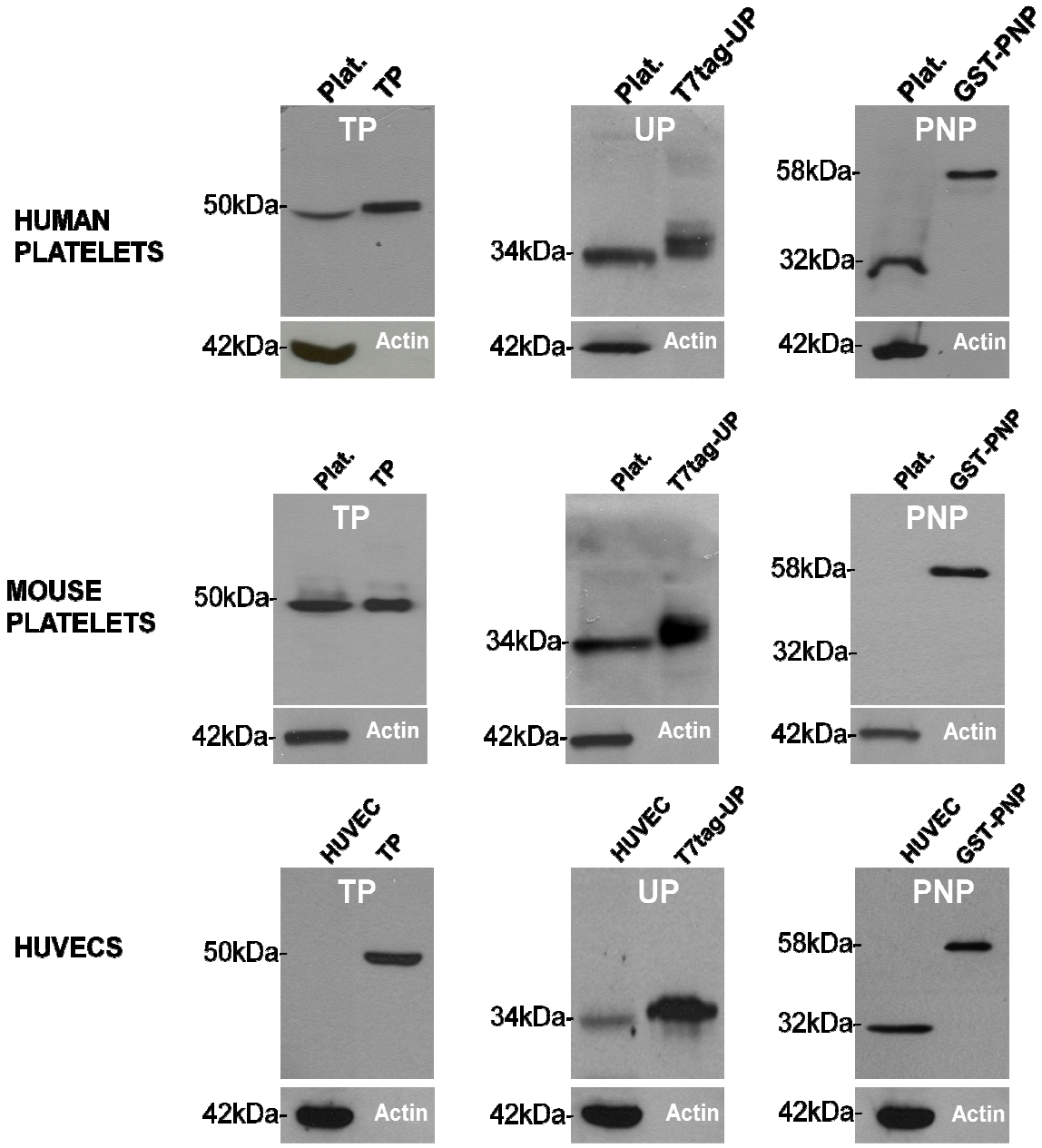
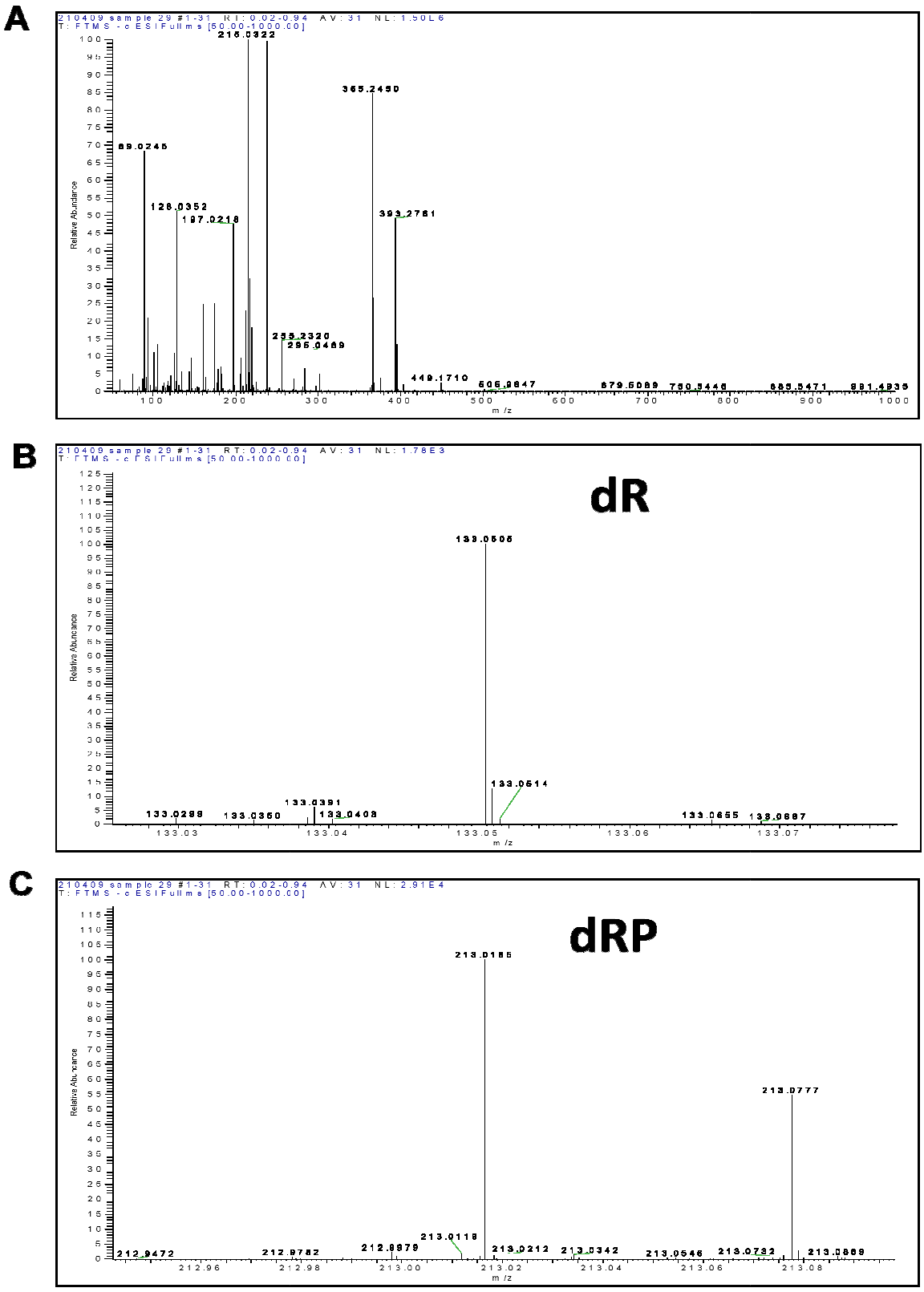
2. Syeda F, Grosjean J, Houliston RA, Keogh RJ, Carter TD, Paleolog E, Wheeler-Jones CP. Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. *J Biol Chem.* 2006;281:11792-11804.

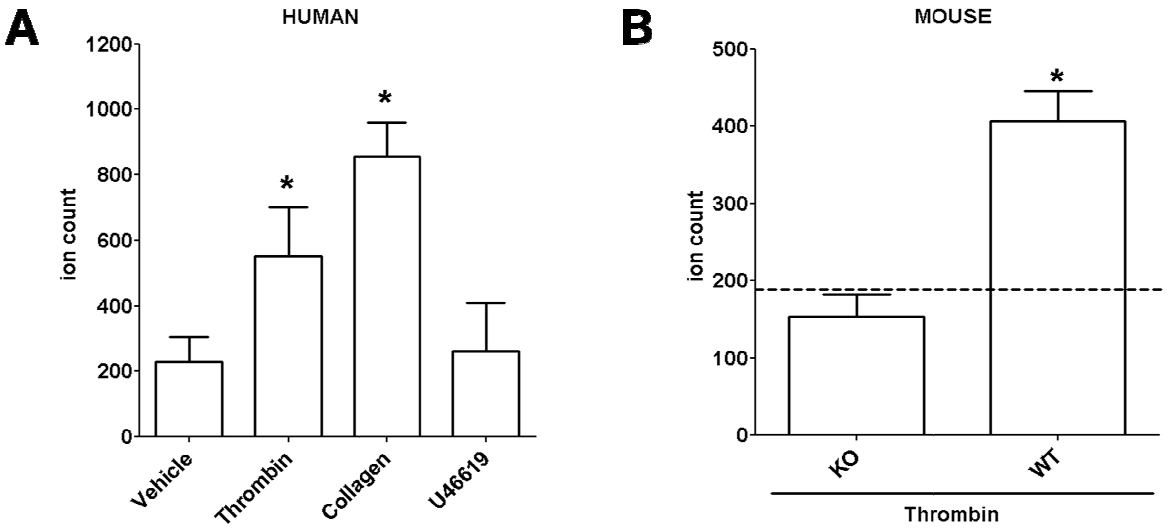
SUPPLEMENTARY FIGURES



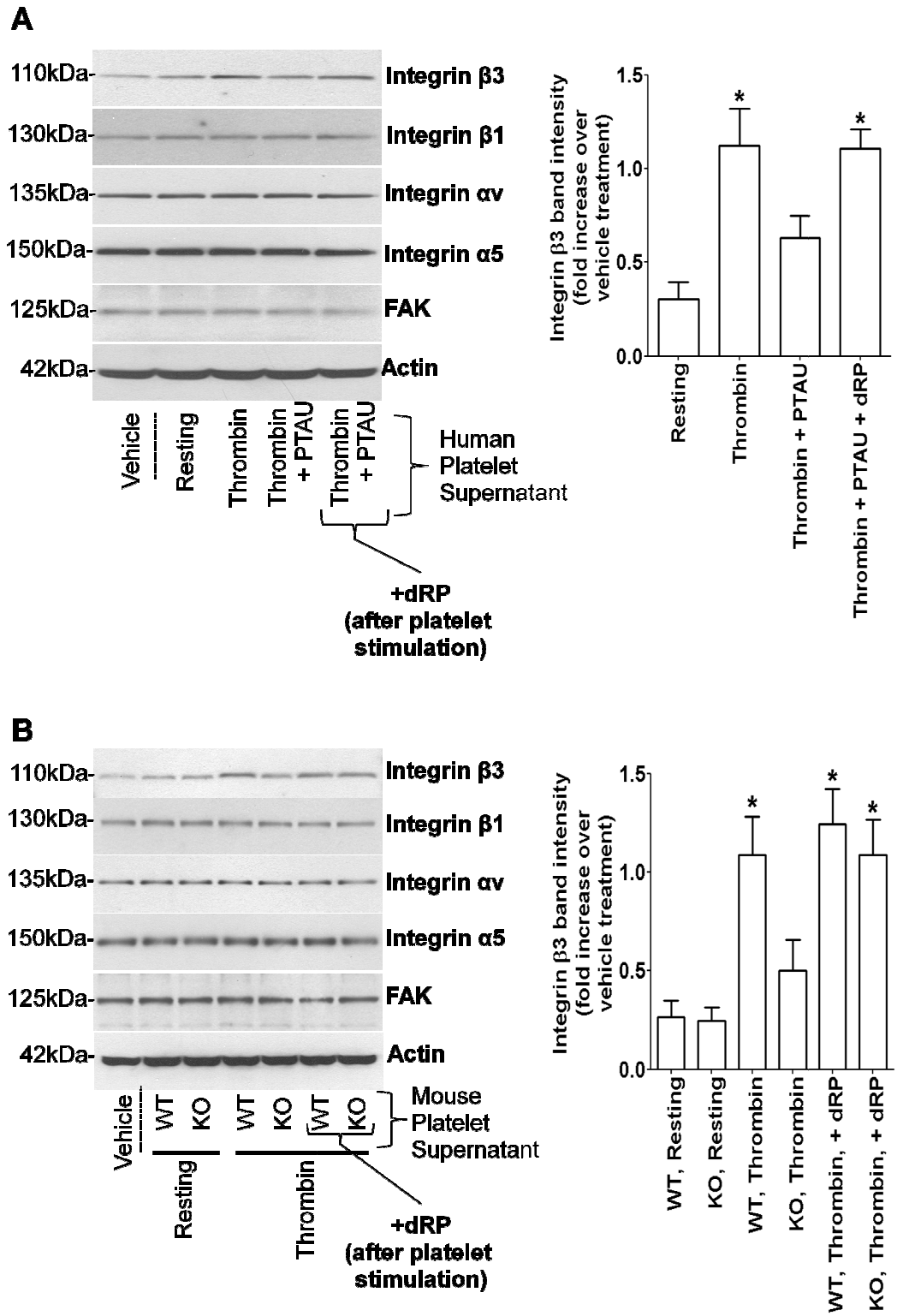
Supplementary figure I. Expression of nucleoside phosphorylases in human platelets, mouse platelets, and HUVECs as detected by immunoblotting. Protein extracts (20µg/lane) were loaded and recombinant TP, T7-tagged UP and GST-tagged PNP were used as positive controls (20µg/lane). The T7 tag has a molecular weight of 1kDa, and the GST tag has a molecular weight of 26kDa, which explains the different molecular weight of native target proteins and the positive control. Actin expression was monitored as a loading control. The data shown are representative of 3 independent experiments.



Supplementary figure II. Mass spectrum acquired by DIMS for resting platelet extracts (A). The specific ionisation spectra for dR (B) and dRP (C) are also shown. Samples were infused for one minute at a flow rate of 5µl/min with data acquired in the mass range 100-1000Da.



Supplementary figure III. DIMS analysis of dRP release by human and mouse platelets. (A) Human platelets (400µl suspension) were stimulated with 0.1unit/ml thrombin, 10µg/ml collagen or 100nM U46619 and their supernatants analysed by DIMS for the presence of dRP. Means ± SEM are reported and compared by one-way ANOVA with Bonferroni post-test (*= $p<0.01$, compared to vehicle treatment). (B) Mouse platelets (400µl suspension) from wild type (WT) or $TP^{-/-}/UP^{-/-}$ animals were stimulated with 1unit/ml thrombin and supernatants analysed by DIMS for the presence of dRP. The average level of dRP in supernatants from resting mouse platelets is indicated by the dashed line. Means ± SEM are given and compared by t-test (*= $p<0.01$).



Supplementary figure IV. Platelet release of dRP induces integrin β3 upregulation in HUVECs. HUVECs were cultured for 6 hours in the presence of vehicle solution or protein-free supernatants from (A) human platelets treated with vehicle (resting), thrombin (0.1unit/ml), or 50μM PTAU plus thrombin (0.1unit/ml), or (B) mouse platelets from wild

type (WT) or $TP^{-/-}/UP^{-/-}$ animals treated with vehicle (resting) or stimulated with 1 unit/ml thrombin. Where indicated, 200µM dRP was added to the supernatants immediately before incubation with HUVEC. HUVECs were lysed and integrin β3 expression was assessed by immunoblotting. Blots from three independent experiments were analysed by densitometry using ImageJ software. Results are expressed as fold-increase over vehicle-incubated HUVECs and analysed by one-way ANOVA or t-test (*= $p<0.05$, compared to resting platelets). The blots were also reprobed for integrin β1, integrin αv, integrin α5, FAK, and actin.