



## Article

# Opposing Roles for the $\alpha$ Isoform of the Catalytic Subunit of Protein Phosphatase 1 in Inside–Out and Outside–In Integrin Signaling in Murine Platelets

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**Abstract:** Platelet activation during hemostasis and thrombosis is facilitated by agonist-induced inside–out and integrin  $\alpha_{IIb}\beta_3$ -initiated outside–in signaling via protein kinases and phosphatases. Pharmacological inhibitor studies suggest that the serine/threonine protein phosphatase 1 (PP1) promotes platelet activation. However, since phosphatase inhibitors block all the isoforms of the catalytic subunit of PP1 (PP1c), the role of specific PP1c isoform in platelet signaling remains unclear. Here, we employed a platelet-specific  $PP1\alpha^{-/-}$  mice to explore the contribution of a major PP1 isoform in platelet functions. Loss of  $PP1\alpha$  moderately decreased activation of integrin  $\alpha_{IIb}\beta_3$ , binding of soluble fibrinogen, and aggregation to low-dose thrombin, ADP, and collagen. In contrast,  $PP1\alpha^{-/-}$  platelets displayed increased adhesion to immobilized fibrinogen, fibrin clot retraction, and thrombus formation on immobilized collagen. Mechanistically, post-fibrinogen engagement potentiated p38 mitogen-activated protein kinase (MAPK) activation in  $PP1\alpha^{-/-}$  platelets and the p38 inhibitor blocked the increased integrin-mediated outside–in signaling function. Tail bleeding time and light-dye injury-induced microvascular thrombosis in the cremaster venules and arterioles were not altered in  $PP1\alpha^{-/-}$  mice. Thus,  $PP1\alpha$  displays pleiotropic signaling in platelets as it amplifies agonist-induced signaling and attenuates integrin-mediated signaling with no impact on hemostasis and thrombosis.

**Keywords:** platelets; protein phosphatase 1 alpha; p38 mitogen-activated protein kinase; fibrinogen



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## 1. Introduction

At the site of vascular injury or ruptured atherosclerotic plaques, exposed extracellular matrix proteins such as collagen and the generated agonist such as thrombin, thromboxane and adenosine diphosphate (ADP) engage with their respective platelet receptors to initiate agonist-induced inside–out signaling [1]. This signaling triggers major platelet integrin  $\alpha_{IIb}\beta_3$  to bind soluble fibrinogen and support platelet aggregation. Fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$  generates a second set of signals referred to as outside–in signaling, which controls the platelet cytoskeletal remodeling and functions such as adhesion and fibrin clot retraction [2,3]. Both inside–out and outside–in signaling contribute to platelet activation and its ability to form stable platelet thrombi.

Protein kinases have held the center stage in our understanding of the agonist and integrin-mediated signaling pathways. As models of signal transduction have been refined to include protein phosphorylation and dephosphorylation, the contribution of the protein phosphatases to platelet signaling and function has garnered fresh attention [4]. Indeed, in quantitative phospho-proteome studies that examined basal, and ADP-stimulated platelets, temporal phosphorylation and dephosphorylation patterns, predominantly in the serine and threonine residues of proteins that engaged the signaling networks governing platelet function were observed [5,6].

Protein phosphatase 1 holoenzyme is among the major serine/threonine phosphatases in eukaryotes and contains a catalytic (PP1c) and regulatory subunit (PP1r) [7]. PP1c has three isoforms PP1 $\alpha$ , PP1 $\beta$ , and PP1 $\gamma$ , all of which are expressed in platelets with the copy number of PP1 $\alpha$  being the highest [8]. Historically, the role of PP1 in platelet biology has been examined using pharmacological agents such as calyculin A and okadaic acid. These agents impaired human platelet functions [9] including aggregation [10,11], secretion [12], the adhesion/spreading on immobilized fibrinogen [13], and clot retraction [14,15] suggesting that PP1 promotes agonist-induced inside-out and integrin-initiated outside-in signaling in platelets. However, since these inhibitors cannot discriminate the different isoforms of PP1c, pharmacological agents are inadequate to inform the isoform-specific role of PP1c in platelet signaling. We have previously generated a platelet-specific PP1 $\alpha$  null mice model and observed decreased thrombin-induced inside-out platelet signaling [16]. Using this genetic model, we explored the impact of PP1 $\alpha$  on inside-out and outside signaling functions in platelets. We report that PP1 $\alpha$  positively regulates agonist-induced inside-out signaling responses such as integrin  $\alpha_{IIb}\beta_3$  activation, soluble fibrinogen binding, and aggregation in response to low doses of agonist. Concurrently, PP1 $\alpha$  negatively regulates integrin outside-in signaling responses such as platelet adhesion and fibrin clot retraction. Consistent with an opposing role for PP1 $\alpha$  in agonist-induced inside-out and integrin-induced outside signaling, loss of PP1 $\alpha$  did not impact in vivo hemostasis and thrombosis.

## 2. Materials and Methods

### 2.1. Materials

The reagents used in this study were mostly obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD41( $\alpha_{IIb}$ ) and anti-CD62 (P-selectin) antibodies tagged to fluorescein isothiocyanate (FITC) were obtained from BD Bioscience (San Jose, CA, USA). Phycoerythrin-tagged antibodies that recognize the active form of murine  $\alpha_{IIb}\beta_3$  (JON/A) were purchased from Emfret Analytics (Eibelstadt, Germany). Thrombin was brought from Hematologic Technologies Inc. (Essex Junction, VT, USA). ADP and collagen were purchased from Helena Laboratories (Beaumont, TX, USA). Thromboxane analog (U44619) and water-soluble p38 SB203580 hydrochloride were ordered from Bio-Techne Corporation (Minneapolis, MN, USA). Fibrinogen and Alexa 488-tagged fibrinogen were purchased from Enzyme Research Laboratories Inc. (South Bend, IN, USA) and Invitrogen, (Carlsbad, CA, USA), respectively. Anti-phospho-p38 and p38 antibodies were obtained from Cell Signaling (Boston, MA, USA).

### 2.2. Mice

Approval from Baylor College of Medicine IACUC (Institutional Animal Care and Use Committee) was sought prior to initiating all animal studies. Mice lacking PP1 $\alpha$  conditionally in platelets were generated as we described previously [16]. Briefly, *ppp1ca-loxP* flox (*fl*) mice [17] were mated with megakaryocyte/platelet-specific *Pf4 Cre* knock-in mice (Jackson Laboratory) [18] to generate PP1 $\alpha$  knock-out mice. For most studies, 8–16-week-old male and female littermates (PP1 $\alpha$  *fl/fl* and *Cre* positive, referred to as PP1 $\alpha$ <sup>-/-</sup>) and PP1 $\alpha$  *fl/fl* and *Cre* negative, referred to as wild type (WT) were employed. The only exception is the use of male mice in intravital microscopy studies on cremaster muscles.

### 2.3. Mice Platelet Preparation

Mice were anesthetized with isoflurane and blood was collected into the anticoagulant acid-citric acid-dextrose (ACD) in a 1:10 (*v/v*) ratio from the inferior vena cava, as we described [19]. Collected blood was then diluted in a 1:1 ratio with Dulbecco's phosphate buffered saline (D-PBS) which contains one part of ACD to nine parts of D-PBS. The first centrifugation at  $68 \times g$  for 10 min provided platelet-rich plasma (PRP). The second centrifugation of PRP at  $754 \times g$  for 10 min yielded a platelet pellet that was subsequently washed with D-PBS. Platelets were then gently resuspended in D-PBS containing 0.005 U/mL apyrase. Coulter counter (Beckman–Coulter (Z1), Miami, FL, USA) was used to count platelets, which were adjusted to the final concentration of  $2.5 \times 10^8$ /mL. All studies with washed platelets were initiated after resting for one hour.

### 2.4. Flow Cytometry Studies for Integrin Activation, Binding of Soluble Fibrinogen, Granule Secretion, and Platelet Aggregation

For these studies, platelets were further diluted to  $2.5 \times 10^7$ /mL with Tyrode's buffer containing  $\text{CaCl}_2$  (1.8 mM) and  $\text{MgCl}_2$  (0.49 mM). Agonist including thrombin (0.02 U/mL, 0.05 U/mL), ADP (2.5  $\mu\text{M}$ , 10  $\mu\text{M}$ ), collagen (0.5, 0.75, 1, 1.5 and 2.5  $\mu\text{g}/\text{mL}$ ) and U46619 (0.25, 0.50  $\mu\text{M}$ ) was used to stimulate platelets. Depending on the assays to be studied, we employed JON/A tagged PE antibody, Alexa 488-tagged soluble fibrinogen, FITC conjugated CD62P antibody and CD41 tagged PE antibody and analyzed platelets in a flow cytometer (EPICS-XL, Beckman Coulter, Miami, FL, USA). FITC or PE-tagged isotype antibodies served as control. To study agonist-induced release of ATP, luciferin/luciferase reagent was added to agonist-challenged washed platelet and evaluated in a lumi-aggregometer (Chrono-Log Corp. Havertown, PA, USA) [20]. To study aggregation, washed platelets (225  $\mu\text{L}$  of  $2.5 \times 10^8$ /mL) were stimulated with different concentrations of agonist under stirring conditions (1200 rpm) in an eight-channel Bio/Data PAP-8 aggregometer (Biodata Corporation, Horsham, PA, USA). Final aggregation after 10 min of agonist stimulation was recorded.

### 2.5. p38 MAPK Immunoblotting Studies

Platelets were left suspended on BSA (5 mg/mL) substrate for 30 min or allowed to adhere to immobilized fibrinogen (100  $\mu\text{g}/\text{mL}$ ) for 15 and 30 min. Following washing, platelet lysate was separated on 10–12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), transferred to nitrocellulose membrane, and then immunoblotted with antibodies to phospho-Thr180, Tyr182 (phospho p38; surrogate marker of p38 activation) and p38 MAPK. HRP-conjugated secondary antibodies were used later, and the membrane was developed using chemiluminescence (ECL). Densitometry of p38 signals was performed using Image J (1.53k/Java/8.0\_172 964 bits).

### 2.6. Platelet Adhesion, Clot Retraction and In Vitro Thrombus Formation

For static adhesion assays, ninety-six well plates were covered with fibrinogen (100  $\mu\text{g}/\text{mL}$ ) and subsequently blocked with 5 mg/mL of Bovine Serum Albumin (BSA). Wells coated with BSA served as controls. Untreated and water-soluble p38 inhibitor (SB203580 10  $\mu\text{M}$ ) treated WT and  $\text{PP1}\alpha^{-/-}$  washed platelets ( $1 \times 10^7$ ) were incubated for 60 min. Following washing, the acid phosphatase activity of bound platelets was used to quantify platelet adhesion. A standard curve of acid phosphatase 405 absorbance with varying platelet counts was used to obtain the adhered platelet number. Percent platelet adhesion as obtained we have previously reported [19,20]. For clot retraction assays, platelet-rich mouse plasma (500  $\mu\text{L}$  of  $2.5 \times 10^8$  platelets/mL) containing 3 mM  $\text{CaCl}_2$  was left untreated or treated with SB203580 (10  $\mu\text{M}$ ) and then challenged with 1 U/mL thrombin for 60 min. The volume of liquid that was not incorporated into the clot was measured. To ascertain the volume of the clot, we subtracted the measured volume from the initial 500  $\mu\text{L}$  volume and expressed it as a percentage of the starting volume. For in vitro platelet adhesion and thrombus under flow conditions, whole blood collected in

PPACK dihydrochloride from wild type (WT) and  $PP1\alpha^{-/-}$  mice were perfused over collagen type 1 at  $1000\text{s}^{-1}$  using a Bio Flux 1000Z Flexion system (Flexion Biosciences, Oakland, CA, USA). Following 4 min of perfusion, epifluorescence microscopy was used to collect multiple images of platelet adhesion and thrombus formation at different sites by an investigator who was blinded to the genotype. Platelet adhesion and thrombus formation were reported as integrated fluorescence intensity using the Bio flux Montage software (Version 7.8.4.0).

### 2.7. Intra Vital Microscopy and Vivo Platelet Thrombus Formation

To study microvascular thrombosis in the venules and arterioles of the cremaster muscle, we used a light/dye-induced endothelial injury model and evaluated it by intravital microscopy as we previously studied [21,22]. Briefly, following a 50 mg/kg phenobarbital sodium-induced anesthesia, male mice were subjected to tracheotomy and cannulation of the internal jugular vein and common carotid arteries. While the former procedure assisted in breathing, the latter enabled the delivery of agents and continuous monitoring of heart rate and blood pressure. The cremaster microvascular bed of the mice was then exposed and equilibrated for at least 30 min with a saline solution buffered with bicarbonate (pH 7.35–7.45) at 35 °C. Through the jugular vein, a 5% FITC-labeled dextran (10 mL/Kg) was injected. Venules and arterioles for the study were then selected and the diameter and velocity of blood flow through these vessels were monitored with a Doppler velocimeter (Microcirculation Research Institute, College Station, TX, USA). 100  $\mu\text{m}$  of the vessels were then exposed to filtered excitation light at  $0.6\text{ W/cm}^2$  from a 175 W xenon lamp to trigger a photochemical injury that triggers thrombosis. After continuously applying epillumination, the time of onset for the initiation of platelet aggregates (thrombus onset) and the time required to occlude the vessel and stop the flow for at least 60 s were monitored. For each mouse, thrombosis was triggered in one or two venules and arterioles, and the average of the results reported. The investigators performing these studies were not aware of the genotype of the mice.

### 2.8. Hemostasis Studies

WT and  $PP1\alpha^{-/-}$  mice were anesthetized and 1 mm of the tip of the tail was clipped with a sterile blade and the time required to stop the bleeding was recorded, as we described before [19,20]. After the clip, the bleeding tail was submerged in a PBS solution for 30 s and then subsequently transferred to a new PBS-containing tube until there was no evidence of blood in the PBS solution. The time from the cut to the termination of blood flow was recorded as tail bleeding time.

### 2.9. Statistics

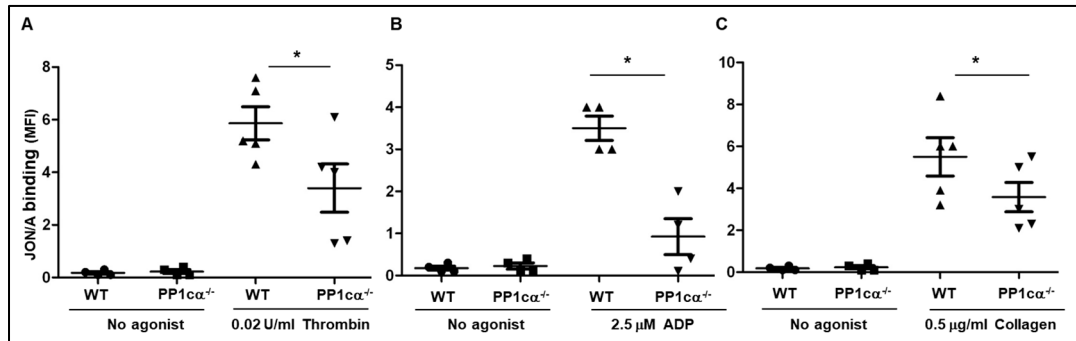
Results are shown as mean  $\pm$  standard deviation. In studies that analyzed two experimental groups, a Students' paired t-test was considered. For the analysis of three or more experimental groups, one-way ANOVA and Tukey multiple comparisons were employed. Analysis was conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and studies were considered significant only if the  $p$ -value was  $<0.05$ .

## 3. Results

### 3.1. Loss of $PP1\alpha$ Moderately Reduced Low Dose Agonist-Induced Integrin Signaling

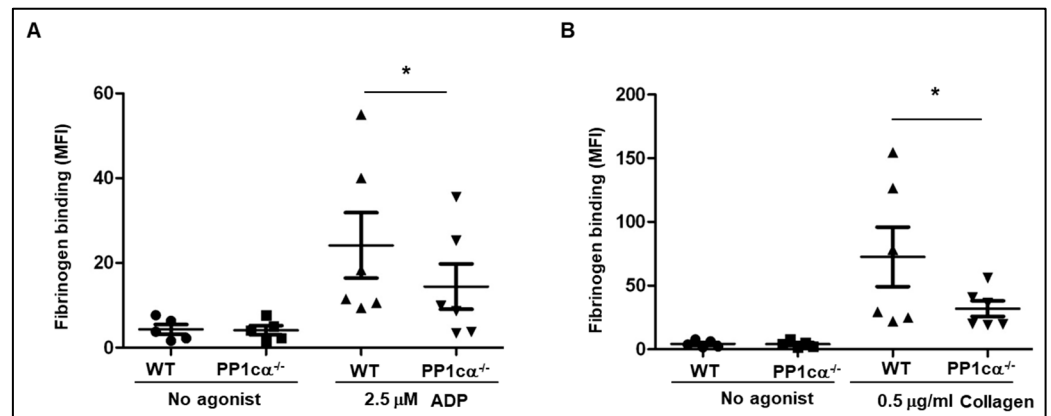
We previously generated a conditional platelet  $PP1\alpha^{-/-}$  mice and observed reduced platelet aggregation and soluble fibrinogen binding to a lower dose of thrombin (0.02 U/mL). At higher thrombin concentration (1 U/mL), aggregation and soluble fibrinogen binding were similar between wild-type (WT) and  $PP1\alpha^{-/-}$  platelets [16]. Like  $PP1\alpha^{-/-}$  platelets,  $PP1\gamma^{-/-}$  platelets also demonstrated moderately decreased aggregation selectively to low but not high doses of thrombin [19]. Due to the potential compensatory effects from other  $PP1c$  isoforms during stimulation with high agonist concentrations, we performed most of our studies in this report with a low dose of agonist.

Since activation of integrin  $\alpha_{IIb}\beta_3$  is a consequence of inside-out signaling triggered by an agonist, we examined the activation status of murine integrin  $\alpha_{IIb}\beta_3$  using JON/A antibody. Compared to the WT, PP1 $\alpha^{-/-}$  platelets bound significantly less JON/A antibody following thrombin (0.02 U/mL) stimulation (Figure 1A). Extending these studies to low doses of other agonists, we observed moderately decreased JON/A binding to PP1 $\alpha^{-/-}$  platelets in response to ADP (2.5  $\mu$ M) (Figure 1B) and collagen (0.5  $\mu$ g/mL) (Figure 1C).



**Figure 1.** Loss of PP1 $\alpha$  moderately decreased agonist-induced integrin  $\alpha_{IIb}\beta_3$  activation. Washed platelets from wild type (WT) and PP1 $\alpha^{-/-}$  mice under basal (no agonist) or challenged with low-dose agonists such as thrombin (A), ADP (B), and collagen (C) were incubated with anti-JON/A-PE (recognizes active murine  $\alpha_{IIb}\beta_3$ ) and subjected to flow cytometry.  $n = 4-5$ . \*  $p < 0.05$ .

We then analyzed the binding of soluble fibrinogen to WT and PP1 $\alpha^{-/-}$  platelets. Compared to the WT platelets, PP1 $\alpha^{-/-}$  platelets revealed a moderate but significant decrease in soluble fibrinogen binding to ADP (2.5  $\mu$ M) (Figure 2A) and collagen (0.5  $\mu$ g/mL) (Figure 2B). We had previously reported a decreased soluble fibrinogen-binding response to 0.02 U/mL thrombin by PP1 $\alpha^{-/-}$  platelets [16].

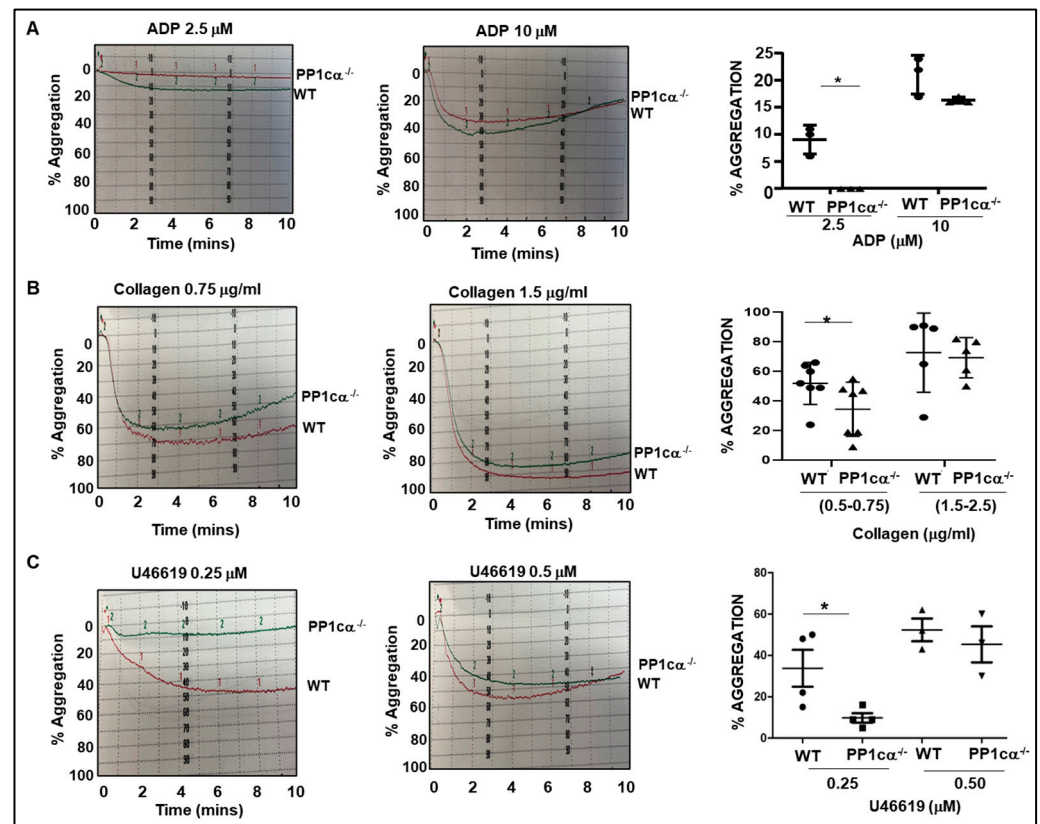


**Figure 2.** Soluble fibrinogen binding was moderately decreased in PP1 $\alpha^{-/-}$  platelets. Washed platelets from wild type (WT) and PP1 $\alpha^{-/-}$  mice were stimulated with low dose agonist (A) ADP, (B) Collagen or left unstimulated (no agonist), and the binding of fluorescent Alexa 488 fibrinogen measured using flow cytometry as mean fluorescence intensity (MFI).  $n = 6$ . \*  $p < 0.05$ .

Because soluble fibrinogen binding facilitates platelet aggregation, we examined agonist-induced aggregatory response. Aggregation of platelets to ADP (2.5  $\mu$ M) (Figure 3A), collagen (0.5–0.75  $\mu$ g/mL) (Figure 3B) and thromboxane analog; U46619 (0.25 mM) (Figure 3C) was moderately decreased in PP1 $\alpha^{-/-}$  platelets. Similarly, we previously reported reduced aggregation to 0.02 U/mL thrombin in PP1 $\alpha^{-/-}$  platelets [16]. Aggregation was comparable in WT and PP1 $\alpha^{-/-}$  platelets at higher agonist concentrations (Figure 3) Taken together, our studies indicate that loss of PP1 $\alpha$  modestly decreased activation of integrin  $\alpha_{IIb}\beta_3$ ,



binding of soluble fibrinogen, and aggregation of platelets. Moreover, the data suggests that PP1 $\alpha$  positively regulates the low-dose agonist-induced inside-out signaling process.

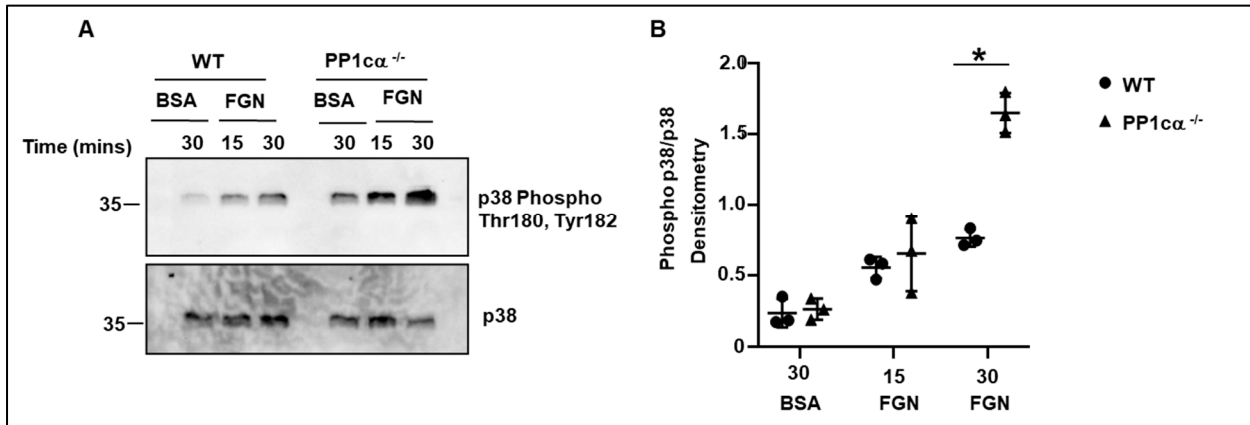


**Figure 3.** Moderately reduced aggregation of PP1 $\alpha^{-/-}$  platelets to low but not to high dose agonists. Washed platelets were subjected to a range of agonist concentrations (A) ADP, (B) Collagen, (C) thromboxane analog U46619, and final percent platelet aggregation was recorded.  $n = 3-7$ . \*  $p < 0.05$ .

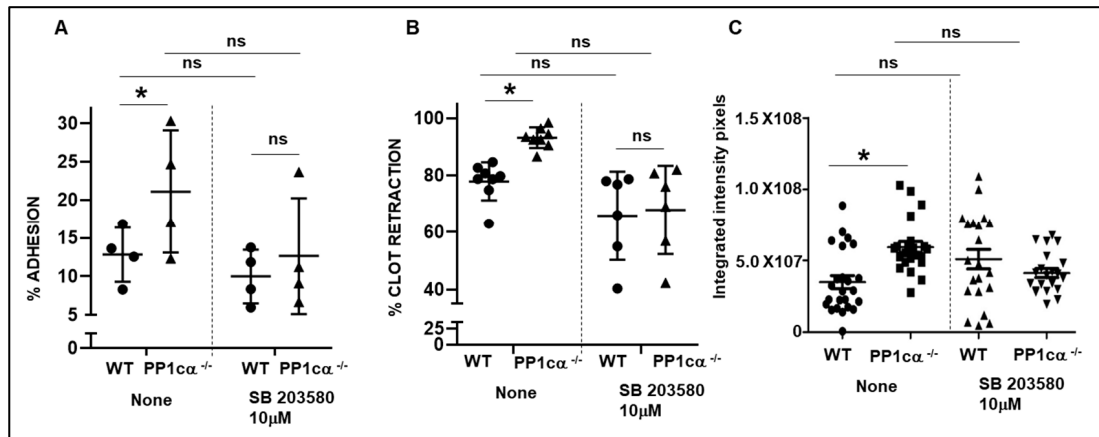
### 3.2. Loss of PP1 $\alpha$ Enhanced Activation of p38 MAPK and Integrin $\alpha_{IIb}\beta_3$ Outside-In Signaling

We previously demonstrated robust activation of p38 mitogen-activated protein kinase (MAPK) in PP1 $\alpha$  depleted heterologous  $\alpha_{IIb}\beta_3$  overexpressing 293 cells. Importantly, this effect was specific to PP1 $\alpha$  but not to other isoforms, and to p38 MAPK but not to other MAPKs [23]. Therefore, in this study, we evaluated the phosphorylation of residues Thr<sup>180</sup> and Tyr<sup>182</sup> of p38 in WT and PP1 $\alpha^{-/-}$  platelets. Phosphorylation of Thr<sup>180</sup> and Tyr<sup>182</sup> of p38 was induced following adhesion to immobilized fibrinogen in both WT and PP1 $\alpha^{-/-}$  platelets. Importantly, the adhesion of PP1 $\alpha^{-/-}$  platelets led to a robust activation of p38 at 30 min (Figure 4A,B). Thus, loss of PP1 $\alpha$  potentiates p38 MAPK activation in fibrinogen-adhered platelets. Following integrin-fibrinogen binding there is a reorganization of the platelet cytoskeleton that impacts platelet functions such as adhesion and clot retraction. Therefore, we studied these functions in WT and PP1 $\alpha^{-/-}$  platelets. Significantly increased adhesion to immobilized fibrinogen was observed with PP1 $\alpha^{-/-}$  platelets when compared to the WT platelets (Figure 5A). p38 inhibitor moderately reduced adhesion in both WT and PP1 $\alpha^{-/-}$  platelets. More importantly, p38 inhibitor SB 203,580 blocked the increased adhesiveness of PP1 $\alpha^{-/-}$  platelets on immobilized fibrinogen (Figure 5A). We also studied another function dependent on outside-in signaling, namely fibrin clot retraction. Compared to the platelet-rich plasma (PRP) from WT mice, PP1 $\alpha^{-/-}$  mice displayed significantly increased clot retraction. The addition of the p38 inhibitor moderately reduced clot reaction in both WT and PP1 $\alpha^{-/-}$  platelets. Importantly, the p38 inhibitor ablated the increased clot retraction by PP1 $\alpha^{-/-}$  platelets (Figure 5B). Since

exposed collagen can support thrombus growth under shear stress, we perfused blood from both mice on immobilized collagen at  $1000\text{ s}^{-1}$  and studied thrombus formation. Significantly increased platelet adhesion and thrombus formation on immobilized collagen shown by  $\text{PP1}\alpha^{-/-}$  mice was blocked by p38 inhibitor (Figure 5C). Collectively, our studies indicate that the loss of  $\text{PP1}\alpha$  in platelets leads to moderately enhanced outside-in  $\alpha_{\text{IIb}}\beta_3$  signaling in part via p38 MAPK.



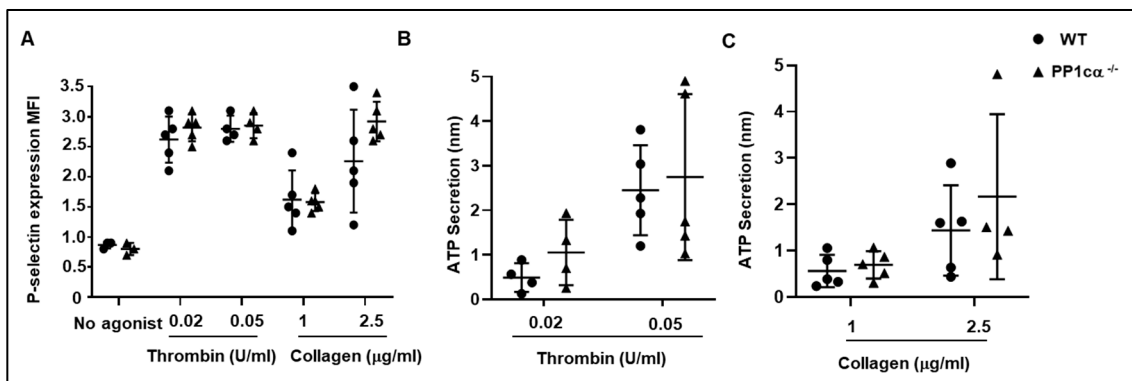
**Figure 4.** Increased activation of mitogen-activated protein kinase (MAPK) p38 in fibrinogen-adhered  $\text{PP1}\alpha^{-/-}$  platelets. (A) Washed platelets were maintained in suspension over bovine serum albumin (BSA) or allowed to adhere to fibrinogen (FGN). Lysate was immunoblotted with anti-phospho Thr180, Tyr182 p38, and anti-p38 antibodies. (B) Densitometry quantification of three independent experiments. \*  $p < 0.05$ .



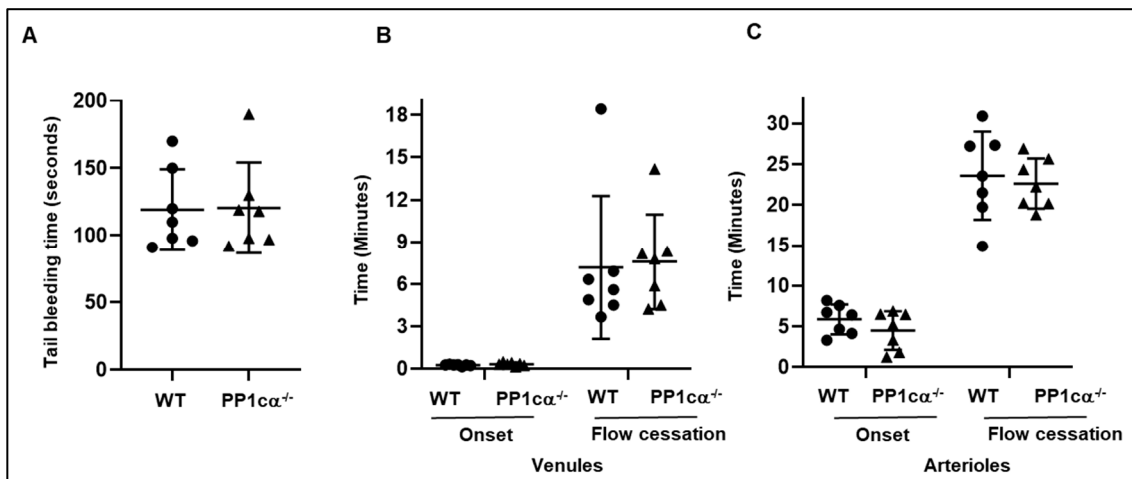
**Figure 5.**  $\text{PP1}\alpha^{-/-}$  platelets showed increased integrin outside-in signaling functions and the p38 inhibitor blocked these functions. (A) Increased adhesion of  $\text{PP1}\alpha^{-/-}$  platelets to fibrinogen at 60 min was blocked by water-soluble p38 inhibitor SB203580.  $n = 4$ . (B) Enhanced fibrin clot retraction from platelet-rich plasma of  $\text{PP1}\alpha^{-/-}$  mice was attenuated with a p38 inhibitor.  $n = 6-8$ . (C) Whole blood from  $\text{PP1}\alpha^{-/-}$  mice perfused over immobilized collagen at  $1000\text{ s}^{-1}$  resulted in an increased in vitro thrombus coverage that was blocked by a p38 inhibitor. Images acquired after 4 min of perfusion were quantified as integrated fluorescence intensity from three independent experiments. \*  $p < 0.05$ . The differences between p38 inhibitor-treated platelets and untreated platelets were not statistically significant ( $p > 0.05$ ; ns).

### 3.3. Loss of PP1 $\alpha$ Does Not Impact Secretion and In Vivo Hemostasis and Thrombosis

Alpha and dense granule secretion is one of the functional consequences of platelet activation and therefore we assessed P-selectin expression and ATP release following agonist stimulation. P-selectin and ATP secretion were comparable between WT and PP1 $\alpha$ <sup>-/-</sup> platelets exposed to low and high concentrations of thrombin and collagen (Figure 6A–C). To study if altered in vitro signaling responses displayed by PP1 $\alpha$ <sup>-/-</sup> platelets impact in vivo hemostasis, we performed mouse tail clip assays. WT and PP1 $\alpha$ <sup>-/-</sup> mice had similar tail bleeding times (Figure 7A). To evaluate if loss of PP1 $\alpha$  impacts thrombosis, we induced microvascular thrombosis in WT and PP1 $\alpha$ <sup>-/-</sup> mice using a light/dye-induced injury model. The time to initiate a thrombus (onset time) and the time to occlude the vessel (occlusion time) in the venules and arterioles of the PP1 $\alpha$ <sup>-/-</sup> mice were similar to that of WT mice (Figure 7B,C). Thus, loss of PP1 $\alpha$  in platelets did not impact in vivo hemostasis and thrombosis.



**Figure 6.** Platelet granule secretion is not affected by the loss of PP1 $\alpha$ . (A) Wildtype (WT) (●) and PP1 $\alpha$ <sup>-/-</sup> platelets (▲) (resting or agonist activated) were challenged with agonists and secretion of a granule was studied using fluorescent anti-P-selectin antibody via flow cytometry as mean fluorescence intensity (MFI). *n* = 5. The release of dense granule content ATP is evaluated in washed platelets in response to (B) thrombin and (C) collagen by studying luciferin/luciferase. *n* = 4.



**Figure 7.** In vivo hemostasis and thrombosis are not affected by the loss of PP1 $\alpha$ . (A) Time to achieve hemostasis after a tail bleeding assay from six wild-type (WT) and PP1 $\alpha$ <sup>-/-</sup> mice were recorded. Light-dye-induced microvascular thrombosis was studied in venules (B) and arterioles (C) of the cremaster muscle from WT and PP1 $\alpha$ <sup>-/-</sup> mice using intravital microscopy. The onset of thrombosis (onset) and cessation of blood flow following injury were studied.



#### 4. Discussion

PP1 positively regulates platelet functions based on previous studies using Ser/Thr phosphatase inhibitors [9–14]. Although such chemical inhibitors represent a useful tool, they lack specificity to discriminate protein phosphatase 1, protein phosphatase 2A, and protein phosphatase 4 and their respective isoforms [24]. Thus, it remains possible that the observed phenotype in pharmacological studies may be due to the combined inhibitory effect of multiple phosphatases. Here using a genetic approach, our studies uncovered a subtle but intriguing phenotype for PP1 $\alpha$  in platelet signaling. Specifically, PP1 $\alpha$  was shown to amplify low-dose agonist-induced inside–out signaling and attenuate integrin  $\alpha_{\text{IIb}}\beta_3$  outside–in signaling functions with no consequence on hemostasis and thrombosis.

Loss of PP1 $\alpha$  modestly reduced activation of integrin, binding of soluble fibrinogen, and aggregation of platelets especially to a low dose of platelet agonist (Figures 1–3). These findings are consistent with our previous report of reduced binding of soluble fibrinogen and aggregation of platelets by PP1 $\alpha^{-/-}$  platelets to low-dose thrombin (0.02 U/mL) [16]. It remains to be understood whether higher agonist concentrations initiate multiple redundant signaling pathways in PP1 $\alpha^{-/-}$  platelets and trigger activation of PP1 $\beta$  or PP1 $\gamma$  isoforms to assist platelet activation. Indeed, in our previous study with PP1 $\gamma^{-/-}$  mice model, we also observed reduced inside–out signaling selectively to a low dose of thrombin [19]. Thus, unlike the robust inhibition of platelet inside–out signaling functions observed in human platelets with pharmacological agents, genetic loss of PP1 $\alpha$  in murine platelets only displayed a subtle reduction in inside–out signaling.

In contrast, loss of PP1 $\alpha$  in murine platelets moderately enhanced integrin  $\alpha_{\text{IIb}}\beta_3$  outside–in signaling functions (Figures 4 and 5). Interestingly, these platelet studies validate our previous observations in 293  $\alpha_{\text{IIb}}\beta_3$  overexpressing heterologous cells, wherein selective depletion of PP1 $\alpha$  caused increased outside–in signaling functional effects (adhesion and clot retraction) [23]. Enhanced outside–in signaling phenotype in PP1 $\alpha^{-/-}$  murine platelets indicates that this phenotype is intrinsic to PP1 $\alpha$  and independent of the cell model we previously employed. Importantly, unlike the inhibition of outside–in signaling functions in human platelets treated with inhibitors of Ser/Thr phosphatases, genetic loss of only PP1 $\alpha$  moderately potentiated integrin  $\alpha_{\text{IIb}}\beta_3$  mediated outside–in signaling function.

How PP1 $\alpha$  can amplify inside–out signaling and attenuate outside–in signals remains unclear. PP1 $\alpha$  spatially and temporally regulate platelet inside–out and outside–in signaling pathways in part by engaging with phosphatase interacting proteins (PIP) and subsequent phospho-modulation of specific effectors within the pathway. Indeed, we previously demonstrated the G $\beta_1$  protein as one PIP downstream of the G protein-coupled receptor. Thrombin stimulation led to the dissociation of PP1 $\alpha$  from G $\beta_1$ , with subsequent association and dephosphorylation of phospholipase C  $\beta_3$  (PLC $\beta_3$ ) at Ser 1105. Since Ser 1105 phosphorylation blocks signaling, dephosphorylation by PP1 $\alpha$  may amplify agonist signaling [16]. In the context of outside–in signaling pathway, integrin  $\alpha_{\text{IIb}}\beta_3$  [25], cytoskeletal protein tensin 1 [26] and p38 MAPK [23] represents some of the PIP. Consistent with the contribution of p38 MAPK in outside–in signaling [27] we observed increased p38 MAPK activation in fibrinogen-adhered PP1 $\alpha^{-/-}$  platelets (Figure 4) and p38 inhibitor to block the enhanced outside-on signaling functions (Figure 5). It is possible that loss of PP1 $\alpha$  may impact tensin 1-induced cytoskeletal reorganization in platelets to potentiate outside–in signaling, as there is precedence for tensin 1 to engage integrin  $\beta_3$  [28] and disruption of PP1 $\alpha$ -tensin interaction enhanced cell migration and invasion of cancer cells [29]. It is important to emphasize here that the distinct phenotype displayed by the loss of PP1 $\alpha$  is not a reflection of perturbation by one effector in a particular pathway. In fact, PP1c interacts with more than 200 phosphatase-interacting proteins (PIP) in a cell. Several of the PIPs represent endogenous PP1 inhibitors, which can be regulated by phosphorylation events [30]. Given the complexity of the PP1c interactome and its ability to integrate with other signaling networks, the functional outcome of PP1 $\alpha$  loss is likely to be more than an additive effect displayed by the phospho-modulation of several PP1 $\alpha$  effectors along the pathway.

Despite the critical role of platelet activation in hemostasis and thrombosis, in vivo hemostasis and thrombosis were unaffected in PP1 $\alpha$ <sup>-/-</sup> mice (Figure 7). Opposing roles for PP1 $\alpha$  in two signaling pathways that contribute to hemostasis and thrombosis along with potential compensation by PP1 $\beta$  and/or PP1 $\gamma$  isoforms in platelets may have contributed to the lack of an in vivo effect. Supporting the issue of compensation by other PP1c isoforms, PP1 $\gamma$ <sup>-/-</sup> global mice had no defect in in vivo hemostasis and only moderately reduced time to occlusion in a light-dye injury model [19]. Despite no differences in the occlusion time in vivo, ex vivo coverage of whole blood on immobilized collagen was enhanced in PP1 $\alpha$ <sup>-/-</sup> mice. This suggests that platelet PP1 $\alpha$  can crosstalk with injured endothelial cells during the development of thrombosis in vivo and modulate outcome.

## 5. Conclusions

Despite the subtle phenotype, our studies suggest that PP1 $\alpha$  contributes to platelet signaling in a fashion that is specific to the mode of signaling. Specifically, PP1 $\alpha$  supports agonist induced inside-out signaling and opposes integrin-mediated outside-in signaling. Interestingly, recent studies suggest a role for PP1 signaling in platelet-cancer cross-talk [31], and alterations in platelet transcriptome including the regulatory subunits of PP1 occur during sepsis [32]. Thus, a contrasting and pleiotropic effect of platelet PP1 $\alpha$  may have relevance to platelet functions beyond hemostasis and thrombosis.

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## References

1. Estevez, B.; Du, X. New Concepts and Mechanisms of Platelet Activation Signaling. *Physiology* **2017**, *32*, 162–177. [[CrossRef](#)] [[PubMed](#)]
2. Shattil, S.J.; Kashiwagi, H.; Pampori, N. Integrin signaling: The platelet paradigm. *Blood* **1998**, *91*, 2645–2657. [[CrossRef](#)]
3. Shattil, S.J. Signaling through platelet integrin  $\alpha_{IIb}\beta_3$ : Inside-out, outside-in, and sideways. *Thromb. Haemost.* **1999**, *82*, 318–325. [[PubMed](#)]
4. Shiravand, Y.; Walter, U.; Jurk, K. Fine-Tuning of Platelet Responses by Serine/Threonine Protein Kinases and Phosphatases—Just the Beginning. *Hamostaseologie* **2021**, *41*, 206–216. [[CrossRef](#)]
5. Beck, F.; Geiger, J.; Gambaryan, S.; Solari, F.A.; Dell’Aica, M.; Loroch, S.; Mattheij, N.J.; Mindukshev, I.; Pötz, O.; Jurk, K.; et al. Temporal quantitative phosphoproteomics of ADP stimulation reveals novel central nodes in platelet activation and inhibition. *Blood* **2017**, *129*, e1–e12. [[CrossRef](#)]

6. Qureshi, A.H.; Chaoji, V.; Maiguel, D.; Faridi, M.H.; Barth, C.J.; Salem, S.M.; Singhal, M.; Stoub, D.; Krastins, B.; Ogihara, M.; et al. Proteomic and phospho-proteomic profile of human platelets in basal, resting state: Insights into integrin signaling. *PLoS ONE* **2009**, *4*, e7627. [[CrossRef](#)]
7. Cohen, P.T. Protein phosphatase 1—Targeted in many directions. *J. Cell Sci.* **2002**, *115*, 241–256. [[CrossRef](#)]
8. Burkhardt, J.M.; Vaudel, M.; Gambaryan, S.; Radau, S.; Walter, U.; Martens, L.; Geiger, J.; Sickmann, A.; Zahedi, R.P. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood* **2012**, *120*, e73–e82. [[CrossRef](#)]
9. Higashihara, M.; Takahata, K.; Kurokawa, K.; Ikebe, M. The inhibitory effects of okadaic acid on platelet function. *FEBS Lett.* **1992**, *307*, 206–210. [[CrossRef](#)]
10. Nishikawa, M.; Toyoda, H.; Saito, M.; Morita, K.; Tawara, I.; Deguchi, K.; Kuno, T.; Shima, H.; Nagao, M.; Shirakawa, S. Calyculin A and okadaic acid inhibit human platelet aggregation by blocking protein phosphatases types 1 and 2A. *Cell Signal* **1994**, *6*, 59–71. [[CrossRef](#)]
11. Hoyt, C.H.; Lerea, K.M. Aggregation-dependent signaling in human platelets is sensitive to protein serine/threonine phosphatase inhibitors. *Biochemistry* **1995**, *34*, 9565–9570. [[CrossRef](#)] [[PubMed](#)]
12. Simon, Z.; Kiss, A.; Erdödi, F.; Setiadi, H.; Beke Debreceni, I.; Nagy, B., Jr.; Kappelmayer, J. Protein phosphatase inhibitor calyculin-A modulates activation markers in TRAP-stimulated human platelets. *Platelets* **2010**, *21*, 555–562. [[CrossRef](#)] [[PubMed](#)]
13. Lerea, K.M.; Cordero, K.P.; Sakariassen, K.S.; Kirk, R.I.; Fried, V.A. Phosphorylation sites in the integrin  $\beta_3$  cytoplasmic domain in intact platelets. *J. Biol. Chem.* **1999**, *274*, 1914–1919. [[CrossRef](#)] [[PubMed](#)]
14. Moscardó, A.; Santos, M.T.; Latorre, A.; Madrid, I.; Vallés, J. Serine/threonine phosphatases regulate platelet  $\alpha_{IIb}\beta_3$  integrin receptor outside-in signaling mechanisms and clot retraction. *Life Sci.* **2013**, *93*, 707–713. [[CrossRef](#)] [[PubMed](#)]
15. Hudák, R.; Vincze, J.; Csernoch, L.; Beke Debreceni, I.; Oláh, T.; Erdödi, F.; Clemetson, K.J.; Kappelmayer, J. The Phosphatase Inhibitor Calyculin-A Impairs Clot Retraction, Platelet Activation, and Thrombin Generation. *BioMed Res. Int.* **2017**, *2017*, 9795271. [[CrossRef](#)]
16. Pradhan, S.; Khatlani, T.; Nairn, A.C.; Vijayan, K.V. The heterotrimeric G protein  $G\beta_1$  interacts with the catalytic subunit of protein phosphatase 1 and modulates G protein-coupled receptor signaling in platelets. *J. Biol. Chem.* **2017**, *292*, 13133–13142. [[CrossRef](#)]
17. Liu, R.; Correll, R.N.; Davis, J.; Vagnozzi, R.J.; York, A.J.; Sargent, M.A.; Nairn, A.C.; Molkenin, J.D. Cardiac-specific deletion of protein phosphatase 1 $\beta$  promotes increased myofilament protein phosphorylation and contractile alterations. *J. Mol. Cell Cardiol.* **2015**, *87*, 204–213. [[CrossRef](#)]
18. Tiedt, R.; Schomber, T.; Hao-Shen, H.; Skoda, R.C. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood* **2007**, *109*, 1503–1506. [[CrossRef](#)]
19. Gushiken, F.C.; Hyojeong, H.; Pradhan, S.; Langlois, K.W.; Alrehani, N.; Cruz, M.A.; Rumbaut, R.E.; Vijayan, K.V. The catalytic subunit of protein phosphatase 1 gamma regulates thrombin-induced murine platelet  $\alpha_{IIb}\beta_3$  function. *PLoS ONE* **2009**, *4*, e8304. [[CrossRef](#)]
20. Khatlani, T.; Pradhan, S.; Da, Q.; Gushiken, F.C.; Bergeron, A.L.; Langlois, K.W.; Molkenin, J.D.; Rumbaut, R.E.; Vijayan, K.V. The  $\beta$  isoform of the catalytic subunit of protein phosphatase 2B restrains platelet function by suppressing outside-in  $\alpha_{IIb}\beta_3$  integrin signaling. *J. Thromb. Haemost.* **2014**, *12*, 2089–2101. [[CrossRef](#)]
21. Patel, K.N.; Soubra, S.H.; Bellera, R.V.; Dong, J.F.; McMullen, C.A.; Burns, A.R.; Rumbaut, R.E. Differential role of von Willebrand factor and P-selectin on microvascular thrombosis in endotoxemia. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 2225–2230. [[CrossRef](#)] [[PubMed](#)]
22. Rumbaut, R.E.; Randhawa, J.K.; Smith, C.W.; Burns, A.R. Mouse cremaster venules are predisposed to light/dye-induced thrombosis independent of wall shear rate, CD18, ICAM-1, or P-selectin. *Microcirculation* **2004**, *11*, 239–247. [[CrossRef](#)] [[PubMed](#)]
23. Alrehani, N.; Pradhan, S.; Khatlani, T.; Kailasam, L.; Vijayan, K.V. Distinct roles for the  $\alpha$ ,  $\beta$  and  $\gamma_1$  isoforms of protein phosphatase 1 in the outside-in  $\alpha_{IIb}\beta_3$  integrin signalling-dependent functions. *Thromb. Haemost.* **2013**, *109*, 118–126. [[PubMed](#)]
24. McCluskey, A.; Sim, A.T.; Sakoff, J.A. Serine-threonine protein phosphatase inhibitors: Development of potential therapeutic strategies. *J. Med. Chem.* **2002**, *45*, 1151–1175. [[CrossRef](#)] [[PubMed](#)]
25. Vijayan, K.V.; Liu, Y.; Li, T.T.; Bray, P.F. Protein phosphatase 1 associates with the integrin  $\alpha_{IIb}$  subunit and regulates signaling. *J. Biol. Chem.* **2004**, *279*, 33039–33042. [[CrossRef](#)]
26. Eto, M.; Kirkbride, J.; Elliott, E.; Lo, S.H.; Brautigan, D.L. Association of the tensin N-terminal protein-tyrosine phosphatase domain with the alpha isoform of protein phosphatase-1 in focal adhesions. *J. Biol. Chem.* **2007**, *282*, 17806–17815. [[CrossRef](#)]
27. Flevaris, P.; Li, Z.; Zhang, G.; Zheng, Y.; Liu, J.; Du, X. Two distinct roles of mitogen-activated protein kinases in platelets and a novel Rac1-MAPK-dependent integrin outside-in retractile signaling pathway. *Blood* **2009**, *113*, 893–901. [[CrossRef](#)]
28. McCleverty, C.J.; Lin, D.C.; Liddington, R.C. Structure of the PTB domain of tensin1 and a model for its recruitment to fibrillar adhesions. *Protein Sci.* **2007**, *16*, 1223–1229. [[CrossRef](#)]
29. Hall, E.H.; Daugherty, A.E.; Choi, C.K.; Horwitz, A.F.; Brautigan, D.L. Tensin1 requires protein phosphatase-1alpha in addition to RhoGAP DLC-1 to control cell polarization, migration, and invasion. *J. Biol. Chem.* **2009**, *284*, 34713–34722. [[CrossRef](#)] [[PubMed](#)]
30. Verbinen, I.; Ferreira, M.; Bollen, M. Biogenesis and activity regulation of protein phosphatase 1. *Biochem. Soc. Trans.* **2017**, *45*, 89–99. [[CrossRef](#)] [[PubMed](#)]

31. Haemmerle, M.; Taylor, M.L.; Gutschner, T.; Pradeep, S.; Cho, M.S.; Sheng, J.; Lyons, Y.M.; Nagaraja, A.S.; Dood, R.L.; Wen, Y.; et al. Platelets reduce anoikis and promote metastasis by activating YAP1 signaling. *Nat. Commun.* **2017**, *8*, 310. [[CrossRef](#)] [[PubMed](#)]
32. Middleton, E.A.; Rowley, J.W.; Campbell, R.A.; Grissom, C.K.; Brown, S.M.; Beesley, S.J.; Schwertz, H.; Kosaka, Y.; Manne, B.K.; Krauel, K.; et al. Sepsis alters the transcriptional and translational landscape of human and murine platelets. *Blood* **2019**, *134*, 911–923. [[CrossRef](#)] [[PubMed](#)]

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