The β isoform of the catalytic subunit of protein phosphatase 2B restrains platelet function by suppressing outside-in αIIbβ3 integrin signaling

Tanvir Khatlani[1], **Subhashree Pradhan**[1], **Qi Da**[1], **Francisca C. Gushiken**[1,6], **Angela L. Bergeron**[1], **Kimberly W. Langlois**[2], **Jeffery D. Molkentin**[3], **Rolando E. Rumbaut**[1,2,4], and **K. Vinod Vijayan**[1,2,4,5]

[1]Department of Medicine, Michael E. DeBakey VA Medical Center, Houston, TX 77030, USA

[2]Department of Pediatrics, Molecular Physiology, Michael E. DeBakey VA Medical Center, Houston, TX 77030, USA

[3]Molecular Cardiovascular Biology Program, Howard Hughes Medical Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

[4]Center for Translational Research on Inflammatory Diseases (CTRID), Michael E. DeBakey VA Medical Center, Houston, TX 77030, USA

[5]Department of Biophysics, Baylor College of Medicine, Michael E. DeBakey VA Medical Center, Houston, TX 77030, USA

Abstract

Background—Calcium dependent signaling mechanisms play a critical role in platelet activation. Unlike calcium-activated protease and kinase, the contribution of calcium-activated protein serine/threonine phosphatase in platelet activation is poorly understood.

Objective—To assess the role of catalytic subunit of protein phosphatase 2B (PP2B) or calcineurin in platelet function.

Results—Here, we showed that an increase in PP2B activity was associated with agonist-induced activation of human and murine platelets. Pharmacological inhibitors of the catalytic subunit of protein phosphatase 2B (PP2B-A) such as cyclosporine A (CsA) or tacrolimus (FK506) potentiated aggregation of human platelets. Murine platelets lacking the β isoform of PP2B-A (PP2B-Aβ$^{-/-}$) displayed increased aggregation with low doses of agonist concentrations. Loss of PP2B-Aβ did not affect agonist-induced integrin $\alpha_{IIb}\beta_3$ inside-out signaling, but increased basal

Address correspondence to. K. Vinod Vijayan, Ph.D, Cardiovascular Research Section, Division of Thrombosis, Baylor College of Medicine, One Baylor Plaza, BCM 286, N1319, Houston, TX 77030. Telephone: (713)798-8677; Fax: (713)798-3415; yvijayan@bcm.edu.
[6]Current address: South Texas Veteran Health Care System, San Antonio, TX.

Src activation and outside-in $\alpha_{IIb}\beta_3$ signaling to p38 mitogen activated protein kinase (MAPK), with a concomitant enhancement in platelet spreading on immobilized fibrinogen and greater fibrin clot retraction. Fibrinogen induced increased p38 activation in PP2B-A$\beta^{-/-}$ platelets were blocked by Src inhibitor. Both PP2B-A$\beta^{-/-}$ platelets and PP2B-Aβ depleted human embryonal kidney 293 $\alpha_{IIb}\beta_3$ cells displayed increased adhesion to immobilized fibrinogen. Filamin A, an actin crosslinking phosphoprotein that is known to associate with β_3 was dephosphorylated on Ser^{2152} in fibrinogen adhered wild type but not in PP2B-A$\beta^{-/-}$ platelets. In a FeCl$_3$ injury thrombosis model, PP2B-A$\beta^{-/-}$ mice showed decreased time to occlusion in the carotid artery.

Conclusion—These observations suggest that PP2B-Aβ by suppressing outside-in $\alpha_{IIb}\beta_3$ integrin signaling limits platelet response to vascular injury.

Keywords

Platelet aggregation; Fibrinogen; Platelets; beta(3) Integrin; Protein-Serine-Threonine Phosphatase

Introduction

At the site of injury, platelets respond to activation and inhibition signals (inside-out signals), which facilitate the engagement of integrin $\alpha_{IIb}\beta_3$ and the transmission of information to initiate cytoskeletal reorganization (outside-in signals). Intrinsic to the activation of platelets via the agonist (G protein coupled and collagen) receptors and integrin $\alpha_{IIb}\beta_3$ receptors is the initiation of phospholipase C (PLC β/γ) signaling pathways that increase intracellular calcium (Ca^{2+}) concentration from the internal stores. (Ca^{2+}) also enters into the platelets through the plasma membrane by a store-operated mechanism. Sustained (Ca^{2+}) levels trigger calcium-dependent signaling and contribute to agonist-induced activation of $\alpha_{IIb}\beta_3$, granule exocytosis, platelet aggregation and $\alpha_{IIb}\beta_3$ dependent cytoskeletal reorganization [1]. Mechanistically, (Ca^{2+}) dependent signaling relies on multiple effectors such as (Ca^{2+}) activated -protease, -kinase and -phosphatase to regulate platelet function. Among these effectors, the role of calpain (Ca^{2+} -activated protease) [2] and classical Protein Kinase C (PKC) α, β isoforms (Ca^{2+}) and diacylglycerol-activated kinases) in platelet function is understood [3,4]. In contrast, the contribution of (Ca^{2+}) -activated protein phosphatase remains unexplored.

Protein phosphatase 2B (PP2B) or calcineurin is a (Ca^{2+}) and calmodulin (CaM) activated serine/threonine (Ser/Thr) phosphatase composed of a 58–64 kDa catalytic subunit (PP2B-A) and a 19 kDa regulatory subunit (PP2B-B). Somatic cells express two isoforms of PP2B-A subunit (PP2B-Aα and PP2B-Aβ) and one isoform of PP2B-B subunit (PP2B-Bα; CNB1). Germ cells express the catalytic subunit PP2B-Aγ and the regulatory subunit PP2B-Bγ (CNB2) [5]. The PP2B-A subunit contains an autoinhibitory (AI) domain along with the binding sites for PP2B-B subunit and CaM. Interaction of (Ca^{2+}) with CaM and PP2B-B subunit changes their conformation and facilitates their interaction with the respective binding sites on PP2B-A subunit. Structural changes induced by the binding of CaM and PP2B-B subunit to the PP2B-A subunit causes dissociation of the AI domain from the catalytic groove, thereby activating PP2B [5]. Immunosuppressive agents like cyclosporine A (CsA) and tacrolimus (FK506) form complexes with cyclophilins and FK506 binding proteins and inhibit the catalytic activity of PP2B [6].

PP2B is classically known to dephosphorylate and promote the nuclear translocation of nuclear factor of activated T cells (NFAT) to enhance the transcription of genes in T cells and cardiac tissues [5]. Megakaryocytes express NFAT isoforms and attenuation of PP2B-NFAT pathway increase megakaryocyte and platelet numbers *in vivo* [7]. Although anucleate platelets express PP2B, its contribution in platelet function is unexplored. Here, using a pharmacological and genetic approach, we report that PP2B-Aβ limits platelet reactivity by suppressing cytoskeletal dependent outside-in $\alpha_{IIb}\beta_3$ signaling function.

Materials and method

Reagents

ADP and collagen were purchased from Helena Laboratories (Beaumont, TX), while thrombin was from Hematologic Technologies Inc. (Essex Junction, VT). Protease activated receptor 4 –activating peptide (PAR4-AP) AYPGKF was synthesized by the Protein Core at Baylor College of Medicine (BCM). CsA and FK506 were from Calbiochem-EMD Millipore (Darmstadt, Germany). Alexa 488-conjugated fibrinogen was from Invitrogen-life Technologies (Grand Island, NY). Human fibrinogen was from Enzyme Research Laboratories Inc. (South bend, IN). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD62 (P-selectin), Phycoerythrin (PE)–conjugated anti-mouse CD41 (αIIb) and control IgG tagged to PE or FITC were purchased from BD Bioscience (San Jose, CA). PE-conjugated JON/A antibody was from Emfret Analytics (Eibelstadt, Germany). Antibodies to phospho Src Tyr^{418}, Src, phospho p38 (Thr^{180} Tyr^{182}), p38, phospho filamin (Ser^{2152}), and filamin were from Cell Signaling Technologies (Beverly, MA). Antibodies to PP2B-Aα, PP2B-Aβ were from Santa Cruz Biotechnology (Santa Cruz, CA). Calcineurin cellular assay kit was from Enzo Life Sciences, Inc. (Farmingdale, NY). A preformed mix of four independent (SMART pool) siRNAs targeting human PP2B-Aβ and non-specific control siRNA pool were purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO).

Mice

Animal studies were approved by the Institutional Animal Care and Use Committee at BCM. CnA$\beta^{-/-}$ or PP2B-A$\beta^{-/-}$ mice were generated as previously described [8]. Experimental animals were generated by crossing male and female PP2B-A$\beta^{+/-}$ mice. Wild type (WT) and PP2B-A$\beta^{-/-}$ littermate mice matched for age (8–14 week's old) and gender were used.

Human and mouse platelet preparation

Following an approval from the Institutional Review Board of BCM, blood was drawn in an acid/citrate/dextrose (ACD) anticoagulant at a ratio of 1:9 (vol/vol) from healthy fasting donors. Platelet rich plasma (PRP) was prepared by centrifuging blood at 200 x *g* for 15 minutes at room temperature. PRP supplemented with 75 nM of PGE$_1$ was centrifuged at 1000 x *g* for 10 minutes at room temperature to obtain platelet pellet that was resuspended in Tyrode's and re-centrifuged. Washed platelets were suspended in Tyrode's buffer and the counts adjusted to 2.5×10^8 platelets/ml.

Mouse blood was collected from the inferior vena cava of isoflorane-anesthetized mice into 3.8% sodium citrate at the ratio of 1:10 (vol/vol) for studies with PRP, and into ACD at the ratio of 1:10 (vol/vol) for washed platelet studies. Blood was diluted 1:1 with Tyrode's and PRP was isolated following centrifugation at 68 x g for 10 minutes. Washed platelets were obtained following additional centrifugation of PRP as we have described before [9]. Platelet counts in PRP and washed preparation from the WT and PP2B-Aβ was adjusted to 2.5×10^8/ml with the platelet poor plasma or Tyrode's buffer respectively.

Protein phosphatase 2B assays

PP2B activity in the lysate obtained from platelets treated with various agonists was measured using the calcineurin cellular assay kit as we have performed before [10]. After depleting free phosphate from the lysate by gel filtration using desalting resins, the ability of PP2B in the lysate to dephosphorylate RII phosphopeptide was measured using Biomol Green reagent. Absorbance read at 620 nm was converted to amount of phosphate released using a phosphate standard curve.

Platelet aggregation, soluble fibrinogen binding, secretion and flow cytometry studies

Washed platelets and PRP from humans and mice were challenged with varying concentrations of agonist. Platelet aggregation was measured under stirring conditions using an eight channel Bio/Data PAP-8C aggregometer (Biodata Corporation, Horsham, PA). Resting and activated murine platelets diluted to 2.5×10^7/ml in Tyrode's buffer with 1.8 mM $CaCl_2$ and 0.49 mM $MgCl_2$, was incubated with either Alexa 488-conjugated fibrinogen, FITC conjugated CD62P, PE conjugated CD41, PE conjugated JON/A (recognizes active murine $\alpha_{IIb}\beta_3$), FITC or PE tagged isotype control antibodies and measured by flow cytometry using EPICS-XL flow cytometer (Beckman Coulter, Miami, FL). In some experiments, washed platelets were stimulated with agonist and ATP release was measured by adding luciferin/luciferase reagent in a lumi-aggregometer (Chrono-Log Corp. Havertown, PA).

Platelet spreading, fibrin clot retraction and adhesion assays

Fibrinogen coated coverslips were incubated with ~1×10^7/ml platelets for varying time points at 37 °C. Adhered platelets were fixed with 3.7 % paraformaldehyde, permeabilized with 0.01 % Triton X-100 and the staining of F-actin by rhodamine phallodin was visualized using a fluorescence microscope. Quantification of the surface area was performed using NIH Image J software. For fibrin clot retraction assays, PRP containing 2.5×10^8 platelets/ml and supplemented with 3 mM $CaCl_2$ was incubated with 1 U/ml thrombin. The amount of liquid not incorporated into the clot was subtracted from the initial volume (150 μl) to ascertain the volume of clot over time. Clot volume was expressed as a percentage of the initial volume. For adhesion assays, washed murine platelets or human embryonal kidney 293 cells expressing $\alpha_{IIb}\beta_3$ [11] transfected with 100 nM siRNAs (targeting PP2B-Aβ and nonspecific control) were added to ninety six well plates coated with fibrinogen and blocked with bovine serum albumin for varying time points at 37 °C. After washing, adhesion was quantified by assaying for acid phosphatase activity at 405 nM. Percent adhesion was calculated as number of fibrinogen adhered platelets/cells divided by the total

number of platelets/cells added to the well and multiplied by 100 as we have previously reported [9, 12].

Ferric chloride (FeCl$_3$) injury thrombosis model and tail bleeding times

This model was performed using techniques similar to those we previously described [13]. Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), with additional doses (25 mg/kg) given as needed. Mice were then placed on a custom Plexiglas tray and maintained at 37 °C with a homeothermic blanket and a rectal temperature probe (FHC, Inc, Bowdoin, ME). The right carotid artery was isolated through a midline cervical incision, and an ultrasonic flow probe (Transonic Systems, Ithaca, NY) was used to monitor carotid blood flow. A 1×2 mm piece of filter paper (Whatman #2, Maidstone, United Kingdom) saturated in 10 % FeCl$_3$ (Sigma-Aldrich) was applied to the surface of the carotid artery for 1 minute. Three minutes after the onset of FeCl$_3$ exposure, the vessel was rinsed with warm isotonic saline, and carotid blood flow was monitored continuously until the onset of flow cessation (>1 minute) or 30 minutes. The time taken for flow cessation lasting at least 30 seconds from the time FeCl$_3$ was rinsed (time 0) was recorded. Tail bleeding time(s) were quantified as previously described [9]. Tails of anesthetized mice were severed 2 mm from the tip with a scalpel and immediately immersed in a tube containing PBS at 37 °C. The tails were transferred to a new tube every 30 seconds until bleeding was stopped and time for cessation of blood was recorded. Thrombosis and hemostasis assays were both recorded by investigators blinded to the genotype of the mice.

Statistics

Results were expressed as mean ± SEM. Student's t test was used to analyze significance of the data. Non-parametric Mann Whitney U test was used to analyze time for occlusion in FeCl$_3$ injury model. $P < 0.05$ was considered significant.

Results

Stimulation of human and murine platelets with agonist is associated with an increased PP2B activity

A rise in intracellular platelet calcium level during agonist stimulation is likely to affect the activity of downstream effectors like calcium –activated kinases, proteases and phosphatases. We investigated the activity of the calcium activated protein phosphatase 2B (PP2B) in response to agonist exposure. Compared to the resting human platelets, treatment with thrombin, ADP and collagen enhanced PP2B activity (Fig. 1A). Similar findings were noticed with wild type murine platelets (Fig. 1B). PP2B activation in human and murine platelets was noticed as early as 2 minutes after agonist stimulation (not shown).

Pharmacological inhibitors of PP2B moderately increased agonist-induced human platelet aggregation

Next, we investigated the role of the catalytic subunit of PP2B (PP2B-A) in agonist-induced platelet aggregation. Compared to the control (DMSO), pretreatment of human platelets with cyclosporine A (CsA) or tacrolimus (FK506), resulted in a moderate increase in thrombin, ADP and collagen induced platelet aggregation (Figs. 2A, 2C and 2E). Importantly, the time

to achieve 50% thrombin induced or 20% ADP and collagen induced aggregation was significantly decreased in CsA and FK506 treated platelets (Figs. 2B, 2D and 2F). These studies suggest that CsA and FK506 can potentiate low dose agonist induced platelet aggregation.

PP2B-Aβ$^{-/-}$ platelets exhibit enhanced agonist induced aggregation

Although CsA is a well-established PP2B inhibitor, it can also block the peptidyl-prolyl *cis-trans* isomerase activity [14] of cyclophilins and thereby impact mitochondrial permeability transition pore and platelet activation [15], an effect independent of PP2B. To specifically explore the contribution of PP2B in platelet function, we used mice deficient in the β isoform of the catalytic subunit of PP2B (PP2B-Aβ) [8]. Immunoblotting of platelet lysate with anti-PP2B-A isoform specific antibodies confirmed the absence of PP2B-Aβ, but not PP2B-Aα isoform in PP2B-Aβ$^{-/-}$ mice (Fig. 3A). Platelet count for PP2B-Aβ$^{-/-}$ mice (mean ± SEM 724.8 × 10^3/mm^3 ± 55.1) were comparable to wild type (WT) mice (700.65 × 10^3/mm^3 ± 35.63).

We studied agonist induced PRP aggregation to assess the role of PP2B-Aβ in platelet function. Compared to the WT, PP2B-Aβ$^{-/-}$ platelets displayed increased aggregation to a low dose of protease-activated receptor 4 activating peptide (PAR4-AP), ADP and collagen (Figs. 3B, 3D and 3F). PP2B-Aβ$^{-/-}$ platelets also showed increased aggregation to collagen related peptide (CRP), an agonist for GPVI (not shown). Final aggregation to a range of low but not high concentrations of each agonist was significantly increased in PP2B-Aβ$^{-/-}$ platelets (Figs. 3C, 3E and 3G). Hyper aggregation response to additional agonist was also noted when studies were performed using washed platelets (Supplementary Fig. 1), suggesting that the enhanced phenotype was intrinsic to platelets. Integrin α$_{IIb}$β$_3$ levels were comparable in resting and agonist-activated WT and PP2B-Aβ$^{-/-}$ washed platelets and could not account for the increased platelet function in PP2B-Aβ$^{-/-}$ platelets (Fig. 4C).

Comparable inside-out signaling response in PP2B-Aβ$^{-/-}$ platelets

We evaluated soluble fibrinogen binding to assess whether the loss of PP2B-Aβ affected agonist-induced integrin α$_{IIb}$β$_3$ inside-out signaling. Agonist-induced soluble fibrinogen binding was comparable between the WT and the PP2B-Aβ$^{-/-}$ platelets (Fig. 4A). Consistent with these findings, the activation status of integrin α$_{IIb}$β$_3$, as assessed by murine α$_{IIb}$β$_3$ activation specific JON/A antibody was not altered between WT and the PP2B-Aβ$^{-/-}$ platelets (Fig. 4B). To evaluate if secretion of platelet granule contents was altered in PP2B-Aβ$^{-/-}$ platelets, we assessed agonist induced P-selectin (α granule) expression and ATP (dense granule) release. Expression of P-selectin induced by agonist stimulation was not significantly altered between the WT and the PP2B-Aβ$^{-/-}$ platelets (Fig. 4D). Likewise, the release of ATP was not significantly altered by the loss of PP2B-Aβ, despite a slight decreased trend in thrombin and collagen induced ATP secretion (Fig. 4E). Taken together, these studies indicate that agonist-induced α$_{IIb}$β$_3$ expression, α$_{IIb}$β$_3$ activation, soluble fibrinogen binding and secretion of both P-selectin and ATP was relatively unaffected by the loss of PP2B-Aβ.

Loss of PP2B-Aβ enhanced outside-in signaling

Next, we evaluated whether post-fibrinogen engagement events were altered in PP2B-Aβ^{−/−} platelets by assessing functional responses dependent on platelet cytoskeletal rearrangement. Compared to the WT platelets, PP2B-Aβ^{−/−} platelets displayed increased filopodial extensions on immobilized fibrinogen (Fig. 5A). Quantification of platelets from multiple studies revealed significant increase in the surface area for PP2B-Aβ^{−/−} platelets (Fig. 5B). To validate these findings, fibrin clot retraction, an independent outside-in signaling function was also examined. Compared to the WT, PP2B-Aβ^{−/−} platelets displayed enhanced clot retraction (Fig. 5C). Given the crucial role of Src in facilitating outside-in $\alpha_{IIb}\beta_3$ signaling [16, 17], we evaluated the phosphorylation of Src Tyr[418] within the kinase domain that promotes Src activity. Compared to platelets suspended over the BSA substrate, fibrinogen engagement stimulated Src Tyr[418] phosphorylation in WT platelets. In contrast, loss of PP2B-Aβ robustly stimulated basal Src Tyr[418] phosphorylation independent of integrin engagement, as evident by an increased Src phosphorylation of PP2B-Aβ^{−/−} platelets on BSA (Figs. 5D and 5E). Increased basal Src phosphorylation was also noticed in unstimulated PP2B-Aβ^{−/−} platelets (not shown). Since clot retraction and/or platelet spreading are dependent on p38 activation [18, 19], we also evaluated p38 signaling. Compared to the WT platelets, fibrinogen adhered PP2B-Aβ^{−/−} platelets displayed increased phosphorylation of residues Thr[180] and Tyr[182] of P38, a surrogate marker of its activation (Figs. 5F and 5G). To assess the relationship between the increased basal Src and fibrinogen-induced p38 activation in PP2B-Aβ^{−/−} platelets, we tested p38 activation in the presence of PP2, a Src inhibitor. Pretreatment with control DMSO did not alter fibrinogen-induced increased p38 activation in PP2B-Aβ^{−/−} platelets (Figs. 6A and 6B). However, compared to the control DMSO, pretreatment with PP2 blocked the increased p38 activation of PP2B-Aβ^{−/−} platelets (Figs. 6C and 6D).

Since platelet deposition at thrombus sites may involve platelet adhesion to immobilized fibrinogen, we studied platelet adhesiveness. Adhesion of PP2B-Aβ^{−/−} platelets to immobilized fibrinogen was enhanced when compared to WT platelets (Figure 7A). siRNA mediated depletion of PP2B-Aβ in human kidney embryonal 293 $\alpha_{IIb}\beta_3$ cells (Fig. 7B) also resulted in an increased adhesion to immobilized fibrinogen (Fig. 7C). Previous studies have identified that PP2B can dephosphorylate recombinant filamin A (FLNa) fragment at the C-terminal Ser[2152] [20]. FLNa is an actin cross linking protein that participates in cell adhesion, spreading, migration [21] and can also interact with integrins β_{1A}, β_2, β_3 and β_7 [22, 23, 24]. Therefore, we examined FLNa Ser[2152] phosphorylation in PP2B-Aβ^{−/−} platelets. Adhesion of WT platelets to immobilized fibrinogen led to the dephosphorylation of FLNa Ser[2152]. In contrast, the dephosphorylation of FLNa Ser[2152] was not evident in fibrinogen engaged PP2B-Aβ^{−/−} platelets (Figs. 7D and 7E). Taken together, these studies indicate that loss of PP2B-Aβ leads to an enhanced outside-in $\alpha_{IIb}\beta_3$ signaling via the Src-p38 axis.

PP2B-Aβ^{−/−} mice display prothrombotic phenotype in a FeCl$_3$ induced thrombosis model

Tail bleeding assays were performed to assess the consequence of the loss of PP2B-Aβ on hemostasis. Tail bleeding time(s) were not significantly different between the WT and PP2B-Aβ^{−/−} mice (Fig. 8A). Lastly, the contribution of PP2B-Aβ in *in vivo* thrombosis was

examined using a FeCl$_3$ induced carotid artery thrombosis model. Carotid artery of the WT and PP2B-Aβ was injured with 10% FeCl$_3$ and the time for complete occlusion was monitored by a probe that measures blood flow. PP2B-Aβ$^{-/-}$ mice revealed a moderate but significant reduction in the time required for complete and stable vessel occlusion (Fig. 8B). Thus, the loss of PP2B-Aβ potentiates thrombosis in a FeCl$_3$ injury model.

Discussion

Increased risk of thromboembolic complications in renal transplant patients on immunosuppressant drugs like CsA and FK506 has prompted investigators to examine the contribution of these immunosuppressants on platelet function. Enhanced platelet aggregation to adrenaline, ADP and collagen was noticed in CsA (250–500 ng/ml) treated PRP [25]. Platelet suspension devoid of plasma in the presence of 600 ng/ml of CsA or FK506 displayed increased aggregation and greater serotonin release to ADP, while CsA augmented ADP induced binding of radiolabelled fibrinogen to platelets [26]. CsA (200 μg/ml) also enhanced collagen induced platelet procoagulant response [27]. The hyper functional platelet phenotype in these studies was observed with high concentrations of CsA. Since the apparent IC$_{50}$ of CsA for PP2B inhibition is in the range of 10–100 nM, we performed studies with 10 nM CsA (~12 ng/ml) in washed platelets and noticed potentiation of aggregation response (Fig. 2). Although a prothrombotic phenotype for CsA or the ability of CsA to block PP2B is not disputed, the contribution of non-PP2B target of CsA (cyclophilins) cannot be ruled out in these studies. In a converse approach, addition of recombinant human PP2B-B regulatory subunit to rabbit or sheep PRP suppressed ADP-induced platelet aggregation [28]. To specifically identify the contribution of PP2B in platelet function, we utilized mice deficient in the β isoform of the catalytic subunit of PP2B. Other PP2B genetic models were not considered here because mice lacking the α isoform of the catalytic subunit PP2B (PP2B-Aα) die within three weeks of birth [29] and mice deficient in the α isoform of the regulatory subunit of PP2B (PP2B-Bα; Cnb1) are embryonically lethal [30].

Loss of PP2B-Aβ did not recapitulate the increased platelet counts displayed by the attenuation of NFAT-PP2B pathway [7]. Perhaps, this reflects a potential redundancy by the α isoform of PP2B. Although PP2B-Aβ$^{-/-}$ platelets displayed enhanced agonist-induced platelet aggregation (Fig. 3), agonist-induced α$_{IIb}$β$_3$ expression, activation, soluble fibrinogen binding and secretion of α and dense granule contents were not significantly altered in PP2B-Aβ$^{-/-}$ platelets (Fig. 4). These findings suggest that the β isoform of PP2B is not involved in agonist induced inside-out α$_{IIb}$β$_3$ signaling. Alternatively, the α isoform of PP2B may have compensated for the loss of PP2B-Aβ. Interestingly, PP2B-Aβ$^{-/-}$ platelets exhibited enhanced spreading, clot retraction and adhesion (Figs. 5 and 7). The ability of PP2B-Aβ to negatively regulate α$_{IIb}$β$_3$ adhesiveness was also recapitulated in α$_{IIb}$β$_3$ expressing model cells (Fig. 7), suggesting that this phenotype is intrinsic to PP2B-Aβ. Selective potentiation of outside-in but not inside-out signaling in PP2B-Aβ$^{-/-}$ platelets may suggest that the loss of PP2B-Aβ is unlikely to globally alter calcium homeostasis. Indeed, cyclophilin A but not PP2B was identified to be critical for Ca^{2+} reuptake by sarcoendoplasmic Ca^{2+} adenosine triphosphatase in platelets [31]. In contrast, loss of PP2B-

Aβ likely affects the cytoskeletal compartment by altering signaling that is initiated by the engagement of integrin $\alpha_{IIb}\beta_3$.

One such $\alpha_{IIb}\beta_3$ proximal signaling molecule is Src, whose activation facilitates anchorage dependent cellular function [16, 17]. Although integrin engagement activated Src in WT platelets, a substantial enhancement in basal Src activation was observed in the PP2B-Aβ$^{-/-}$ platelets. (Fig. 5). Activation of p38, which participates in platelet clot retraction and/or spreading [18, 19], was also markedly up regulated in fibrinogen engaged PP2B-Aβ$^{-/-}$ platelets (Fig. 5). Consistent with this observation, activation of PP2B-Aβ in cardiac myocytes was associated with down regulation of p38 activation [32]. Interestingly, the increased p38 activation in fibrinogen adhered PP2B-Aβ platelets was blocked by Src inhibitor (Fig. 6). These studies suggest that deregulated Src signaling caused by the loss of PP2B-Aβ was relayed to the p38 MAPK following integrin ligation.

Since outside-in signaling often regulates cytoskeletal rearrangement, we considered whether one or more cytoskeletal proteins, including FLNa may represent targets of PP2B-Aβ. Patients with filaminopathy A caused by truncating FLNa mutations displayed low levels of FLNa and increased platelet spreading on fibrinogen [33]. Consistent with the reports that cAMP dependent PKA phosphorylates FLNa Ser^{2152} [34, 35], we observed basal phosphorylation of FLNa Ser^{2152} in unstimulated platelets. Importantly, fibrinogen engagement for 30 minutes in WT but not PP2B-Aβ$^{-/-}$ platelet led to filamin dephosphorylation. Ser^{2152} phosphorylation of FLNa a) protects FLNa from calpain cleavage [34, 20], b) may facilitate integrin binding to filamin [36] and c) positively correlates with membrane ruffle formation and migration of melanoma cells [37]. FLNa also serves as a scaffold to recruit proteins such as Rho, Rac, Cdc42 that are involved in the remodeling of the cytoskeleton and Syk, which is required for outside-in signaling [21, 38]. It is possible that the increased FLNa Ser ^{2152} phosphorylation in fibrinogen engaged PP2B-Aβ$^{-/-}$ platelets might stabilize cytoskeleton and thereby support increased adhesion and spreading, by altering its interaction with one of more effectors or the interaction of FLNA with β3.

Two issues highlight potential caveats in our study: a) confounding effect of PP2B-Aα isoform in the PP2B-Aβ$^{-/-}$ platelets, b) global loss of PP2B-Aβ in hematopoietic cells other than platelets may influence the *in vivo* thrombosis studies. In conclusion, our studies support a mechanism whereby the loss of PP2B-Aβ has little effect on direct $\alpha_{IIb}\beta_3$ activation, but rather potentiates prothrombotic phenotype following post-fibrinogen occupancy via the Src-p38 axis. Thus, platelet activation at the site of vascular injury is coupled to a PP2B-Aβ driven signaling machinery, which limits platelet reactivity by dampening outside-in $\alpha_{IIb}\beta_3$ signaling. Harnessing the suppressive role of PP2B-Aβ may offer a new anti-thrombotic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

Human Platelets

B
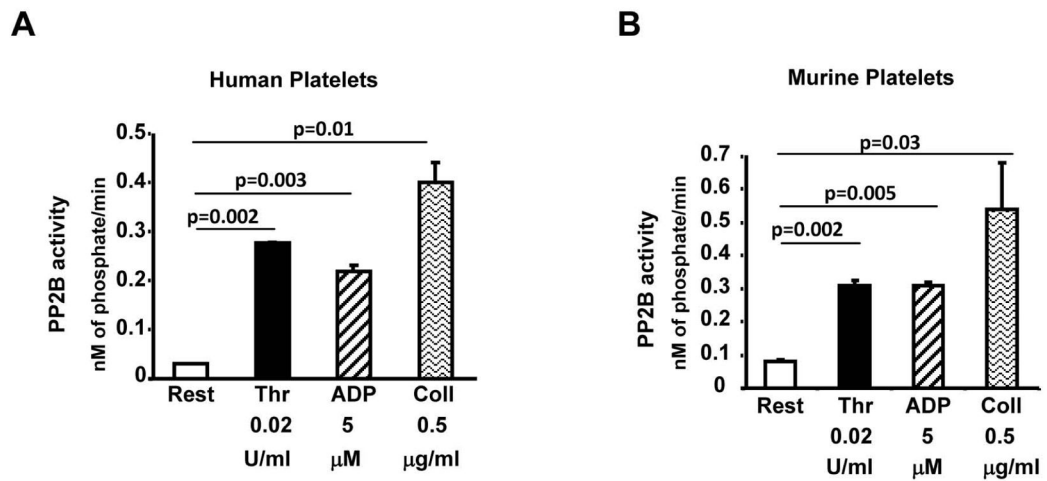
Murine Platelets

**Fig. 1. Agonist stimulation increased PP2B activity in platelets**

Washed platelets from human (**A**) or wild type murine (**B**) were maintained in the resting state (Rest) or stimulated with thrombin (thr; 0.02 U/ml), ADP (5 uM) or collagen (Coll; 0.5 µg/ml) for 10 minutes. PP2B activity in the lysate was evaluated using calcineurin cellular assay kit. Data are mean ± SE from 3–4 experiments.
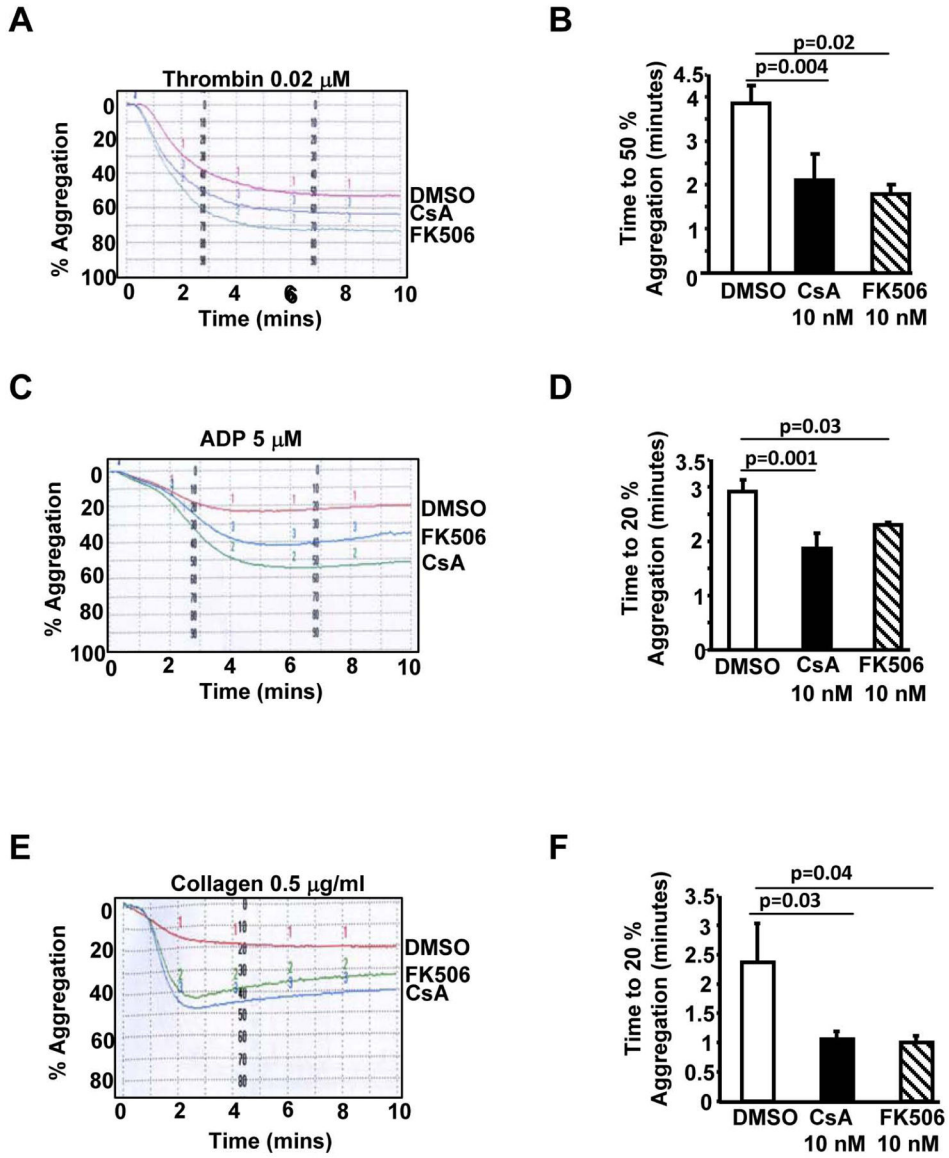
Fig. 2. PP2B inhibitors CsA and FK506 potentiated platelet aggregation
Washed human platelets were pretreated with DMSO, CsA (10 nM) or FK506 (10 nM) for 30 minutes prior to studying aggregation induced by thrombin (**A**) ADP (**C**) or collagen (**E**). Time to achieve 50% thrombin-induced aggregation (**B**) and 20% aggregation for ADP (**D**) or collagen (**F**) is documented. Data are mean ± SE from 4–5 subjects.
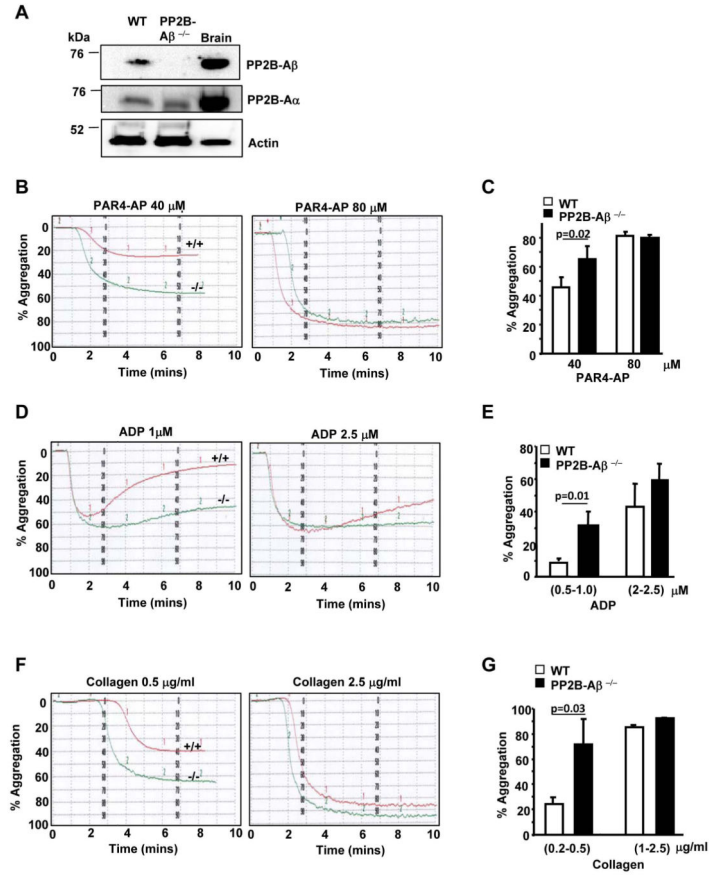
Fig. 3. PP2B-Aβ$^{-/-}$ platelets display enhanced aggregation to low dose agonist
(**A**) Lysate from wild type (WT), PP2B-Aβ$^{-/-}$ platelets or mouse brain (brain; positive control) were immunoblotted with PP2A-β, PP2B-Aα and actin antibodies. (**B**) PRP from WT or PP2B-Aβ$^{-/-}$ mice was challenged with PAR4-AP, ADP (**D**) and collagen (**F**). Final percentage aggregation to varying concentrations of PAR4-AP (**C**), ADP (**E**) and collagen (**G**) is indicated as mean ± SE from 5–6 experiments.
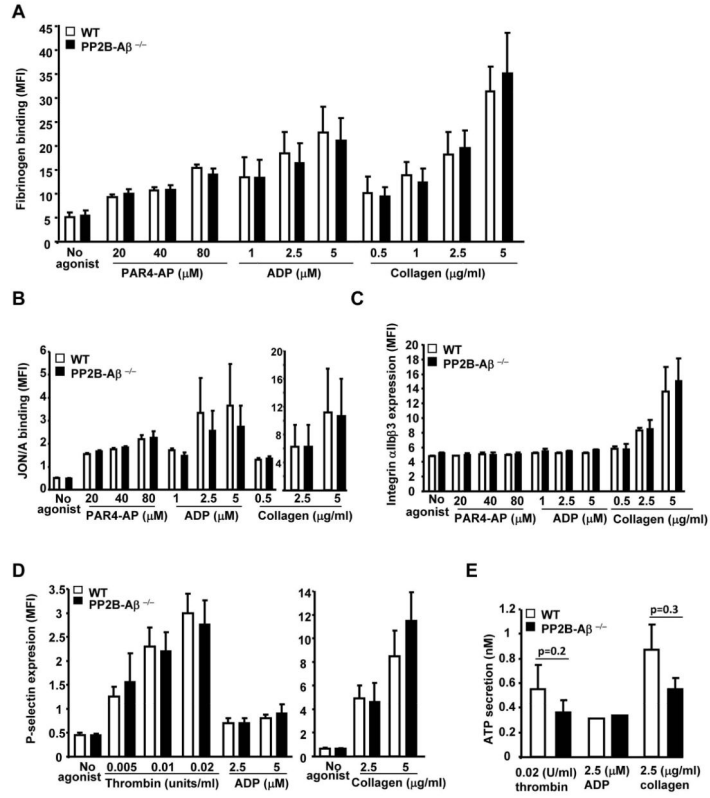
Fig. 4. Inside-out signaling to $\alpha_{IIb}\beta_3$ and granule secretion is normal in PP2B-A$\beta^{-/-}$ platelets
Washed platelets from WT and PP2B-A$\beta^{-/-}$ mice were stimulated with PAR4-AP, ADP and collagen in the presence of (**A**) Alexa 488 fibrinogen. Bound fibrinogen was measured by flow cytometry as mean fluorescence intensity (MFI) (N=6–7). Fibrinogen binding in WT and PP2B-A$\beta^{-/-}$ platelets was blocked by addition of EDTA (not shown). Washed platelets were incubated with anti-JON/A PE antibody (N=4–6) (**B**), anti-αIIb FITC antibody (N=3) (**C**) and anti-P-selectin antibody (N=3–6) (**D**) and MFI measured by flow cytometry. Values from isotype control antibodies were subtracted to obtain MFI. (**E**). ATP release was studied in washed platelets by measuring luciferin/luciferase stimulated with thrombin and collagen (N=3–6), and ADP (N=2).
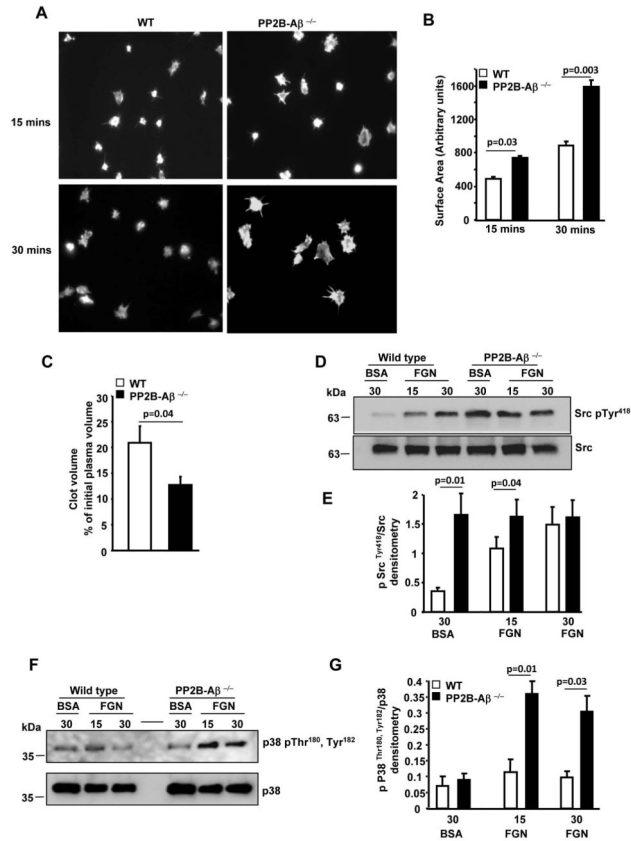
Fig. 5. Outside-in signaling to $\alpha_{IIb}\beta_3$ is enhanced in PP2B-A$\beta^{-/-}$ platelets
(**A**) Washed platelets were added on immobilized fibrinogen for 15 and 30 minutes and actin fibers stained with rhodamine phallodine. (**B**) Surface area of 50 platelets spread on fibrinogen from 5–6 experiments was quantified. (**C**) Fibrin clot retraction was evaluated following addition of thrombin to PRP and volume of the clot expressed as ± SEM of 6 experiments. Lysate of platelets suspended on BSA or adhered on immobilized fibrinogen (FGN) was immunoblotted with anti-phospho Src Tyr^{418} and Src antibodies (**D**) or phospho-Thr^{180}, Tyr^{182} and p38 antibodies (**F**). Quantification of Src activation (**E**) or p38 activation (**G**) from 4–5 experiments.
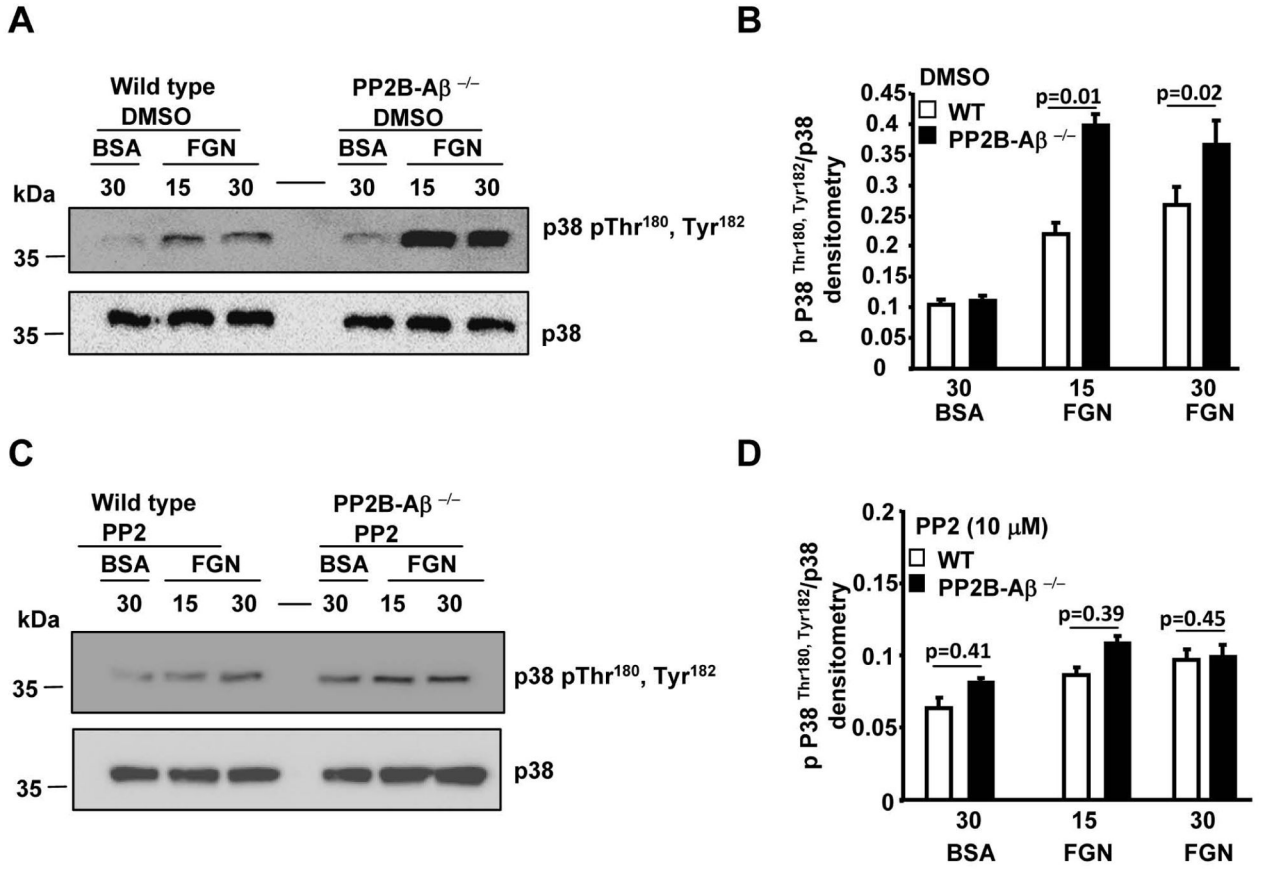
Fig. 6. Increased p38 activation in fibrinogen adhered PP2B-Aβ$^{-/-}$ platelets is blocked by Src inhibitor

Platelets pretreated with DMSO (**A** and **B**) or with 10 μM PP2 (**C** and **D**) were either suspended on BSA or adhered on FGN. Lysate was immunoblotted with anti-phospho-Thr^{180}, Tyr^{182} and p38 antibodies (**A** and **C**). Quantification of p38 activation from (**B** and **D**) from 4 experiments.
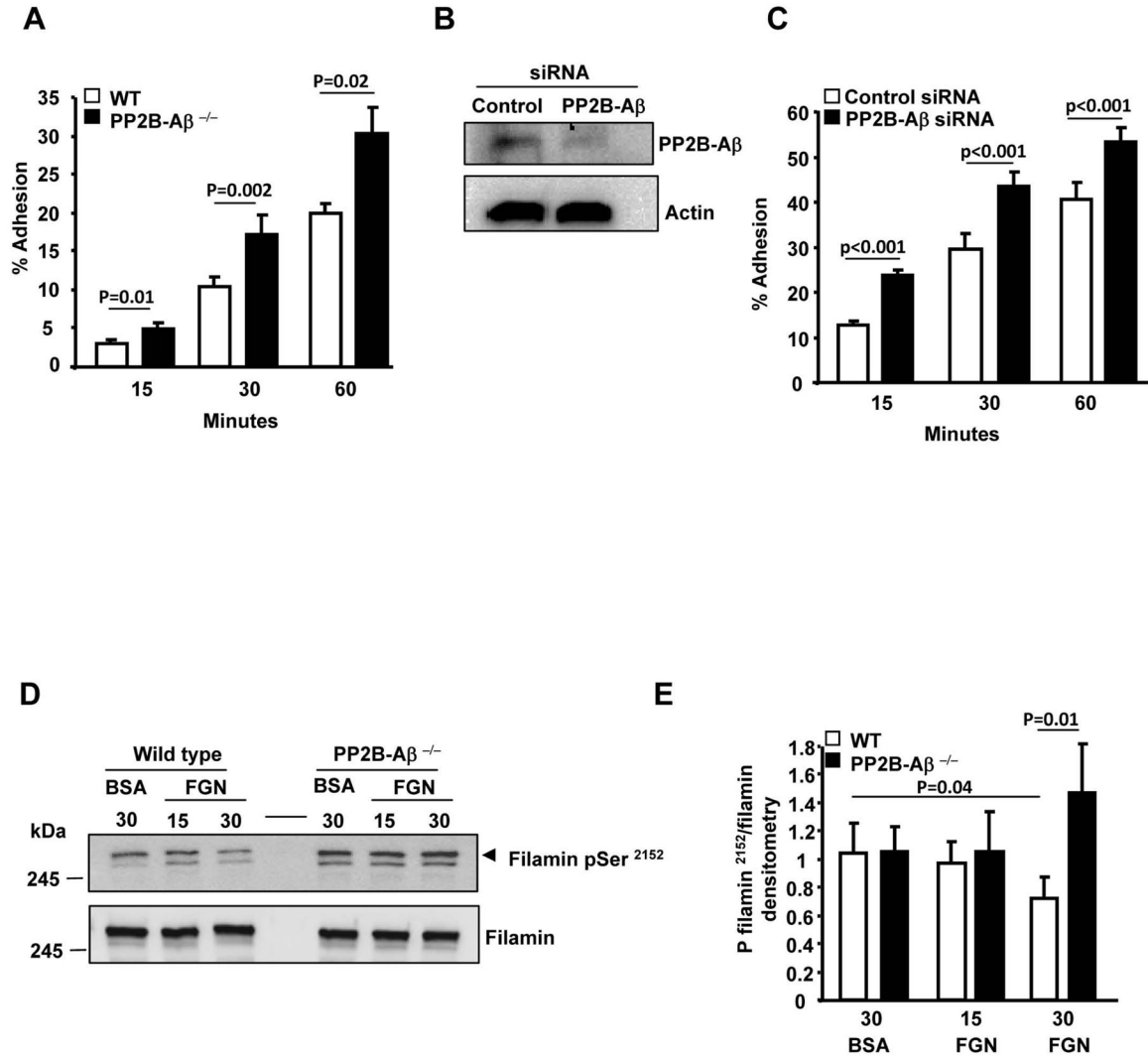
Fig. 7. Increased adhesiveness and phosphorylation of filamin A on Ser^{2152} in PP2B-Aβ$^{-/-}$ platelets

(**A**) Washed platelets were incubated with immobilized fibrinogen and adhesion quantified. Data are expressed as ± SEM of 4 experiments. (**B**) Lysate from the control siRNA and PP2B-Aβ depleted 293 α$_{IIb}$β$_3$ cells were immunoblotted with anti-PP2B-Aβ and anti-actin (loading). (**C**) Adhesion of 293 α$_{IIb}$β$_3$ cells treated with control or PP2B-Aβ siRNA to fibrinogen. Data are expressed as ± SEM of 5 experiments. (**D**) Lysate of platelets suspended on BSA (30 mins) or adhered on immobilized fibrinogen was immunoblotted with anti-phospho FLNa Ser^{2152} and FLNa antibodies (**E**) Quantification of FLNa phosphorylation from 4 experiments.
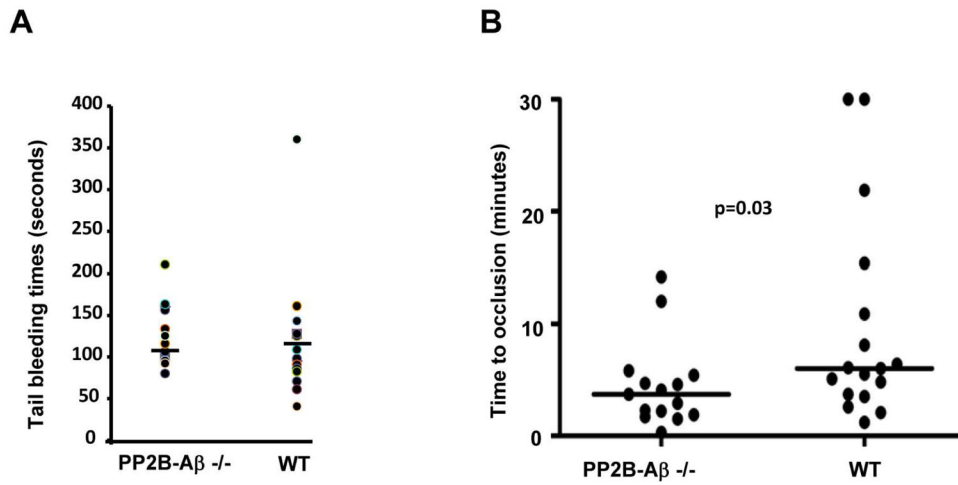
Fig. 8. Contribution of PP2B-Aβ in hemostasis and thrombosis models

(**A**) Tail vein bleeding time(s) from 15 WT and PP2B-Aβ$^{-/-}$ mice. The horizontal line depicts average time (126 ± 9 seconds for PP2B-Aβ$^{-/-}$ and 117 ± 17 seconds for WT mice) (**B**) Carotid arteries from 17 WT and PP2B-Aβ$^{-/-}$ mice were subjected to a FeCl$_3$ injury and the time to vessel occlusion measured. Median time (horizontal line) to occlusion is shown. The mean occlusion time for PP2B-Aβ$^{-/-}$ mice was 4.5 ± 1 minutes while that of WT was 9.6 ± 2.2 minutes.