

RAP1-RHO small GTPase cross-talk mediates integrin-dependent and -independent platelet procoagulant response

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Abstract

Platelet adhesion and procoagulant activity are critical for primary and secondary hemostasis, respectively. The small GTPase RAP1 is a central regulator of platelet aggregation as it controls α IIb β 3 integrin activation through direct interaction with the integrin adapter protein, TALIN1 (TLN1). In addition to their aggregation defect, activated platelets lacking RAP1 (*Rap1*^{mKO}) exhibited a marked impairment in surface exposure of phosphatidylserine (PtdSer), a negatively charged phospholipid with procoagulant activity. However, the mechanisms by which RAP1 regulates PtdSer exposure are unclear. Here we investigated the hypothesis that RAP1 regulates platelet PtdSer exposure through cross-talk with small GTPases of the RHO family. Consistent with their defect in PtdSer exposure, *Rap1*^{mKO} platelets showed reduced procoagulant activity *in vitro* and *in vivo* when compared to controls. Stimulated *Rap1*^{mKO} platelets exhibited elevated RHOA-GTP levels, and inhibition of the RHOA effector, Rho associated coiled-coil kinase (ROCK), partially restored PtdSer exposure in these cells. A milder defect in PtdSer exposure was observed for platelets from *Tln1*^{mR35/118E} mice, i.e., mice with impaired RAP1-TLN1 interaction but otherwise intact RAP1 signaling. ROCK inhibition fully restored PtdSer exposure in *Tln1*^{mR35/118E} platelets. Opening of the mitochondrial permeability transition pore, a cellular response critical to PtdSer exposure, was impaired in *Rap1*^{mKO} platelets and restored by pretreatment of cells with the ROCK inhibitor. Our study provides first evidence that platelet RAP1 signaling affects hemostatic plug formation independent of its key role in platelet adhesion. Additionally, our studies strongly suggest that RAP1 regulates PtdSer exposure and procoagulant activity in a RHOA/integrin-dependent and -independent manner.

Introduction

Within a hemostatic plug there are two classifications of activated platelets: proadhesive and procoagulant.¹ Proadhesive platelets are characterized by high affinity integrin receptors, such as α IIb β 3, which mediate platelet adhesion to the site of injury and platelet aggregation via binding of fibrinogen and other ligands.² Integrin-mediated platelet adhesion is essential to formation of the initial platelet plug to cease bleeding. Procoagulant platelets facilitate thrombin generation and thus enhance the formation of fibrin, a fibrous protein critical for hemostatic plug stability.

Characteristic to procoagulant platelets is exposure of the negatively charged phospholipid, phosphatidylserine (PtdSer), on the cell surface.³ Procoagulant platelet formation is dependent on sustained high cytosolic calcium levels and mitochondrial depolarization, mediated by the opening of the mitochondrial permeability transition pore (MPTP) via its essential adaptor protein cyclophilin D (CypD).^{4,5} Depolarization of the mitochondria ultimately allows the scramblase transmembrane protein 16F (TMEM16F) to flip PtdSer to the outer plasma membrane leaflet.^{6,7} Platelet activation is a tightly controlled process with small GTPases playing a central role.⁸⁻¹⁰ Small GTPases are mo-

lecular switches which are active in their GTP-bound state and inactive in their GDP-bound state. When GTP bound, small GTPases undergo conformational changes to interact with effector proteins.¹¹ The role of small GTPases in α IIb β 3 integrin activation is well-defined, while their activity in procoagulant platelet formation remains less explored.

The Ras family GTPase, RAP1, is the most abundant small GTPase in platelets.⁹ RAP1 is a central regulator of platelet adhesion/aggregation as loss of both isoforms (RAP1a and RAP1b) markedly impairs integrin activation, resulting in significantly prolonged bleeding times.¹² Activation of α IIb β 3 integrin requires direct interaction between RAP1 and its effector protein TLN1.¹³⁻¹⁵ Disruption of the interaction between RAP1 and TLN1 (*Tln1*^{mR35/118E}) results in loss of α IIb β 3 integrin activation while retaining other RAP1 signaling responses.¹³ Ligand binding to α IIb β 3 integrin induces outside-in signaling, an important mechanism to potentiate platelet activation.^{16,17} Loss of RAP1 also results in impaired platelet procoagulant response,¹³ although the mechanism of RAP1-mediated PtdSer exposure is unclear. The RHO family GTPase, RHOA, is primarily known for its role in cytoskeletal rearrangements.^{10,18} Activation of RHOA occurs downstream of G₁₃-coupled receptors, whereas inhibition of RHOA results from integrin outside-in signaling.^{19,20} In contrast to RAP1, inhibition of RHOA-ROCK (Rho associated coiled-coil kinase) signaling leads to increased PtdSer exposure.²¹ Crosstalk between RAP1 and RHOA was demonstrated for other cell types;^{22,23} however, the potential role of RAP1-RHOA crosstalk in platelet procoagulant response remains unexplored.

In the present study, we investigated the mechanism of RAP1-mediated PtdSer exposure in platelets. Our studies demonstrate that RAP1-mediated PtdSer exposure occurs through integrin-dependent and -independent mechanisms, and that the integrin-dependent mechanism occurs through a connection to RHOA.

Methods

Mice

Generation of *Rap1*^{mKO,12}, *Tln1*^{mR35/118E,13}, IL4R α -GPIb α -tg²⁴ and *CypD*^{-/-25} mice has been previously described. Experimental procedures were approved by the Institutional Animal Care and Uses Committee.

Four-dimensional saphenous vein laser injury model

Adoptive transfer of platelets into thrombocytopenic mice was performed as previously described.^{26,27} In brief, blood was collected in phosphate-buffered saline with heparin and platelets were washed.²⁶ Platelets were depleted with α -hIL4R antibody (2.5 μ g/g body weight) in IL4R α /GPIb α -tg mice. Washed platelets were labeled with AlexaFluor (AF)488 or AF647-labeled antibodies to GPIX and administered via retroorbital injection into platelet-depleted

IL4R α /GPIb α -tg mice to a final circulating concentration of 2-5x10⁸ platelets/mL. Four-dimensional (4D) imaging of saphenous vein laser injury was performed as recently described.²⁷ In brief, the saphenous vein was exposed and relevant fluorescently labeled antibodies were administered via retroorbital injection. Laser ablation was used to create a perforating injury in the vein and spinning disk confocal imaging was performed on a Zeiss Axio Examiner Z1 inverted spinning disk confocal microscope (4x4 binning, 7.5 μ m step, 150 μ m total travel). Images were acquired with SLIDEBOOK 6.0 software (Intelligent Imaging Innovations). Image analysis was performed using ImageTank software (Visual Data Tools, Inc) as previously described.²⁸

Human blood collection

Whole blood was collected from healthy subjects (male and female subjects between the ages of 20 and 50 years who had not taken aspirin/non-steroidal anti-inflammatory drugs within 2 weeks) using a 21-gauge needle vacutainer butterfly into 3.2% citrate tubes (BD). Blood collection from healthy donors was performed with informed consent in accordance with a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill.

Flow cytometry

Platelets were washed as previously described¹² and activated with the indicated concentrations of convulxin (CVX; purchased from Kenneth Clemetson, Theodor Kocher Institute, University of Bern, Bern, Switzerland) and PAR4p (GL Biochem) in the presence of 2 μ g/mL JON/A-PE (clone Leo. H4, Emfret Analytics), α -P-selectin-FITC (clone RB40.34; BD Biosciences), and Annexin V-AF647 (generous gift from Sriram Krishnaswamy, Children's Hospital of Philadelphia). Where indicated, samples were incubated with ROCK inhibitor (20 μ M Y-27632, Tocris) for 10 minutes (min) prior to activation. After 15 min of incubation, samples were diluted and analyzed via flow cytometry (Accuri C6 Plus flow cytometer; BD Biosciences).

RAP1 and RHOA activation assays

Washed platelets (260 μ L samples at 8x10⁸/mL) were stimulated with 50 ng/mL CVX and 250 μ M PAR4p in aggregometry. Platelets were lysed with cold 2x lysis buffer (100 mmol/L Tris/HCl pH 7.4, 400 mmol/L NaCl, 5 mmol/L MgCl₂, 2% Nonidet P-40, 20% glycerol and protease inhibitor cocktail lacking ethylenediaminetetraacetic acid; Roche). Lysates were incubated with RalGDS-RBD beads for RAP1-GTP (Millipore, Billerica, MA) or Rhotekin-RBD beads for RHOA-GTP (Cytoskeleton) for 1 hour at 4°C. Beads were washed three times then resuspended in 2x Laemmli buffer for detection of RAP1-GTP or RHOA-GTP via standard western blotting procedure. For loading controls, 50 μ L of the platelet sample was combined with 50 μ L 2x Laemmli buffer (75 mmol/L Tris/HCl, pH 6.8, 2% sodium dodecyl

sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue). Antibodies to RAP1 (clone 121; Santa Cruz, cat # sc-65) or RHOA (clone 55; Sigma, cat # 05-778) were used for detection of GTP-bound and total RAP1 or RHOA, respectively.

Thrombin generation assay

Thrombin generation assay was performed in a 96-well plate; 0.5 pM TF (Innovin) and 200 ng/mL CVX were added, and wells were brought to volume (30 μ L) with Tyrodes buffer containing 1 mM CaCl₂. Platelet-poor plasma (PPP) or platelet-rich plasma (PRP, platelet count 5x10⁸/mL) was added to initiate the reaction. Each sample was calibrated with α 2-macroglobulin-thrombin complex calibrator (Diagnostica Stago Inc Fluca Kit). Fluorescence was measured on a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Waltham, MA) with the Ascent Software (version 2.6, Thermo Fisher Scientific) at 37°C.

JC-1 mitochondrial depolarization assay

Platelets were washed and adjusted to 7.5x10⁸/mL. JC-1 dye (1 μ L; Invitrogen,) and washed platelets (20 μ L) were added to 180 μ L of Tyrode's buffer with bovine-serum albumin and incubated at 37°C for 10 min; 30 μ L of labeled platelets were transferred to 70 μ L of Tyrode's buffer containing 2 mM CaCl₂. Platelets were activated with 100 μ L of 2x agonist and 2.5 μ g/mL Annexin V-AF647 in Tyrode's buffer containing 2 mM CaCl₂, and fluorescence intensities were recorded on a BD Accuri C6 Flow Cytometer. Analysis was completed using FlowJo (FlowJo LLC).

Fluo-4 calcium mobilization assay

Platelets were washed and adjusted to 1x10⁹/mL. Fluo-4 dye (Life Technologies) was diluted to a concentration of 0.5 mM in dimethyl sulfoxide (DMSO). Platelets (1x10⁸/mL in 200 μ L Tyrode's buffer) were labeled with 1 μ L of Fluo-4

dye for 30 min at 37°C. After incubation, 800 μ L of Tyrode's buffer was added to labeling reaction. Labeled platelets were diluted (1:1) in Tyrode's buffer with 4 mM CaCl₂. Cellular stimulation was induced by addition of a 2x agonist solution. Fluorescence intensity (Fl1) was recorded on a BD Accuri C6 Flow Cytometer. Analysis was completed using FlowJo (FlowJo LLC).

Statistics

Results are shown as mean \pm standard error of the mean (SEM). Unless otherwise indicated, statistical significance was analyzed via Welch's *t* test.

Results

Decreased phosphatidylserine exposure and thrombin generation potential in RAP1-deficient platelets *in vitro*

Dual-agonist stimulation of platelet glycoprotein (GP)VI and protease-activated receptor (PAR)4 results in robust PtdSer exposure *in vitro*.³ Therefore, procoagulant response was stimulated with convulxin (CVX; GPVI) and PAR4-activating peptide (PAR4p) and measured via Annexin V binding using flow cytometry (% PtdSer+ events). Compared to controls, exposure of PtdSer was significantly impaired in platelets lacking RAP1 (*Rap1*^{mKO}) activated with CVX/PAR4p (Figure 1A). Using a modified thrombin generation assay²⁹ in which activated procoagulant platelets in PRP provide the negatively charged phospholipid surface required for coagulation factor assembly, we evaluated the contribution of RAP1-mediated PtdSer exposure to thrombin generation *in vitro*. Thrombin generation was minimal in PPP when compared to PRP samples (Figure 1B, C). Congruent with the defect in PtdSer exposure, thrombin generation, defined as peak thrombin (nM), in *Rap1*^{mKO} PRP was significantly reduced when compared to control PRP (Figure 1B,

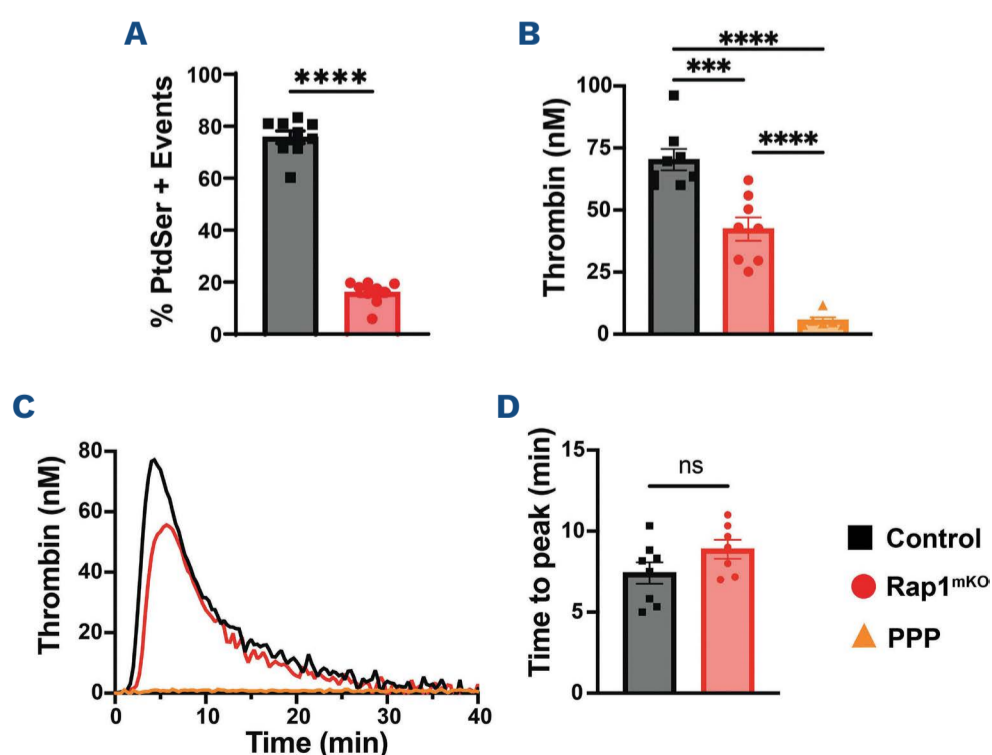


Figure 1. RAP1-mediated phosphatidylserine exposure contributes to thrombin generation *in vitro*.

(A) Flow cytometry analysis of procoagulant response (percent [%] phosphatidylserine-positive [PtdSer+] events [Annexin V binding]) in control or *Rap1*^{mKO} platelets stimulated with 50 ng/mL CVX + 250 μ M Par4p; N=9. (B-D) Thrombin generation. (B) Peak thrombin (nM) levels for control platelet-rich plasma (PRP), *Rap1*^{mKO} PRP, and platelet-poor plasma (PPP). (C) Representative curves for indicated samples. (D) Time to peak thrombin generation for control and *Rap1*^{mKO} PRP; N=7-8. *****P*<0.0001; NS: not significant; mKO: megakaryocyte/platelet-specific knockout.

C); however, there was no significant difference in time to peak (Figure 1D).

Impaired procoagulant activity of RAP1-deficient platelets *in vivo*

We recently developed a new imaging model to quantify platelet procoagulant activity during hemostatic plug formation *in vivo*.²⁷ In this model, perforating injuries ~50 μ m in diameter are induced to the saphenous vein by laser injury, and the three-dimensional accumulation of platelets and fibrin is monitored in real time by spinning disk confocal microscopy. Using this model, we were able to demonstrate that fibrin accumulation is significantly impaired in mice lacking CypD in platelets only, demonstrating that platelets are the main procoagulant cellular surface during hemostatic plug formation.²⁷ One limitation of the model is difficulties visualizing and measuring hemostatic plug components in mice with excessive bleeding, including *Rap1*^{mKO} mice. To circumvent this limitation and determine the contribution of RAP1 to platelet procoagulant activity *in vivo*, we used an adoptive platelet transfer strategy to generate mice with specific defects in platelet function without excess bleeding: (i) mice that received a mixture of *CypD*^{-/-} and wild-type (WT) platelets at a ratio of three to one, and (ii) mice that received a mixture of *CypD*^{-/-} and *Rap1*^{mKO} platelets at a ratio of three to one. *CypD*^{-/-} platelets were co-transfused to facilitate hemostatic plug formation in the context of RAP1-deficiency (*Online Supplementary Figure S1*). *CypD*^{-/-} platelets, however, have minimal procoagulant activity in this model.²⁷ Thus, fibrin accumulation at sites of injury in co-transfused mice would depend on the procoagulant activity of WT or *Rap1*^{mKO} platelets. As shown

in Figure 2, fibrin accumulation was significantly reduced in mice transfused with *CypD*^{-/-}/*Rap1*^{mKO} platelets when compared to mice transfused with *CypD*^{-/-}/WT platelets. Together, these studies demonstrate a critical role for RAP1 in platelet procoagulant activity *in vitro* and *in vivo*.

Integrin-dependent and -independent mechanisms of RAP1-mediated phosphatidylserine exposure

Given the documented role of integrin outside-in signaling in PtdSer exposure,³⁰ we next determined whether the defect in platelet procoagulant response observed in *Rap1*^{mKO} platelets is secondary to their defect in α IIb β 3 integrin activation. We compared the response to dual-agonist stimulation in *Rap1*^{mKO} platelets to platelets from mice with impaired RAP1-TLN1 interaction (*Tln1*^{mR35/118E}). Consistent with previous studies with single agonists, both *Rap1*^{mKO} and *Tln1*^{mR35/118E} platelets exhibited a marked defect in α IIb β 3 integrin activation (JON/A-PE binding) in response to dual-agonist stimulation (Figure 3A, D), while granule secretion (CD62P surface expression) was not impaired (Figure 3B, E). Dual agonist-induced PtdSer exposure was also significantly impaired in *Rap1*^{mKO} and *Tln1*^{mR35/118E} platelets (Figure 3C, F). Interestingly, PtdSer exposure was reduced by 85% in *Rap1*^{mKO} platelets, while only a 48% reduction in PtdSer-positive events was observed for *Tln1*^{mR35/118E} platelets compared to controls. Thus, these studies suggested that RAP1 facilitates platelet PtdSer exposure by integrin-dependent and -independent mechanisms.

Integrin outside-in signaling negatively regulates the activation state of the small GTPase RHOA,³¹ and previous work demonstrated that inhibition of RHOA signaling leads to increased PtdSer exposure in platelets.²¹ Thus, we hy-

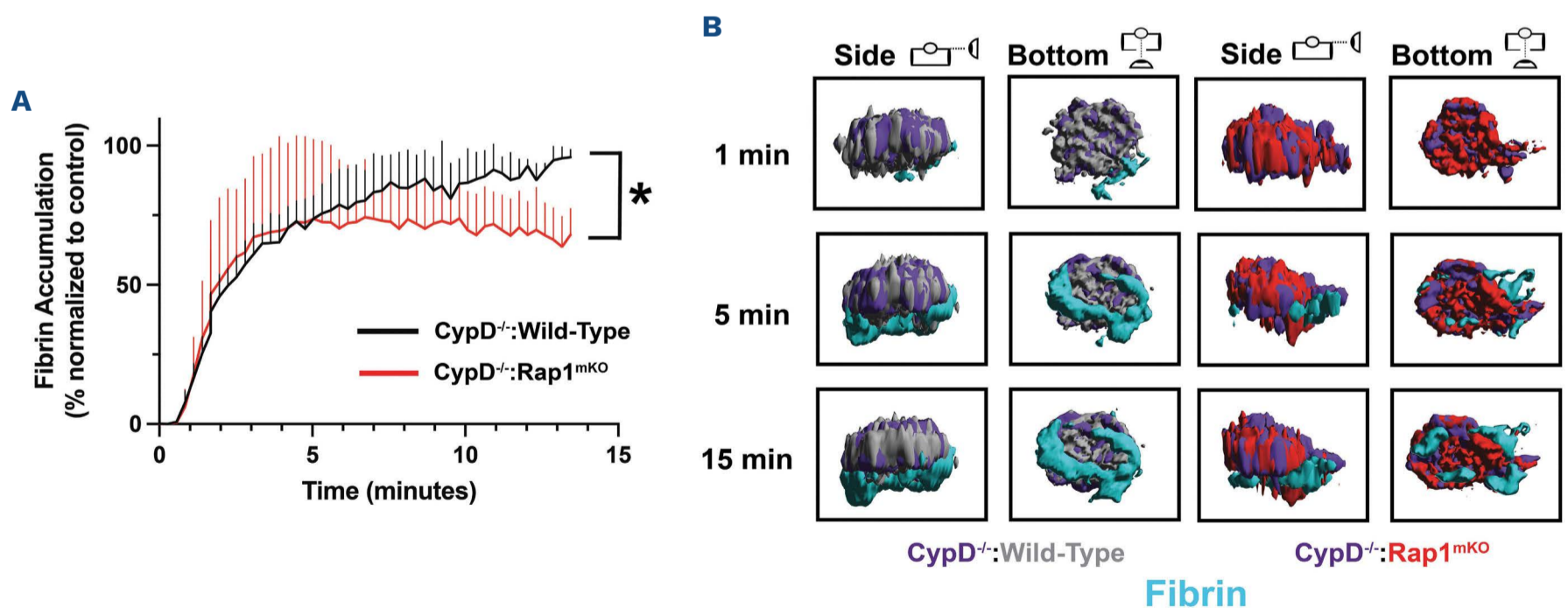


Figure 2. Reduced procoagulant activity of *Rap1*^{mKO} platelets *in vivo*. (A) Fibrin accumulation at sites of laser injury in mice transfused with *CypD*^{-/-} and *Rap1*^{mKO} platelets (N=21 injuries; 3 mice) compared to mice transfused with *CypD*^{-/-} and wild-type platelets (N=20; 3 mice). Data are shown as sum fluorescence intensity \pm standard error of the mean; **P*<0.05. (B) Representative images of hemostatic plugs in mice transfused with *CypD*^{-/-} (purple) and wild-type (grey) or *CypD*^{-/-} (purple) and *Rap1*^{mKO} (red) platelets. Fibrin is shown in cyan. Images are presented with side and bottom views at indicated time points. min: minutes; mKO: megakaryocyte/platelet-specific knockout.

pothesized that impaired PtdSer exposure in *Rap1^{mKO}* platelets results, at least in part, from elevated RHOA activity. Consistent with this hypothesis, we observed significantly increased RHOA-GTP levels in *Rap1^{mKO}* and *Tln1^{mR35/118E}* platelets at 2 min after addition of agonists (Figure 4A, B). We next studied dual agonist-induced platelet activation in *Rap1^{mKO}* and *Tln1^{mR35/118E}* platelets pretreated with an inhibitor of the RHOA effector protein, ROCK.²¹ Treatment with the ROCK inhibitor (Y-27632) did not significantly affect granule secretion or α IIb β 3 activation in controls, *Rap1^{mKO}*, or *Tln1^{mR35/118E}* platelets (Online Supplementary Figure S2). However, inhibition of ROCK partially recovered PtdSer exposure in *Rap1^{mKO}* platelets (Figure 4C). Importantly, ROCK inhibition fully restored PtdSer exposure in *Tln1^{mR35/118E}* platelets (Figure 4D). Previous studies demonstrated that sustained RAP1 activation in human and murine platelets depends on feedback activation via the ADP receptor, P2Y₁₂.⁹ We thus studied PtdSer exposure in platelets pretreated with the P2Y₁₂ inhibitor, 2-MeSAMP. Following dual agonist stimulation, PtdSer exposure was significantly reduced in murine (Online Supplementary Figure S3A) and human (Online Supplemental Figure S3B) platelets pretreated with 2-MeSAMP when compared to vehicle treated controls. The defect in PtdSer exposure induced by 2-MeSAMP was reversed by preincubation of platelets with Y-27632.

Calcium mobilization is not affected in *Rap1^{mKO}* platelets

One of the key factors to successful platelet procoagulant response is a robust and sustained increase in cytosolic

calcium levels, which is required to trigger mitochondrial depolarization.^{3,5} To determine cytosolic calcium levels, control or *Rap1^{mKO}* platelets were labeled with the calcium-sensitive dye, Fluo-4,⁵ and activated in the presence or absence of ROCK inhibitor. Dual-agonist stimulation resulted in sustained high calcium levels in control and *Rap1^{mKO}* platelets, both in the presence and absence of ROCK inhibitor (Figure 5A). The integrated calcium signal (area under the curve) was comparable between *Rap1^{mKO}* and control platelets. Slightly increased calcium levels were observed for both control and *Rap1^{mKO}* platelets activated in the presence of ROCK inhibitor (Figure 5B). Under these experimental conditions, *Rap1^{mKO}* platelets had decreased PtdSer exposure compared to controls; and inhibition of RHOA/ROCK signaling led to a significant increase in PtdSer-positivity for control and *Rap1^{mKO}* platelets, similar to Figure 4C. Together, these studies suggest that altered calcium mobilization does not account for the observed differences in PtdSer exposure observed in *Rap1^{mKO}* platelets.

Mitochondrial depolarization is decreased in *Rap1^{mKO}* platelets and partially recovered by inhibition of RHOA/ROCK signaling

Opening of the MPTP and mitochondrial depolarization are critical events for platelet procoagulant response.^{4,32} We next studied mitochondrial depolarization in dual agonist-stimulated platelets using the JC-1 flow cytometry-based assay.³³ Mitochondrial depolarization was significantly impaired in *Rap1^{mKO}* platelets when compared to controls (Figure 6A,

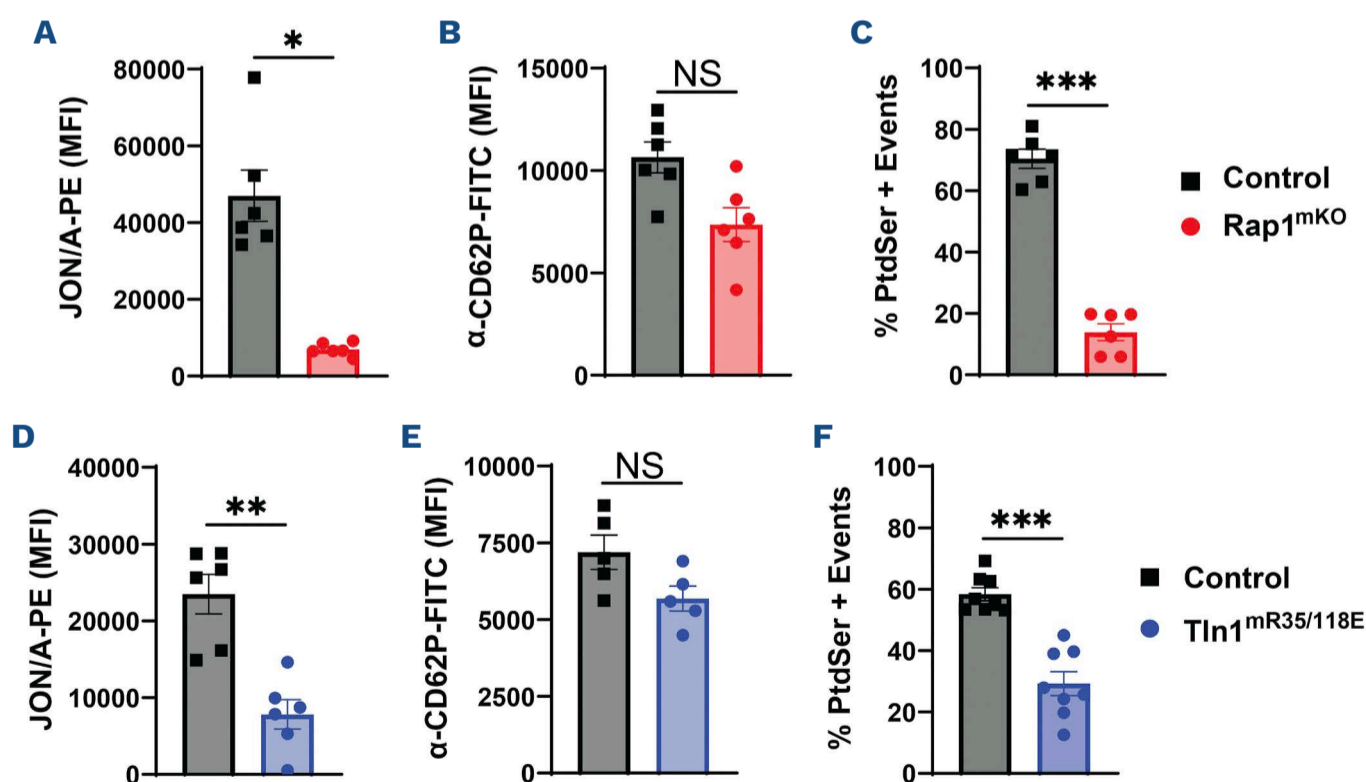


Figure 3. RAP1 affects platelet phosphatidylserine exposure via integrin independent and dependent mechanisms. (A-C) Flow cytometry analysis of α IIb β 3 activation (JON/A-PE MFI normalized to platelet size) (A), granule secretion (α -CD62P-FITC mean fluorescence intensity (MFI) normalized to platelet size) (B), and phosphatidylserine (PtdSer) exposure (Annexin V-AF647, % positive events) (C) for control or *Rap1^{mKO}* platelets stimulated with 50 ng/mL CVX + 250 μ M Par4p (N=6). (D-F) Flow cytometry analysis of α IIb β 3 activation (JON/APE) (D), granule secretion (α -CD62P-FITC) (E), and PtdSer exposure Annexin V-AF647 (F) for control or *Tln1^{mR35/118E}* platelets stimulated with 50 ng/mL CVX + 250 μ M Par4p (N=8). * P <0.05; ** P <0.01; *** P <0.001; NS: not significant; mKO: megakaryocyte/platelet-specific knockout.

B). Pretreatment with ROCK inhibitor led to markedly increased mitochondrial depolarization in both control and *Rap1^{mKO}* platelets (Figure 6A, B).

Discussion

Procoagulant platelets, i.e., platelets exposing PtdSer on their outer plasma membrane, are critical to thrombin/fibrin generation and hemostatic plug stability. Small GT-

Pases of the RAS (RAP1) and RHO (RHOA, RAC1) families are known regulators of PtdSer exposure in platelets. Our study provides first evidence for RAP1-RHO cross-talk required for platelet PtdSer exposure and platelet-dependent thrombin generation, both *in vitro* and *in vivo* (Figure 7). RAP1 is an essential regulator of α IIb β 3 integrin-mediated platelet adhesion and hemostatic plug formation.^{12,13} While no patients with loss-of-function mutations in RAP1 have been identified, mutations in CalDAG-GEFI (*RASGRP2*), an important regulator of RAP1 activation, are known. These

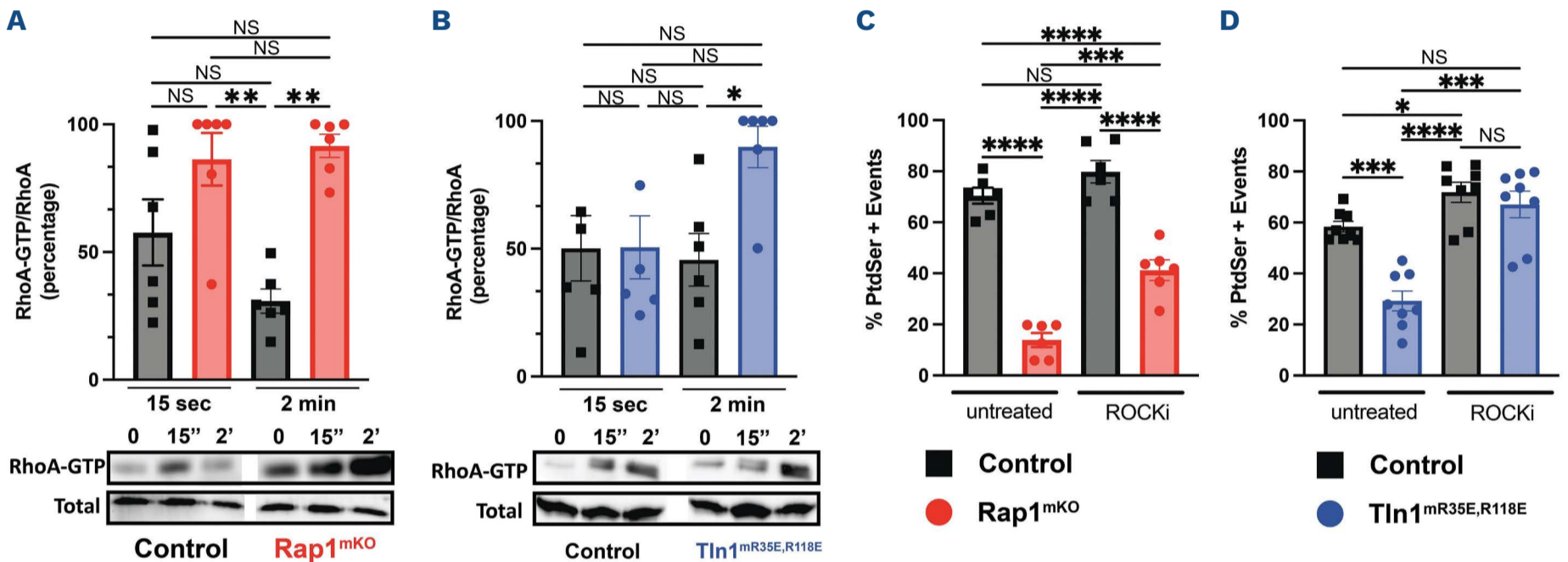


Figure 4. Integrin-dependent RAP1-RHOA connection regulates phosphatidylserine exposure. (A) Active (GTP-bound) RHOA over total RHOA in control (black bars) or *Rap1^{mKO}* platelets (red bars) activated for the indicated times with 50 ng/mL CVX + 250 μ M Par4p (N=5). Percentages normalized to highest value. (B) Active (GTP-bound) RHOA over total RHOA in control (black bars) or *Tln1^{mR35E/118E}* platelets (blue bars) activated for the indicated times with 50 ng/mL CVX + 250 μ M Par4p (N=5). Percentages normalized to highest value. (C) Flow cytometry analysis of Annexin V binding (% PtdSer-positive events) on control or *Rap1^{mKO}* platelets pretreated with ROCK inhibitor (ROCKi, 20 μ M Y-27632) and then stimulated with 50 ng/mL CVX + 250 μ M Par4p (N=6). (D) Flow cytometry analysis of Annexin V binding (% PtdSer+ events) on control or *Tln1^{mR35E/118E}* platelets pretreated with ROCKi and then stimulated with 50 ng/mL CVX + 250 μ M Par4p (N=8). * P <0.05; ** P <0.01; *** P <0.001; NS: not significant. Data shown for untreated samples in panels (C) and (D) are the same as those in Figures 3C and F, respectively. They were included in Figure 4 to better illustrate the effect of ROCKi on PtdSer exposure. sec: seconds; min: minutes; mKO: megakaryocyte/platelet-specific knockout.

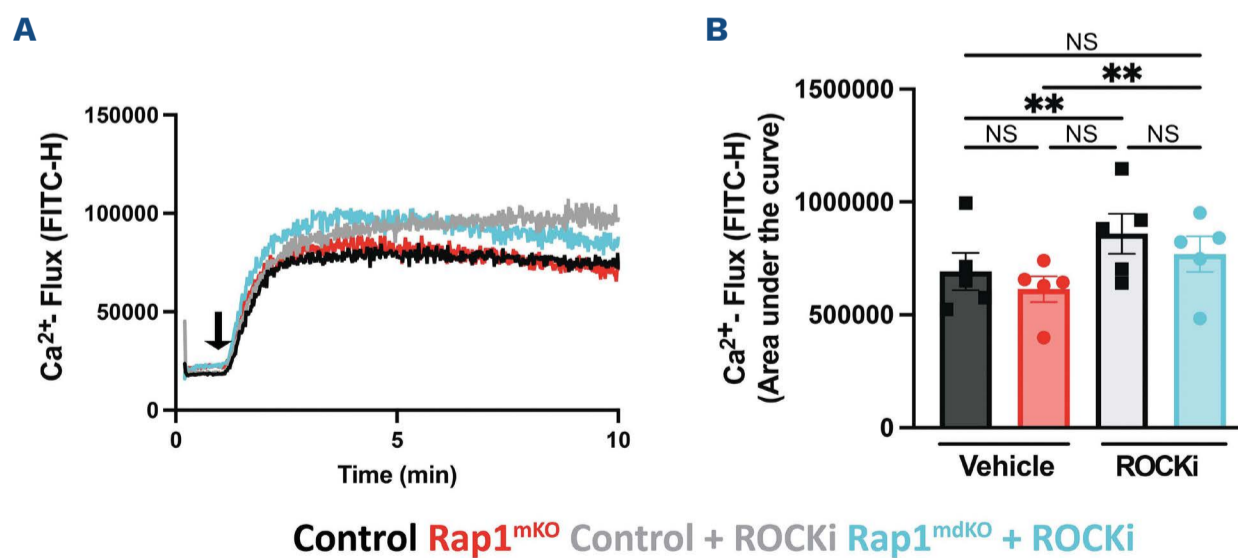


Figure 5. Calcium mobilization is not affected in *Rap1^{mKO}* platelets. (A) Representative curves for Fluo-4 fluorescence (FITC-H) in control (black and grey curves) or *Rap1^{mKO}* platelets (red and teal curves) activated in the absence or presence of ROCK inhibitor (ROCKi, Y-27632) with 50 ng/mL CVX + Par4p 150 μ M (added at 30 seconds; indicated by arrow). (B) Area under the curve analysis for Fluo-4 fluorescence traces described in (A) (N=5). ** P <0.01; NS: not significant; mKO: megakaryocyte/platelet-specific knockout.

mutations cause moderate to severe bleeding in humans,³⁴⁻³⁶ similar to what was shown for mice deficient in RAP1 or CalDAG-GEFI.^{12,37} As outlined above, RAP1 signaling is also important for procoagulant platelet formation. Importantly, bleeding is also observed in patients with Scott syndrome, i.e., in patients with a defect in procoagulant platelet formation but not α IIb β 3 integrin activation.³⁸ Scott syndrome is attributed to mutations in *TMEM16F*, a phospholipid scramblase that mediates PtdSer translo-

cation to the outer membrane layer.^{7,39} *TMEM16F* is highly expressed in platelets but is also found in other cell types including erythrocytes and endothelial cells (EC).⁴⁰⁻⁴² The similar bleeding phenotype between *TMEM16F* germline and megakaryocyte/platelet-specific knockout mice suggests that impaired procoagulant activity in platelets is the main reason for bleeding observed in Scott syndrome patients.^{6,7,40} Furthermore, we recently demonstrated a key role of procoagulant platelets, but not EC for throm-

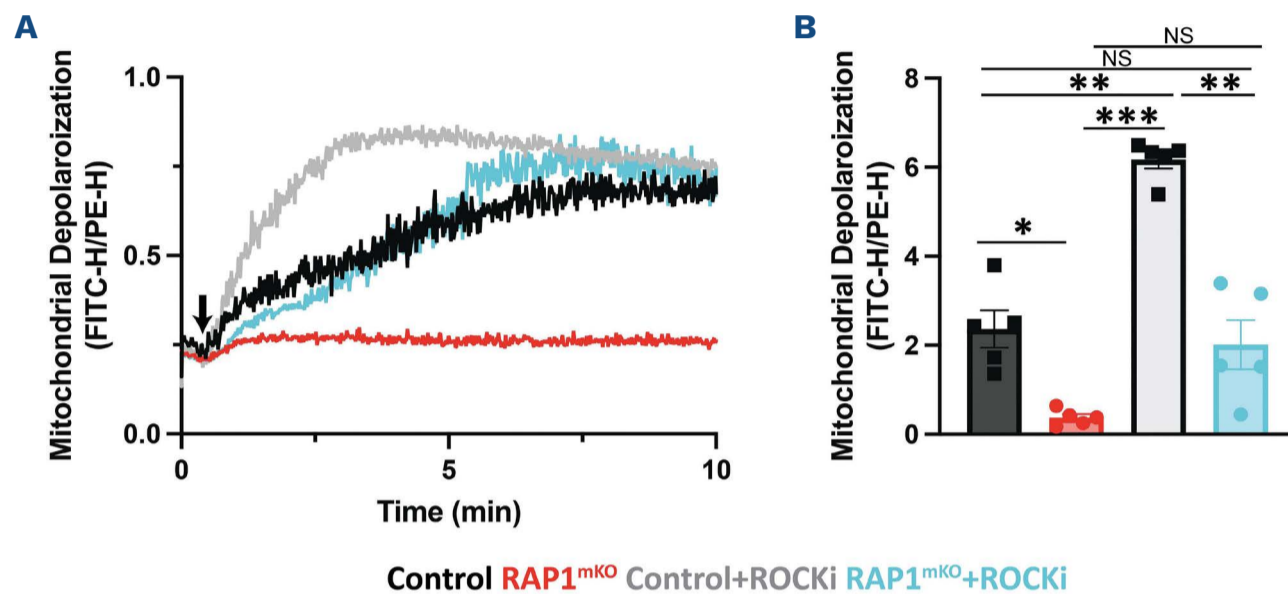


Figure 6. Mitochondrial depolarization is decreased in *Rap1*^{mKO} platelets and partially recovered with ROCK inhibitor. (A) JC-1 fluorescence ratio (FITC-H/PE-H) for control (black and grey curves) or *Rap1*^{mKO} platelets (red and teal curves) activated in the absence or presence of ROCK inhibitor (ROCKi, Y-27632) with 50 ng/mL CVX + Par4p 150 μ M (added at 10 seconds [sec]; indicated by arrow). (B) Area under the curve (AUC) analysis (N=5). * P <0.05; ** P <0.01; *** P <0.001; NS: not significant. min: minutes; mKO: megakaryocyte/platelet-specific knockout.

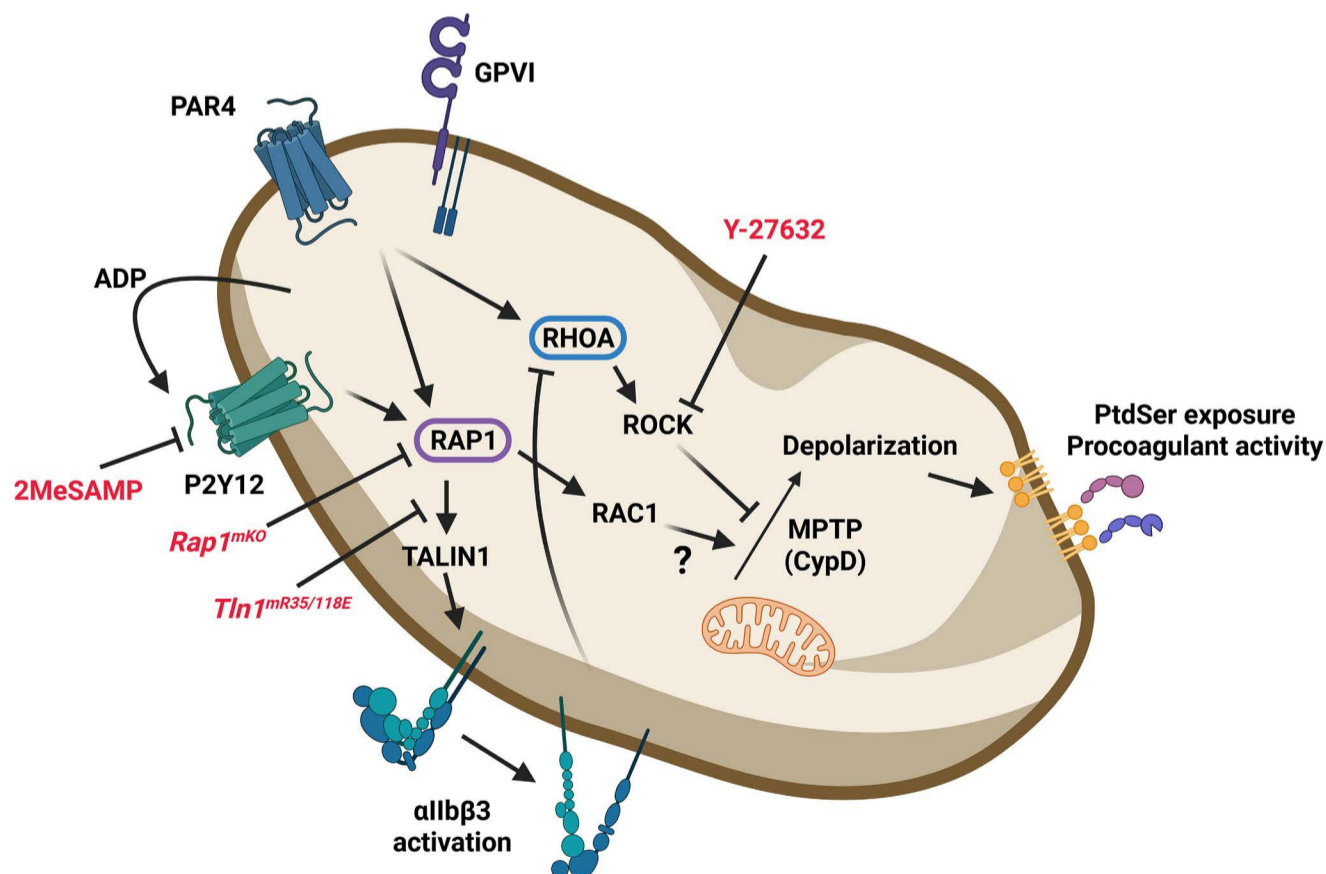


Figure 7. Summary diagram illustrating the crosstalk between signaling pathways discussed in this manuscript. PAR4, P2Y12, GPVI: agonist receptors; RAP1, RHOA, RAC1: small GTPases; Y-27632: ROCK inhibitor; 2MeSAMP: P2Y12 inhibitor; CypD: cyclophilinD; PtdSer: phosphatidylserine; ADP: adenosine diphosphate; *Rap1*^{mKO}: megakaryocyte/platelet-specific knockout of *Rap1a* and *Rap1b* isoforms; *Tln1*^{mR35/118E}: mice expressing Talin1 variant in megakaryocytes/platelets only. This figure was created in BioRender.

bin/fibrin formation during hemostatic plug formation.²⁷ Here we provide the first evidence that RAP1-mediated platelet PtdSer exposure contributes to thrombin/fibrin generation *in vitro* and *in vivo*. These studies suggest that bleeding in patients with impaired RAP1 signaling may result in part from reduced thrombin/fibrin formation at sites of vascular injury.

The RHO family of GTPases plays an important role in platelet procoagulant response. Kunzelmann *et al.* showed that inhibition of RHOA reduces store-operated calcium entry and PtdSer exposure.⁴³ As discussed in their paper, this effect of RHOA inhibition is through the reorganization of actin cytoskeleton, but not through the ROCK pathway. Dasgupta *et al.* described increased procoagulant function for platelets deficient in ROCK1, but no alterations in other cellular functions, including calcium response and shape change. Thus, the RHOA/ROCK pathway inhibits PtdSer exposure^{21,30,44} and needs to be downregulated by integrin outside-in signaling during platelet procoagulant response.^{30,31} We observed prolonged RHOA activation in *Rap1^{mKO}* and *Tln1^{mR35/118E}* platelets, i.e., platelets with markedly impaired integrin inside-out activation. Our studies further established that inhibition of RHOA/ROCK signaling fully restored PtdSer exposure in *Tln1^{mR35/118E}* platelets, which are defective in RAP1-TLN1 interaction. However, only a partial recovery of PtdSer exposure by inhibition of RHOA/ROCK signaling was observed in *Rap1^{mKO}* platelets, a finding that suggests a TLN1/integrin/RHOA-independent contribution of RAP1 to platelet procoagulant response. We previously showed that in platelets RAP1 positively regulates the activity of RAC1,^{12,45} another RHO GTPase with a documented role in PtdSer exposure.²¹ Importantly, RAC1 and RHOA are also known to negatively regulate each other's activation state.⁴⁶ Together, these studies suggest an important role for cross-talk between RAP1 and RHO GTPases in integrin-dependent and -independent PtdSer exposure.

High sustained cytosolic calcium levels and mitochondrial depolarization are two critical steps in procoagulant platelet formation. Inhibition of RHOA/ROCK signaling led to a small but significant increase in cytosolic calcium and a marked increase in mitochondrial depolarization in both control and *Rap1^{mKO}* dual agonist-stimulated platelets. Compared to controls, a significant decrease in mitochondrial depolarization but not calcium mobilization was observed for dual agonist-stimulated RAP1-deficient

platelets. Given that calcium mobilization was not altered in *Rap1^{mKO}* platelets, another mechanism must account for the decreased mitochondrial depolarization. Reactive oxygen species also affect mitochondrial depolarization, and both RHOA and RAC1 have been shown to regulate ROS production.⁴⁷ Whether impaired mitochondrial depolarization and PtdSer exposure in *Rap1^{mKO}* platelets are caused by dysregulated ROS production will be a topic of future studies.

In conclusion, our studies demonstrate that impaired RAP1 signaling leads to decreased platelet procoagulant response and thrombin generation *in vitro* and *in vivo*. RAP1 affects PtdSer exposure via integrin-dependent and -independent mechanisms, which likely involve cross-talk with RHO GTPases and the depolarization of the MPTP. This study improves our understanding of the role of small GTPases in platelet procoagulant response and thus may have important implications for the development of better therapies to prevent bleeding or thrombosis.

Disclosures

No conflicts of interest to disclose.

Contributions

AB-K designed research, performed research, analyzed data and wrote manuscript. NZ and WS performed research and analyzed data. MHG contributed vital reagents. AS designed and performed research. RHL designed research, performed research and analyzed data. WB designed research and wrote manuscript.

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Data sharing statement

Data are available on request from the corresponding author

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