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The Role of Rac1 in Glycoprotein Ib-IX–Mediated Signal Transduction and Integrin Activation

M. Keegan Delaney, Junling Liu, Yi Zheng, Michael C. Berndt, Xiaoping Du

Objective—The platelet receptor for von Willebrand factor, the glycoprotein Ib-IX (GPIb-IX) complex, mediates platelet adhesion at sites of vascular injury and transmits signals leading to platelet activation. von Willebrand factor/GPIb-IX interaction sequentially activates the Src family kinase Lyn (SFK), phosphoinositide 3-kinase (PI3K), and Akt, leading to activation of integrin $\alpha_{IIb}\beta_3$ and integrin-dependent stable platelet adhesion and aggregation. It remains unclear how Lyn activates the PI3K/Akt pathway after ligand binding to GPIb-IX.

Methods and Results—Using platelet-specific Rac1^{-/-} mice and the Rac1 inhibitor NSC23766, we examined the role of Rac1 in GPIb-IX–dependent platelet activation. Rac1^{-/-} mouse platelets and NSC23766-treated human platelets were defective in GPIb-dependent stable adhesion to von Willebrand factor under shear stress, integrin activation, thromboxane A₂ synthesis, and platelet aggregation. Interestingly, GPIb-induced activation of Rac1 and the guanine nucleotide exchange factor for Rac1, Vav, was abolished in both Lyn^{-/-} and SFK inhibitor-treated platelets but was unaffected by the PI3K inhibitor LY294002, indicating that Lyn mediates activation of Vav and Rac1 independently of PI3K. Furthermore, GPIb-induced activation of Akt was abolished in Rac1-deficient platelets, suggesting that Rac1 is upstream of the PI3K/Akt pathway.

Conclusion—A Lyn-Vav-Rac1-PI3K-Akt pathway mediates von Willebrand factor–induced activation of integrin $\alpha_{IIb}\beta_3$ to promote GPIb-IX–dependent platelet activation. (*Arterioscler Thromb Vasc Biol.* 2012;32:2761–2768.)

Key Words: glycoprotein Ib-IX ■ platelet ■ platelet adhesion ■ Rac1 ■ von Willebrand factor

Under the high shear-rate flow conditions present in arteries and capillaries, platelet adhesion to the site of vascular injury is mediated by the interaction between subendothelial-bound von Willebrand factor (VWF) and its platelet receptor, the glycoprotein Ib-IX (GPIb-IX) complex.^{1–3} The interaction between VWF and GPIb-IX not only mediates transient platelet adhesion to the injured vessel wall but also initiates a signal transduction cascade culminating in the activation of integrin $\alpha_{IIb}\beta_3$, leading to stable platelet adhesion, spreading, and aggregation.^{2,4–6} This process is essential for thrombosis under high shear conditions. GPIb-IX–mediated platelet activation signaling is known to involve several intracellular signaling molecules and pathways, including the Src family kinase (SFK) Lyn, the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the cGMP and mitogen-activated protein kinase (MAPK) pathways.^{2,7–16} GPIb-IX signaling is also greatly amplified by the activation of the immunoreceptor tyrosine-based activation motif, thromboxane (TX) A₂, and ADP signaling pathways.^{2,8,17,18} Although the importance of GPIb-IX–mediated platelet activation to arterial thrombosis is well understood, the signaling pathway that regulates GPIb-IX–dependent platelet activation is poorly characterized. Thus far, the identified most proximal step to GPIb-IX that propagates platelet activation signals is the activation of Lyn and Lyn-dependent activation of the PI3K/Akt pathway. Interestingly, although the PI3K/Akt

pathway is activated downstream of Lyn and is critical for promoting GPIb-IX–mediated platelet activation, the exact molecular mechanisms governing this process are unclear.

The Rho-family small GTPase, Rac1, has been shown to be important for arteriole thrombosis in vivo.^{19,20} Rac1 is activated by binding to GTP and plays roles in multiple cellular processes, including actin polymerization,²¹ lamellipodia formation,^{19,22–24} and cell retraction.²⁵ However, the role of Rac1 in GPIb-IX–mediated signal transduction has not been explored.

Here, we show that Rac1 is a critical mediator of GPIb-IX–dependent early signaling, leading to integrin activation, stable platelet adhesion under shear stress, TXA₂ production, and platelet aggregation. Most importantly, we demonstrate that Rac1 mediates Lyn-dependent activation of the PI3K/Akt signaling pathway during GPIb-IX–dependent platelet activation. Thus, our study reveals an important new link in the GPIb-IX signaling pathway and a novel mechanism of Rac1-dependent PI3K/Akt activation.

Materials and Methods

Generation of Mice With Rac1^{-/-} Platelets

Mice containing the Rac1 conditional allele (Rac1^{loxP/loxP})²⁶ were crossed with mice carrying the Pf4-Cre transgene (Pf4-Cre⁺).²⁷ Pf4-Cre⁺/Rac1^{loxP/loxP} mice are notated as Rac1^{-/-} and Pf4-Cre⁻/Rac1^{loxP/loxP}.

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mice as wild type (WT). Mice were maintained on a mixed SV129/C57/BL-6 background, and littermates were used as control. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago.

Platelet Preparation and Adhesion Under Flow

Human and murine platelets were prepared, as previously described, and used at $3 \times 10^8/\text{mL}$.^{28,29} Analysis of platelet adhesion under flow was performed as described previously.^{11,12} Data are representative of ≥ 3 experiments, and statistical significance was determined via ANOVA and posttest.

Fibrinogen Binding Assay

Buffers contained 1% BSA and 2 mmol/L MgCl_2 . Washed human ($1 \times 10^8/\text{mL}$) and mouse ($2 \times 10^8/\text{mL}$) platelets were stimulated with ristocetin/biotroctin±VWF in the presence of 5 or 2 $\mu\text{g}/\text{mL}$ Oregon Green–labeled fibrinogen (Molecular Probes) for 20 or 10 minutes, respectively. Integrilin and Arg-Gly-Asp-Ser peptide were used to determine the level of nonspecific binding of Oregon Green–labeled fibrinogen to the platelet surface. Platelets were diluted 1:20 in modified Tyrode buffer and analyzed with a BD Accuri C6 flow cytometer. Specific fibrinogen binding was determined by subtracting the geometric means of fluorescence intensity of the nonspecific binding (integrin antagonist present) from the total binding (integrin antagonist absent).

VWF-Binding Assay

To all buffers, 2 mmol/L MgCl_2 , 1% BSA, and 10 mmol/L EDTA were added, and 1×10^8 platelets/mL were used. Washed human and mouse platelets were incubated 5 or 10 minutes with VWF±ristocetin/biotroctin, respectively. Platelets were fixed with a final concentration of 1% paraformaldehyde in HEPES-saline buffer for 20 minutes, centrifuged at 100g for 1 minute, and the pellet stained for 30 minutes in 0.1 mL modified Tyrode buffer containing 4 $\mu\text{g}/\text{mL}$ SZ-29-fluorescein isothiocyanate. Samples were diluted 1:20 with modified Tyrode buffer and analyzed using a BD Accuri C6 flow cytometer. Specific VWF binding was determined by subtracting the geometric means of fluorescence intensity of VWF binding in the presence of biotroctin/ristocetin alone from the VWF binding in the absence of biotroctin/ristocetin.

Platelet Aggregation and TXB_2 Generation Assay

Platelet aggregation³⁰ and measurement of the TXA_2 metabolite, TXB_2 ,^{15,17} were performed as described previously. Briefly, after platelet aggregation for 8 minutes, the reaction was stopped with EDTA and aspirin, the platelets briefly spun down in a microfuge, and the resultant supernatant used in a TXB_2 ELISA (Assay Designs). TXB_2 is used to indicate levels of TXA_2 . Data are from ≥ 3 experiments and expressed as mean±SEM. Statistical significance was determined using the Student *t* test.

Immunoblot Detection of SFKs, Vav, Akt, and p38 MAPK

SDS-PAGE analysis of phosphorylated signaling proteins was performed as described previously.¹² Anti-pan phospho SFK (No. 2101, Tyr416), which reacts with phosphorylated Lyn, anti-phospho P38 (No. 9211, Thr180/Tyr182), anti-phospho Akt (No. 4058, Ser473), and anti-pan Akt (No. 2920) antibodies are from Cell Signaling Technology. Anti-pan phospho Vav (sc-16408-R), anti-pan Vav (sc-55482), anti-Lyn (sc-7274), and anti-P38 (sc-535) antibodies are from Santa Cruz Biotechnology.

Immunoblot Detection of Small GTPases

Rac1 activation assays were performed, as described previously, to detect levels of GTP-bound Rac1.^{26,31} Anti-Rac1 antibody (No. 61051; BD Biosciences) was used for Western Blot.

Results

Generation of Megakaryocyte- and Platelet-Specific Conditional Rac1 Knockout Mice

To study the role of Rac1 in GPIb-IX–induced platelet activation, we generated mice that lack expression of Rac1 exclusively in megakaryocytes and platelets and used a small-molecule inhibitor of Rac1, NSC23766. To obtain megakaryocyte- and platelet-specific conditional Rac1 knockout mice, Pf4-Cre transgenic mice²⁷ were crossbred with mice containing the Rac1^{loxP/loxP} allele.²⁶ Deletion of Rac1 in Rac1^{−/−} mouse platelets was verified via Western Blot analysis of platelet lysates (Figure 1A). Rac1^{−/−} mouse platelets did not show any noticeable difference in routine hematologic parameters, including morphology and counts, compared with WT platelets. This is similar to previous reports, where Rac1^{−/−} mouse platelets were generated using the Mx1-Cre transgene.^{19,20}

Role of Rac1 in the Early GPIb-IX–Mediated Integrin Activation Signaling Pathway Leading to Stable Platelet Adhesion to VWF Under Flow Conditions

We initially evaluated the effect of Rac1 deficiency on GPIb-IX–induced, integrin-dependent stable platelet adhesion to VWF under shear stress, which was created using a cone–plate rheometer. As shown in Figure 1, stable adhesion of human and mouse platelets to VWF was completely inhibited by the integrin antagonist Arg-Gly-Asp-Ser peptide (Figure 1B–1D). This is consistent with our previous results that stable platelet adhesion to VWF under shear flow conditions requires not only GPIb-IX but also GPIb-IX–dependent activation of integrin $\alpha_{\text{IIb}}\beta_3$.^{11,12} Stable platelet adhesion to VWF under flow is partially inhibited by the cyclooxygenase inhibitor aspirin, which is also consistent with our previous data that the cyclooxygenase pathway plays an amplifying role in promoting platelet adhesion under shear stress.^{11,12} Interestingly, stable platelet adhesion to VWF was dramatically impaired in Rac1^{−/−} mouse platelets or NSC23766-treated human platelets (Figure 1B–1D). These data indicate that Rac1 plays a critical role in GPIb-IX–mediated, integrin-dependent platelet adhesion to VWF under shear stress. Importantly, deficiency of Rac1 led to significantly greater inhibition of stable platelet adhesion than aspirin (Figure 1B–1D), indicating that the role of Rac1 in GPIb-IX–mediated platelet activation exceeds the cyclooxygenase-dependent amplification signaling pathway.

Rac1 Is Important for GPIb-IX–Induced Integrin Activation

We next investigated whether Rac1 is important for GPIb-IX–induced integrin activation. WT and Rac1^{−/−} mouse platelets were stimulated with VWF in the presence of biotroctin and analyzed for the binding of Oregon Green–labeled fibrinogen using flow cytometry. VWF/biotroctin-induced fibrinogen binding was diminished in Rac1^{−/−} mouse platelets compared with WT platelets, indicating that Rac1^{−/−} mouse platelets were defective in GPIb-IX–induced activation of $\alpha_{\text{IIb}}\beta_3$ (Figure 2A and 2B). These results were recapitulated in human platelets, where VWF/ristocetin-induced fibrinogen binding was abolished by Rac1 inhibitor treatment compared with the control solvent dimethyl sulfoxide (Figure 2C and 2D). These results

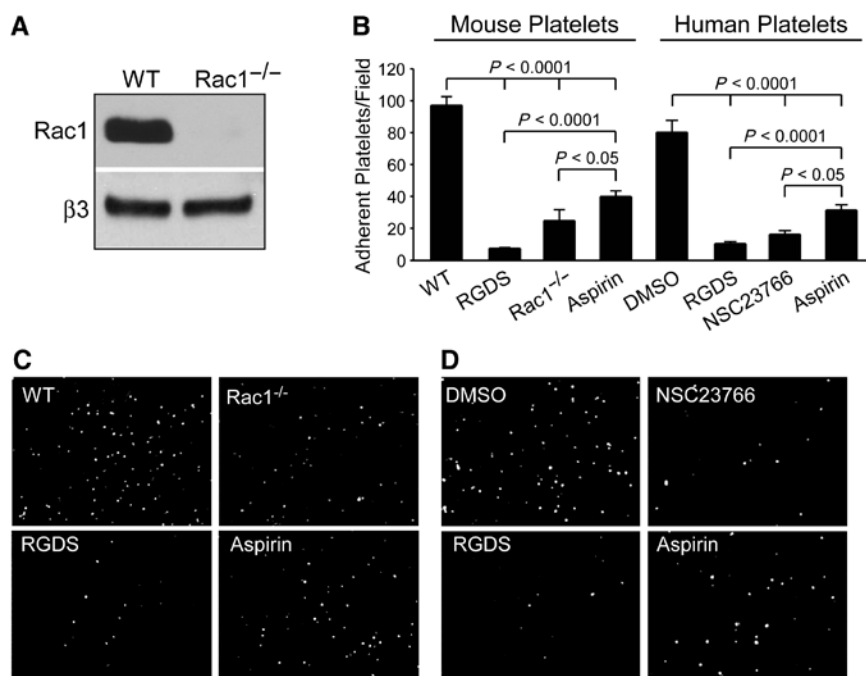


Figure 1. The role of Rac1 in integrin-dependent stable platelet adhesion to von Willebrand factor (VWF) under flow.

A, Washed wild-type (WT) and Rac1^{-/-} mouse platelets were solubilized in SDS buffer, and protein expression of Rac1 and β_3 was analyzed via SDS-PAGE and Western blot. **B**, Washed WT and Rac1^{-/-} mouse platelets, pretreated with 2 mmol/L Arg-Gly-Asp-Ser peptide (RGDS) or 1 mmol/L aspirin for 5 minutes, or washed human platelets, pretreated with 0.1% dimethyl sulfoxide (DMSO), 100 μ mol/L NSC23766, 2 mmol/L RGDS, or 1 mmol/L aspirin for 5 minutes, were loaded onto VWF-coated glass slides in the presence of 10 μ mol/L mepacrine. Platelets were subjected to a constant shear rate for 5 minutes. After washing, adherent platelets were viewed and photographed. The number of adherent platelets per field was quantified and shown as mean \pm SEM. **C**, Representative images of adherent WT and Rac1^{-/-} mouse platelets from **B**. **D**, Representative images of adherent human platelets from **B**.

demonstrate that Rac1 plays an important role in the signaling pathway of GPIb-IX-dependent integrin activation.

Platelet Deficiency in Rac1 Does Not Affect the VWF-Binding Function of GPIb-IX

To exclude the possibility that the functional defects observed in Rac1-deficient platelets derive from a defect in the VWF-binding function of GPIb-IX, washed WT and Rac1^{-/-} mouse platelets or washed human platelets treated with dimethyl sulfoxide or NSC23766 were incubated with human VWF in the presence of botrocetin or ristocetin, respectively, to induce the binding of VWF to GPIb-IX. The platelets were then fixed, stained with a fluorescein isothiocyanate-labeled anti-VWF antibody, and analyzed via flow cytometry. As expected, botrocetin or ristocetin induced the binding of soluble VWF to platelets. There was no difference in botrocetin-induced binding of VWF to WT and Rac1^{-/-} mouse platelets (Figure 3A and 3B) or ristocetin-induced binding of VWF to dimethyl sulfoxide-treated and NSC23766-treated human platelets (Figure 3C and 3D). Therefore, Rac1 is not involved in regulating the VWF-binding function of GPIb-IX but rather is important in GPIb-IX-dependent signal transduction.

Rac1 Is Required for GPIb/VWF-Mediated Platelet Aggregation and TXA₂ Production

To further confirm the role of Rac1 in GPIb-IX-mediated platelet activation, we also evaluated the effect of Rac1 deficiency on VWF-induced platelet aggregation. VWF-induced platelet aggregation is characterized by 2 distinct waves.^{2,8} The first wave mainly comprises VWF/GPIb-IX-dependent platelet agglutination and an often minor component of GPIb-IX-induced integrin-dependent platelet aggregation.^{9,32} The second wave represents TXA₂, secretion-, and integrin-dependent platelet aggregation. It requires GPIb-IX-induced activation of integrin $\alpha_{IIb}\beta_3$ and subsequent TXA₂ synthesis, which initiates

integrin- and TXA₂-dependent granule secretion and secretion-dependent amplification of platelet activation. As expected, stimulation of WT mouse platelets with VWF/botrocetin or stimulation of human platelets with VWF /ristocetin lead to 2 waves of platelet aggregation (Figure 4A and 4B). However, Rac1^{-/-} platelets and WT mouse platelets treated with NSC23766 had a defect in the second wave of VWF-induced aggregation (Figure 4A). Similarly, NSC23766-treated human platelets were also defective in the second wave of VWF-induced platelet aggregation (Figure 4B). Thus, these results further verify that Rac1 is required for VWF/GPIb-IX-mediated platelet activation in both human and mouse platelets. In addition, Rac1^{-/-} mouse platelets (Figure 4C) or NSC23766-treated human platelets (Figure 4D) were defective in GPIb-IX-mediated TXA₂ synthesis, indicating that Rac1 is important in the early GPIb-IX-mediated signaling pathway that is upstream of TXA₂ synthesis.

Rac1 Is Required for GPIb-IX-Mediated Activation of the PI3K/Akt Pathway

Previous studies from our laboratory and others have shown that GPIb-IX-mediated early signaling leading to integrin activation involves GPIb-IX-dependent activation of the SFK Lyn and Lyn-dependent activation of the PI3K/Akt pathway, which subsequently stimulates the cGMP and MAPK pathways.^{9-12,32,33}

To determine how Rac1 is involved in GPIb-IX-mediated platelet activation, we determined whether and how loss of function of Rac1 affected this signaling pathway by evaluating VWF/GPIb-IX-mediated phosphorylation of SFKs, Akt, and p38 MAPK during the early agglutination phase of VWF-induced platelet aggregation (2-minute time point) before the second wave of platelet aggregation.^{2,8} VWF/GPIb-IX-induced phosphorylation of Akt was abolished in Rac1^{-/-} mouse platelets (Figure 5A) or NSC23766-treated human platelets (Figure 5B), indicating that Rac1 is upstream of Akt in the GPIb-IX signaling pathway. Furthermore, platelet deficiency in Rac1 also abolished

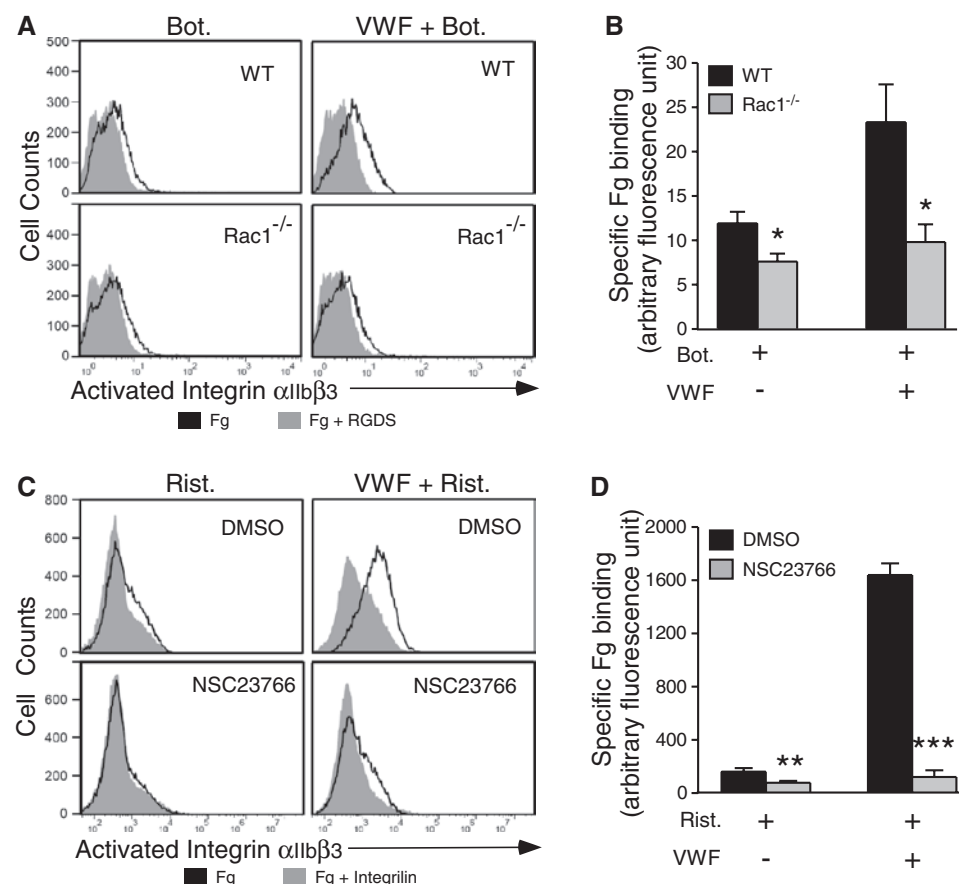


Figure 2. Rac1 is important for glycoprotein Ib-IX-induced integrin activation. **A**, Flow cytometric analysis of the binding of Oregon Green-conjugated fibrinogen (Oregon Green-Fg) to washed wild-type (WT) and Rac1^{-/-} mouse platelets stimulated with 2 µg/mL botrocetin (Bot.) ± 10 µg/mL von Willebrand factor (VWF) in the presence (gray filled) or absence (black line) of 2 mmol/L of the integrin receptor antagonist Arg-Gly-Asp-Ser peptide (RGDS). **B**, The quantitative results from 3 independent experiments as described in **A**. **C**, Flow cytometric analysis of the binding of Oregon Green-Fg to washed human platelets treated with 0.1% dimethyl sulfoxide (DMSO) or 100 µmol/L NSC23766 for 5 minutes and subsequently stimulated with 0.6 mg/mL ristocetin (Rist.) and 10 µg/mL VWF in the presence (gray filled) or absence (black line) of 40 µg/mL of the integrin receptor antagonist integrilin. **D**, The quantitative results from 3 independent experiments as described in **C**. ***, **, and * represent statistical significance ($P < 0.0001$, $P < 0.001$, and $P < 0.05$, respectively) as determined by Student *t* test. Data are shown as mean ± SEM.

GPIb-IX-mediated phosphorylation of p38 MAPK (Figure 5A and 5B). These data suggest that Rac1 is required for activation of the PI3K/Akt pathway as well as the downstream p38 MAPK pathway. Consistent with the observation that Rac1 functions upstream of the TXA₂ synthesis pathway (Figure 4), aspirin had no effect on GPIb-IX-mediated phosphorylation of Akt and P38, which was fully inhibited by NSC23766 in the presence of aspirin. In contrast, VWF/GPIb-IX-induced phosphorylation of SFKs was not negatively affected by deficiency of Rac1 (Figure 5A and 5B). Thus, Rac1 is not required for GPIb-IX-dependent activation of SFKs.

Activation of Vav and Rac1 During GPIb-IX Signaling Requires the SFK Lyn

To assess whether Lyn and PI3K were involved in GPIb-IX-mediated activation of Rac1, the levels of GTP-bound Rac1 in human platelets, treated with the SFK inhibitor PP2 or PI3K inhibitor LY294002, or in Lyn^{-/-} mouse platelets were assayed after ligation of GPIb-IX. Rac1 was activated within 1 minute and remained active throughout VWF-induced platelet aggregation in WT platelets (Figure 6A). However, VWF-induced GTP loading of Rac1 was abolished in Lyn^{-/-} (Figure 6A) and PP2-treated platelets (Figure 6B), indicating that Rac1 is activated downstream of Lyn in the GPIb-IX signaling pathway. Interestingly, VWF-induced GTP loading of Rac1 remained unaffected by LY294002 (Figure 6B and 6C), indicating that Rac1 activation occurs independent of PI3K. In fact, under the same conditions, LY294002- and NSC23766-treated platelets were both defective in GPIb-IX-mediated activation of Akt

and p38 MAPK but not SFKs (Figure 5B). Taken together, these results indicate not only that Rac1 becomes activated during GPIb-IX-dependent platelet activation but also that Rac1 is activated downstream of Lyn and functions upstream of the PI3K/Akt/MAPK pathway.

Rac1 can be activated by multiple guanine nucleotide exchange factors, including Vav. Recent studies suggest that Vav can be activated by Lyn-dependent phosphorylation at Tyr¹⁷⁴.³⁴ To evaluate whether Lyn may mediate GPIb-IX-dependent activation of Rac1 via phosphorylation of Vav, we determined whether ligation of GPIb-IX induces Vav phosphorylation and whether GPIb-IX-dependent Vav phosphorylation is affected in Lyn^{-/-} mouse platelets or PP2-treated platelets during the early phase of VWF-induced platelet aggregation/aggregation before the second wave of platelet aggregation. Indeed, ligation of GPIb-IX induced Vav activation, which was abolished in Lyn^{-/-} mouse platelets (Figure 6D) or PP2-treated human platelets (Figure 6E). In contrast, activation of Vav was unaffected by either LY294002 or NSC23766 (Figure 6E). Thus, Lyn mediates GPIb-IX-induced activation of Vav and Rac1, independent of the PI3K pathway. Collectively, we have demonstrated that a novel Lyn-Vav-Rac1-PI3K-Akt signaling pathway is important in the early phase of GPIb-IX-mediated signal transduction leading to platelet activation.

Discussion

In this study, we demonstrate that Rac1 plays a critical role in stimulating GPIb-IX-dependent platelet activation. We further

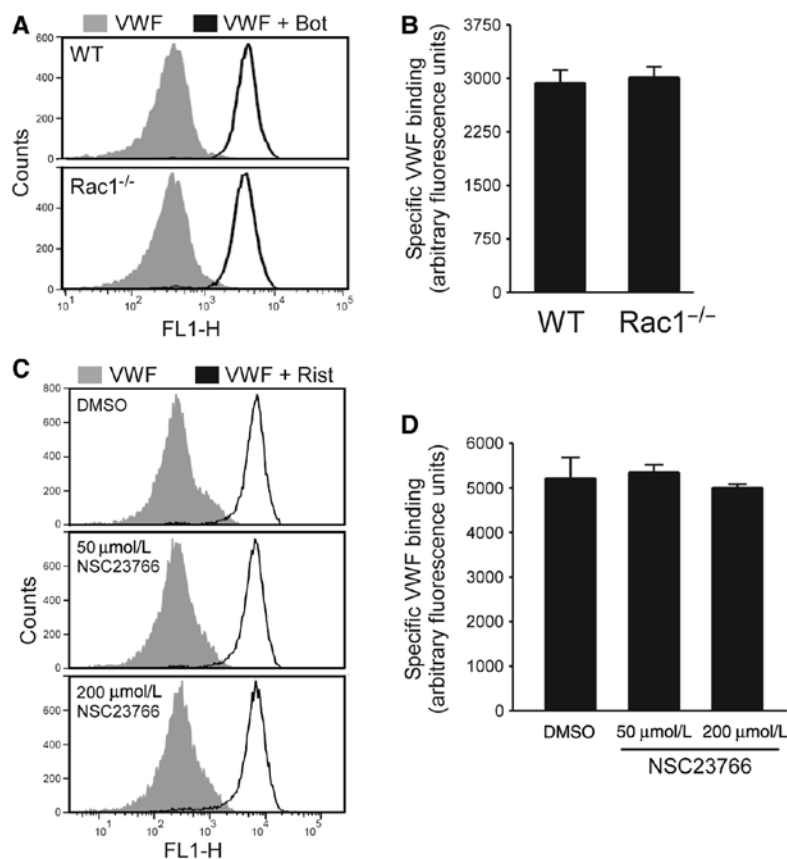


Figure 3. Platelet deficiency in Rac1 does not affect the von Willebrand factor (VWF)-binding function of glycoprotein Ib-IX. **A**, Flow cytometric analysis of VWF binding, as determined by the binding of fluorescein isothiocyanate-labeled anti-VWF antibody, to washed wild-type (WT) and Rac1^{-/-} mouse platelets after addition of 20 μg/mL VWF in the presence (black line) or absence (gray filled) of 1.25 μg/mL botrocetin for 10 minutes. **B**, Quantification of specific VWF binding from **A**. **C**, Flow cytometric analysis of VWF binding to washed human platelets that were pretreated with either 0.1% dimethyl sulfoxide (DMSO) or 50 μmol/L or 200 μmol/L NSC23766 and stimulated with 20 μg/mL VWF in the presence (black line) or absence (gray filled) of 0.5 mg/mL ristocetin for 5 minutes. **D**, Quantification of specific VWF binding from **C**. Data are shown as mean±SEM and are from 3 experiments.

show that Rac1 is important in the early GPIb-IX signaling pathway, leading to activation of integrin $\alpha_{IIb}\beta_3$ and stable platelet adhesion under flow. Importantly, we have discovered that Rac1 and its guanine nucleotide exchange factor, Vav, are activated downstream from Lyn and that Rac1 is required for activating the PI3K/Akt pathway. Thus, our study not only reveals a novel role for Rac1 in platelet activation but also as an important mediator of GPIb-IX-induced, Lyn-dependent activation of the PI3K/Akt signaling pathway, leading to integrin activation.

We conclude that Rac1 is required for GPIb-IX-mediated platelet activation. This conclusion is supported by the data that Rac1^{-/-} mouse platelets and NSC23766-treated human platelets were defective in GPIb-IX-dependent integrin activation and stable platelet adhesion to VWF under flow. Furthermore, the TXA₂-, secretion-, and integrin-dependent second wave of platelet aggregation was abolished in Rac1-deficient platelets without affecting VWF binding, further supporting an important role for Rac1 in GPIb-IX-mediated platelet activation signaling. The platelet activation process induced by GPIb-IX can be divided into early-phase GPIb-IX-specific signaling events and late-phase amplification signaling pathways shared by all other platelet agonists.^{2,8} Several signaling molecules and events have been shown to be important in the early GPIb-IX signaling pathway leading to integrin activation and stable platelet adhesion, including the SFKs c-Src and Lyn, the PI3K/Akt pathway, intracellular calcium oscillation, cGMP-dependent protein kinase (still controversial), and p38/extracellular-signal-regulated kinase MAPKs.^{2,8,10,13,14,18,35–37} Some other signaling molecules, such as

components of the immunoreceptor tyrosine-based activation motif signaling pathway, have been shown to be important in the late signal amplification phase of GPIb-IX-induced platelet activation, which facilitates aggregation and recruitment of additional platelets to the growing thrombus.² However, the distinct role of many other signaling molecules in either the early or late phase of GPIb-IX-induced platelet activation remains poorly characterized. This is because the aggregation response of platelets to GPIb-IX-specific early phase receptor signaling is often masked by VWF-mediated platelet agglutination, and the second phase of VWF-induced platelet aggregation is complicated by the platelet response to amplification signals induced via the integrin, immunoreceptor tyrosine-based activation motif, TXA₂, and ADP signaling pathways. It is thus difficult to use the routine platelet aggregation assay to effectively specify the role of a particular signaling molecule in GPIb-IX-specific platelet responses. We thus determined the specific role of Rac1 in early GPIb-IX signaling by analyzing the effect of Rac1 deficiency in platelet adhesion to VWF under flow conditions. Under shear stress, stable platelet adhesion to immobilized VWF requires the early GPIb-IX-mediated signaling pathway that stimulates the inside-out activation of integrin, leading to integrin-dependent stable platelet adhesion. Stable platelet adhesion under shear stress does not require molecules important in the secondary signaling pathways, such as Syk and immunoreceptor tyrosine-based activation motif,^{11,12} although it is amplified by TXA₂. Our data demonstrate that stable platelet adhesion to VWF under shear stress was dramatically impaired in Rac1^{-/-} mouse platelets

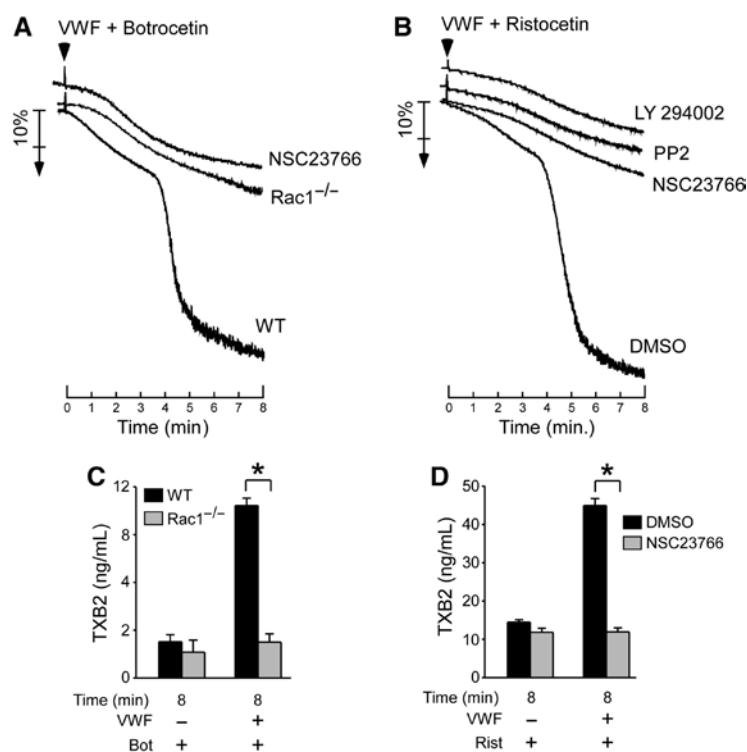


Figure 4. Rac1 is required for von Willebrand factor (VWF)-induced platelet aggregation and thromboxane (TX) A₂ synthesis. **A**, Washed wild-type (WT) platelets, treated with 100 μ M NSC23766 or Rac1^{-/-} platelets, were stimulated with 5 μ g/mL and 1 μ g/mL botrocetin to induce aggregation in a lumi-aggregometer. **B**, Washed human platelets, preincubated with 0.1% dimethyl sulfoxide (DMSO), 100 μ M NSC23766, 10 μ M PP2, or 20 μ M LY294002, were stimulated with 5 μ g/mL VWF and 0.3 mg/mL ristocetin to induce aggregation in a lumi-aggregometer. **C** and **D**, The amount of TXB₂ was determined in WT and Rac1^{-/-} mouse platelets or human platelets treated with 0.1% DMSO or 100 μ M NSC23766 after platelet aggregation stimulated by 1 μ g/mL botrocetin or 0.3 mg/mL ristocetin, respectively, \pm 5 μ g/mL VWF.

and NSC23766-treated human platelets. Furthermore, the impairment in Rac1^{-/-} mouse platelets and NSC23766-treated platelets is significantly greater than the inhibitory effects of saturable concentrations of aspirin. These data indicate that the role of Rac1 in stable platelet adhesion is not limited to its role in TXA₂ synthesis and signal amplification but rather that Rac1 plays an important role in the early, TXA₂-independent GPIb-IX-dependent signaling pathway that activates $\alpha_{IIb}\beta_3$. This conclusion is further supported by the data showing that Rac1 stimulates activation of Akt and p38 MAPK independent of the TXA₂ pathway (Figure 5B) and that Rac1 is also upstream of the TXA₂ synthesis pathway (Figure 4C and 4D). Importantly, we have provided direct evidence that Rac1 is critical for GPIb-IX-dependent integrin activation (Figure 2). These results, for the first time, indicate a new small GTPase-dependent signaling mechanism in the early GPIb-IX signaling pathway, leading to integrin activation and stable platelet adhesion under shear stress. These results,

however, do not exclude an important role for Rac1 also in the secondary platelet amplification pathways, such as granule secretion and integrin outside-in signaling, which have been demonstrated previously.^{26,38}

It is established that the SFK Lyn and the PI3K/Akt pathway are important in early GPIb-IX signaling.^{11–13,15,16} It remains unclear, however, as to how GPIb-IX activates these molecules and how these molecules are linked into a signaling pathway or network that mediates GPIb-IX signaling leading to integrin activation. Previous studies have shown the coimmunoprecipitation of GPIb-IX with PI3K³⁹ and Lyn,⁴⁰ and it was postulated that the GPIb-associated p85 subunit of PI3K functions as a scaffold that recruits SFKs to GPIb, thereby facilitating the activation of SFKs.⁴⁰ Here, we show that GPIb-IX-mediated activation of the PI3K/Akt signaling pathway requires Rac1. We also show that GPIb-IX-mediated activation of Rac1 requires Lyn and likely involves Lyn-dependent phosphorylation

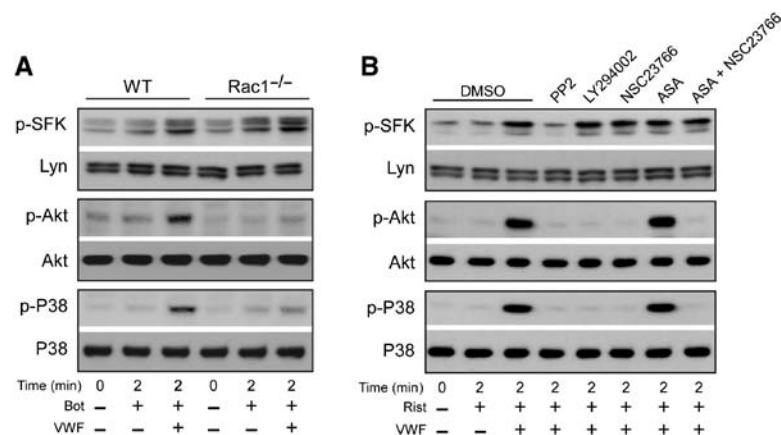


Figure 5. Rac1 is required for glycoprotein Ib-IX-mediated activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. **A**, Washed wild-type (WT) and Rac1^{-/-} mouse platelets or **B** washed human platelets treated with 0.1% dimethyl sulfoxide (DMSO), 10 μ M PP2, 20 μ M LY294002, 100 μ M NSC23766, 1 mmol/L aspirin (ASA), or 100 μ M NSC23766+1 mmol/L ASA were stimulated with 1 μ g/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist), respectively, with or without 5 μ g/mL von Willebrand factor (VWF) in a lumi-aggregometer. **A** and **B**, The amount of phosphorylated Src family kinase (SFK) (Tyr⁴¹⁶), Akt (Ser⁴⁷³), and P38 mitogen-activated protein kinase (MAPK) (Thr¹⁸⁰/Tyr¹⁸²) was determined using SDS-PAGE and Western blot with appropriate antibodies. Total Lyn, Akt, and P38 are shown as loading controls.

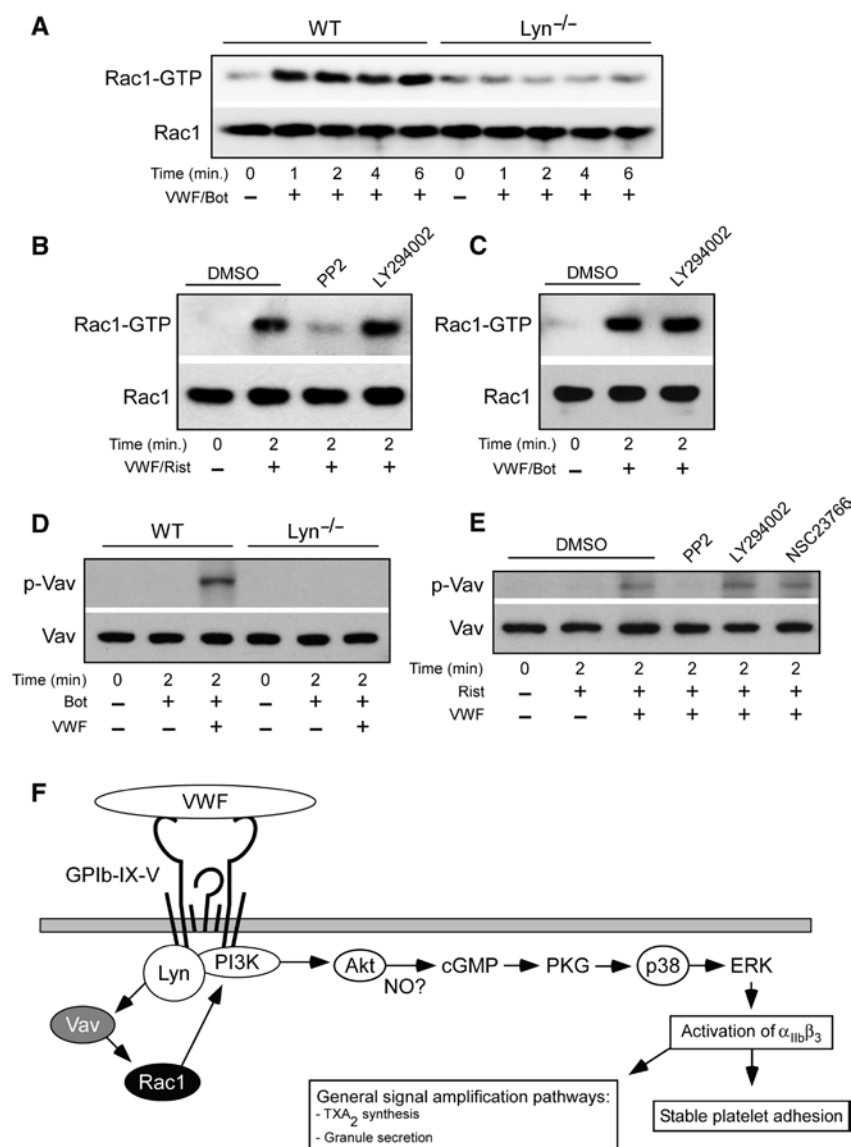


Figure 6. Activation of Vav and Rac1 during glycoprotein Ib-IX (GPIb-IX) signaling requires the Src family kinase (SFK) Lyn. **A**, Washed wild-type (WT) and *Lyn*^{-/-} mouse platelets, **(B)** washed human platelets treated with 0.1% dimethyl sulfoxide (DMSO), 10 μ mol/L PP2, or 20 μ mol/L LY294002, and **(C)** washed WT mouse platelets treated with 0.1% DMSO or 20 μ mol/L LY294002 were stimulated with 5 μ g/mL von Willebrand factor (VWF) together with 1 μ g/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist) in a lumi-aggregometer. The amount of GTP-bound Rac1 was determined using the Rac1 activation assay, SDS-PAGE, and Western blot. Total Rac1 is shown as loading control. **D**, Washed WT and *Lyn*^{-/-} mouse platelets or **(E)** human platelets treated with 0.1% DMSO, 10 μ mol/L PP2, 20 μ mol/L LY294002, or 100 μ mol/L NSC23766 were stimulated with 1 μ g/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist), respectively, with or without 5 μ g/mL VWF. The level of phosphorylated Vav (Tyr¹⁷⁴) was determined using SDS-PAGE and Western blot. Total Vav is shown as loading control. **F**, A schematic of the GPIb-IX signaling pathway. VWF binding to GPIb-IX sequentially activates Lyn, Vav, Rac1, PI3K, and Akt. Akt stimulates cGMP-dependent activation of mitogen-activated protein kinases (MAPKs; there are still controversies), leading to integrin activation and stable platelet adhesion. This further leads to activation of signal amplification pathways, in which Rac1 may also play a role. Encircled signaling molecules were investigated in this study. TXA₂ indicates thromboxane A₂.

of Vav. Furthermore, the catalytic function of PI3K is not required for GPIb-IX-mediated activation of Rac1, because LY294002 had no effect on GPIb-IX-dependent Rac1 activation. Thus, although we do not exclude the role of PI3K as a scaffold, our study reveals a novel signaling mechanism in which ligand binding to GPIb-IX induces the sequential activation of the SFK Lyn, the guanine nucleotide exchange factor Vav, and Rac1. Rac1 then mediates activation of the PI3K/Akt signaling pathway. Thus, this study represents a significant advance by identifying an important molecule in the early GPIb-IX signaling pathway leading to integrin activation.

The identification of Rac1 as a downstream effector of Lyn that stimulates the PI3K/Akt pathway is a novel finding not only to the GPIb-IX signaling pathway in platelets but also may have general implications to the mechanisms regulating PI3K activation. The SFKs Lyn/Fyn and Rac1 have both been implicated in stimulating the activity of PI3K by binding to the p85 subunit of PI3K.⁴¹⁻⁴³ Our data, however, indicate that Lyn activates the Rac1 guanine nucleotide exchange factor

Vav and thus Rac1, and requires Rac1 to mediate activation of PI3K. It will be interesting to further investigate whether this new model of Lyn- and Rac1-dependent PI3K activation is a common mechanism in other cell types.

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Disclosures

None.

References

- López JA. The platelet glycoprotein Ib-IX complex. *Blood Coagul Fibrinolysis*. 1994;5:97–119.
- Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. *Curr Opin Hematol*. 2007;14:262–269.
- Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. *Circ Res*. 2007;100:1673–1685.
- De Marco L, Girolami A, Zimmerman TS, Ruggeri ZM. Interaction of purified type IIB von Willebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and initiates aggregation. *Proc Natl Acad Sci USA*. 1985;82:7424–7428.
- Grainick HR, Williams SB, Collier BS. Asialo von Willebrand factor interactions with platelets. Interdependence of glycoproteins Ib and IIb/IIIa for binding and aggregation. *J Clin Invest*. 1985;75:19–25.
- Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*. 1998;94:657–666.
- Li Z, Zhang G, Liu J, Stojanovic A, Ruan C, Lowell CA, Du X. An important role of the SRC family kinase Lyn in stimulating platelet granule secretion. *J Biol Chem*. 2010;285:12559–12570.
- Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol*. 2010;30:2341–2349.
- Li Z, Xi X, Du X. A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin alpha IIb beta3. *J Biol Chem*. 2001;276:42226–42232.
- Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, Hofmann F, Du X. A stimulatory role for cGMP-dependent protein kinase in platelet activation. *Cell*. 2003;112:77–86.
- Yin H, Liu J, Li Z, Berndt MC, Lowell CA, Du X. Src family tyrosine kinase Lyn mediates VWF/GPIb-IX-induced platelet activation via the cGMP signaling pathway. *Blood*. 2008;112:1139–1146.
- Yin H, Stojanovic A, Hay N, Du X. The role of Akt in the signaling pathway of the glycoprotein Ib-IX induced platelet activation. *Blood*. 2008;111:658–665.
- Yap CL, Anderson KE, Hughan SC, Doppeide SM, Salem HH, Jackson SP. Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/V/IX and integrin alpha(IIb) beta(3). *Blood*. 2002;99:151–158.
- Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A2 generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005;106:3410–3414.
- Liu J, Pestina TI, Berndt MC, Jackson CW, Gartner TK. Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIb beta3- and aggregation-independent manner. *Blood*. 2005;106:2750–2756.
- Gu M, Xi X, Englund GD, Berndt MC, Du X. Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin alpha(IIb) beta(3) using a reconstituted mammalian cell expression model. *J Cell Biol*. 1999;147:1085–1096.
- Liu J, Pestina TI, Berndt MC, Steward SA, Jackson CW, Gartner TK. The roles of ADP and TXA in botrocetin/VWF-induced aggregation of washed platelets. *J Thromb Haemost*. 2004;2:2213–2222.
- Wu Y, Suzuki-Inoue K, Satoh K, Asazuma N, Yatomi Y, Berndt MC, Ozaki Y. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. *Blood*. 2001;97:3836–3845.
- McCarty OJ, Larson MK, Auger JM, Kalia N, Atkinson BT, Pearce AC, Ruf S, Henderson RB, Tybulewicz VL, Machesky LM, Watson SP. Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *J Biol Chem*. 2005;280:39474–39484.
- Pleines I, Elvers M, Strehl A, Pozgajova M, Varga-Szabo D, May F, Chrostek-Grashoff A, Brakebusch C, Nieswandt B. Rac1 is essential for phospholipase C-gamma2 activation in platelets. *Pflugers Arch*. 2009;457:1173–1185.
- Falet H, Hoffmeister KM, Neujahr R, Italiano JE Jr, Stossel TP, Southwick FS, Hartwig JH. Importance of free actin filament barbed ends for Arp2/3 complex function in platelets and fibroblasts. *Proc Natl Acad Sci USA*. 2002;99:16782–16787.
- Soulet C, Gendreau S, Missy K, Benard V, Plantavid M, Payrastre B. Characterisation of Rac activation in thrombin- and collagen-stimulated human blood platelets. *FEBS Lett*. 2001;507:253–258.
- Suzuki-Inoue K, Yatomi Y, Asazuma N, Kainoh M, Tanaka T, Satoh K, Ozaki Y. Rac, a small guanosine triphosphate-binding protein, and p21-activated kinase are activated during platelet spreading on collagen-coated surfaces: roles of integrin alpha(2)beta(1). *Blood*. 2001;98:3708–3716.
- Vidal C, Geny B, Melle J, Jandrot-Perrus M, Fontenay-Roupie M. Cdc42/Rac1-dependent activation of the p21-activated kinase (PAK) regulates human platelet lamellipodia spreading: implication of the cortical-actin binding protein cortactin. *Blood*. 2002;100:4462–4469.
- 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.
- Akbar H, Kim J, Funk K, Cancelas JA, Shang X, Chen L, Johnson JF, Williams DA, Zheng Y. Genetic and pharmacologic evidence that Rac1 GTPase is involved in regulation of platelet secretion and aggregation. *J Thromb Haemost*. 2007;5:1747–1755.
- Tiedt R, Schomber T, Hao-Shen H, Skoda RC. 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.
- O'Brien KA, Gartner TK, Hay N, Du X. ADP-Stimulated Activation of Akt During Integrin Outside-In Signaling Promotes Platelet Spreading by Inhibiting Glycogen Synthase Kinase-3B. *Arterioscler Thromb Vasc Biol*. 2012;32:2232–2240.
- O'Brien KA, Stojanovic-Terpo A, Hay N, Du X. An important role for Akt3 in platelet activation and thrombosis. *Blood*. 2011;118:4215–4223.
- Marjanovic JA, Li Z, Stojanovic A, Du X. Stimulatory roles of nitric oxide synthase 3 and guanylyl cyclase in platelet activation. *J Biol Chem*. 2005;280:37430–37438.
- Gong H, Shen B, Flevaris P, Chow C, Lam SC, Voyno-Yasenetskaya TA, Kozasa T, Du X. G protein subunit Galpha13 binds to integrin alphaIIb beta3 and mediates integrin "outside-in" signaling. *Science*. 2010;327:340–343.
- Li Z, Zhang G, Feil R, Han J, Du X. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. *Blood*. 2006;107:965–972.
- Stojanovic A, Marjanovic JA, Brovkovich VM, Peng X, Hay N, Skidgel RA, Du X. A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. *J Biol Chem*. 2006;281:16333–16339.
- Pearce AC, Senis YA, Billadeau DD, Turner M, Watson SP, Vigorito E. Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen. *J Biol Chem*. 2004;279:53955–53962.
- Kasirer-Friede A, Cozzi MR, Mazzucato M, De Marco L, Ruggeri ZM, Shattil SJ. Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors. *Blood*. 2004;103:3403–3411.
- Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. von Willebrand factor binding to platelet GPIb initiates signals for platelet activation. *J Clin Invest*. 1991;88:1568–1573.
- Ozaki Y, Satoh K, Yatomi Y, Miura S, Fujimura Y, Kume S. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. *Biochim Biophys Acta*. 1995;1243:482–488.
- Aslan JE, Tormoen GW, Loren CP, Pang J, McCarty OJ. S6K1 and mTOR regulate Rac1-driven platelet activation and aggregation. *Blood*. 2011;118:3129–3136.
- Mu FT, Cranmer SL, Andrews RK, Berndt MC. Functional association of phosphoinositide-3-kinase with platelet glycoprotein Ibalph, the major ligand-binding subunit of the glycoprotein Ib-IX-V complex. *J Thromb Haemost*. 2010;8:324–330.
- Wu Y, Asazuma N, Satoh K, Yatomi Y, Takafuta T, Berndt MC, Ozaki Y. Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase. *Blood*. 2003;101:3469–3476.
- Pleiman CM, Hertz WM, Cambier JC. Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science*. 1994;263:1609–1612.
- Bokoch GM, Vlahos CJ, Wang Y, Knaus UG, Traynor-Kaplan AE. Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. *Biochem J*. 1996;315:775–779.
- Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem*. 1995;270:17656–17659.