haematologica
Journal of the Ferrata Storti Foundation

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Received: June 12, 2026.
Accepted: August 24, 2026.

Citation: Justine Suffit, Lionel Galicier, Theo Pigaglio, Marie-Christine Alessi, Lucie Derrida, Claire Poggi, Romaric Lacroix, Denis Bernot, Chloé Dol, Manal Ibrahim-Kosta and Marjorie Poggi.
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Haematologica. 2026 Sept 3. doi: 10.3324/haematol.2026.301418 [Epub ahead of print]

Normal platelet function in a patient with ARHGEF1 deficiency.

Justine Suffit[1], Lionel Galicier[2], Theo Pigaglio[1], Marie-Christine Alessi[1,3,4], Lucie Derrida[2], Claire Poggi[2], Romaric Lacroix[1,3], Denis Bernot[3], Chloé Dol[2], Manal Ibrahim-Kosta[1,3,4]* and Marjorie Poggi[1]*

* Equally contribution

[1]Aix-Marseille University, INSERM, INRAE, C2VN, Marseille, France.

[2]Department of Internal Medicine, La Timone Hospital, APHM, Marseille, France

[3]Laboratory of Hematology, Biogénopôle, CHU La Timone, APHM, Marseille, France.

[4]French Reference Center on Inherited Platelet Disorders, Laboratory of hematology, AP-HM, Marseille, France.

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Corresponding authors : Marjorie Poggi, C2VN, Faculté de Médecine, Aix-Marseille University, 27 Boulevard Jean Moulin, 13385 Marseille, France. *Email :* marjorie.poggi@univ-amu.fr
Manal Ibrahim-Kosta, C2VN, Faculté de Médecine, Aix-Marseille University, 27 Boulevard Jean Moulin, 13385 Marseille, France. *Email*: MANAL.IBRAHIM@ap-hm.fr

Author contributions: J.S., T.P., D.B, M-C. A and M.P. performed the functional experiments and analyzed the data. G.L., L.D., and C.P. were responsible for patient management. R.L. performed the extracellular vesicle analyses. D.B M.I.-K. and M.P. conceived and supervised the project and wrote the manuscript.

Data-sharing statement: the dataset generated and analyzed are available from the corresponding authors on request.

Disclosure: there are no competing interests to disclose.

Trial registration: ClinicalTrials.gov identifier: NCT04419987 - EVGPP: study of the functionality of new genetic variants at the origin of constitutional platelet pathologies, Application Number: 2019-A03054-53.

Funding: the authors declare no source of funding.

Main text

Rho GTPases are critical regulators of platelet function. These proteins cycle between an inactive GDP-bound and an active GTP-bound states through tightly regulated mechanisms, central to which are guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)[1]. Platelets express a wide range of RhoGAPs and RhoGEFs, as revealed by proteomic analyses to date[2,3]. In contrast to RhoGAPs, comparatively fewer studies have characterized the roles of specific RhoGEFs in platelet function, and detailed functional analyses remain lacking.

ARHGEF1 is a member of the GEF family, predominantly expressed in hematopoietic cells, and is specific for the small RhoA GTPase, which plays a key role in cytoskeletal signaling. In platelets, ARHGEF1 is involved in signaling downstream of G protein-coupled receptors coupled to Gα12/13 heterotrimeric G proteins[4,5], such as thrombin and thromboxane A_2 receptors.

In an ARHGEF1 knockout (Arhgef1$^{-/-}$) mouse model, prolonged tail bleeding time and severe platelet activation defect were observed[6]. These mice exhibited impaired platelet aggregation in response to low-dose thrombin (0.05 and 0.1 U/mL); U46619 (2.5 µM) and collagen (5 µg/mL). They also showed defects in granule secretion, GPIIb/IIIa activation, clot retraction and spreading associated with an absence of RhoA activation in platelets. The authors concluded that ARHGEF1 regulates platelet function, hemostasis, and thrombogenesis in mice, and proposed ARHGEF1 as a potential therapeutic target for thrombotic disorders. However, its role in human platelet remains unknown. Importantly, murine and human platelet biology are not always fully comparable, and findings from mouse models cannot be directly extrapolated to humans. For example, several lines of evidence from the literature suggest that ARHGEF3 plays a role in human megakaryocytes and platelets[7–9]. In contrast, ARHGEF3-deficient mice display normal megakaryocyte development and platelet function, indicating that ARHGEF3 may have a species-specific role in humans that is not conserved in mice[7].

ARHGEF1 deficiency has been described in two female siblings (P1 and P2) carrying compound heterozygous germline variants in *ARHGEF1* (Figure 1A), resulting in primary antibody deficiency and recurrent severe respiratory tract infections with bronchiectasis[10]. T and B lymphocytes from both patients displayed reduced RhoA activity and decreased steady-state actin polymerization. As a consequence of dysregulated signaling downstream of RhoA, including altered activity of Rho-associated kinase (ROCK I/II), the patients' lymphocytes failed to properly restrain AKT phosphorylation.

To date, platelet function has not been investigated in these patients. In the present study, platelet function was assessed exclusively in patient P2, for whom biological samples were available, in order to determine whether ARHGEF1 deficiency also affects human platelet activation and hemostatic function.

The study was approved by the institutional Ethics Committee, and written informed consent was obtained from the patient and matched healthy controls (1 or 2 controls per experiment) in accordance with the Declaration of Helsinki. The study was registered at

ClinicalTrials.gov (identifier: NCT04419987) under the protocol "EVGPP: Study of the Functionality of Novel Genetic Variants Responsible for Inherited Platelet Disorders" (Application Number: 2019-A03054-53).

Patient P2, a 36-year-old woman, had no personal or familial history of bleeding, including during multiple hemostatic challenges (lung lobectomy, ovarian cyst excision, breast implant placement, two childbirths, and elective pregnancy terminations). Platelet count (200-325 $x10^9$/L) and morphology were normal. Western-blot showed the absence of ARHGEF1 expression in the patient's platelets, in contrast to control platelets (Figure 1B and Supplemental Figure S1).

Light transmission aggregometry in response to several agonists (ADP 5 and 10 µM (Helena), collagen 2 and 10 µg/mL (Hyphen Biomed), epinephrine 5µmol/L (Helena), thrombin receptor-activating peptide 6 (TRAP6) 10, 20 and 50 µM (Stago), ristocetin 1.25 mg/mL (Stago), and arachidonic acid 1mM Helena)) was normal as summarized in Table 1.

Platelet glycoprotein (GP) expression, including GPIIb/IIIa (αIIbβ3, CD41) and GPIb (CD42b), was assessed by flow cytometry (Navios EX, Beckman Coulter) at baseline and following activation with TRAP6 (50 µM, Stago) and ADP (10 µM, Helena). No abnormalities were observed in GP expression or in GPIIb/IIIa activation, as measured by PAC-1 binding, in patient P2 (Table 1).

No defect in dense or α-granule content or secretion was observed in platelets from patient P2. This was demonstrated by normal mepacrine uptake and release by flow cytometry, as well as normal CD63 and P-selectin (CD62P) expression following activation with TRAP6 (50 µM) and ADP (10 µM). In addition, intraplatelet content of nucleotides (ATP/ADP ratio), serotonin and, PAI-1 were within the normal range (Table 1).

GPIIb/IIIa outside-in signaling was assessed by platelet spreading on fibrinogen following activation with ADP (10 µM) and TRAP6 (20 µM) (Figure 1C–D), as well as by a clot retraction assay in the presence of 0.1 U/mL thrombin (Figure 1E). ARHGEF1-deficient platelets spread efficiently and form filopodia and lamellipodia similarly to control platelets (Figure 1C–D). Clot retraction was also preserved and appeared even earlier compared to control platelets (Figure 1E). This experiment was independently reproduced using another control donor, yielding consistent results, with clot retraction again appearing enhanced in patient's platelets (not shown).

Platelet ($CD41^+$- extracellular vesicles (EV)), erythrocyte ($CD235^+$-EV), granulocyte ($CD15^+$-EV), and monocyte ($HLA\text{-}DR^+$-EV) expressing phosphatidylserine (PS^+) were quantified by Annexin-V labeling using flow cytometry (Cytoflex, standardized with Megamix SSC 0.16–0.9)[7]. No abnormalities were observed in the number or cellular origin of circulating EV (Table 1).

Production of PS^+ platelet-derived EV in platelet-rich plasma after activation with thrombin 1U/mL and collagen 10µg/mL was also normal. For reference, control values ranged from 100–700 EV/µL at baseline and >10,000 EV/µL after thrombin/collagen activation, whereas

patient P2 had 210 EV/µL at baseline and 22,500 EV/µL after activation, consistent with preserved platelet procoagulant function.

Further assessment of platelet procoagulant activity was performed using thrombin generation assays in platelet-rich plasma in presence of 1 pM tissue factor, measured by the Calibrated Automated Thrombography method[11]. Patient P2 exhibited a shorter lag time and time to peak compared to normal pooled plasma (Figure 2A–B). The area under the thrombin generation curve (endogenous thrombin potential, ETP, in nM.min), calculated using Thrombinoscope software, was slightly lower than the control but remained within the normal range. Thrombin generation in platelet-poor plasma triggered with 1 pM tissue factor was similar between patient and control; however, both lag time and time to peak were shorter in the patient's plasma, and the peak thrombin level was higher (Figure 2A–B). Altogether, these results argue against a defect in platelet-dependent thrombin generation and support preserved hemostatic function.

Although the absence of a bleeding phenotype, the accelerated clot retraction, and the shorter lag time and time to peak, and higher peak thrombin in PPP could collectively suggest enhanced hemostasis activity, these findings should be interpreted with caution. Neither the patient nor the affected sister reported a personal or a family history of venous or arterial thrombosis.

Because ARHGEF1 is a RhoA-specific guanine nucleotide exchange factor, platelet lysates were analyzed using a G-LISA RhoA activation assay, which specifically detects the active RhoA-GTP form but not the inactive RhoA-GDP form. ARHGEF1-deficient platelets exhibited RhoA activation of a similar magnitude to control platelets under identical activation conditions (Figure 2C), indicating no defect in RhoA activation.

Given that RhoA is a key regulator of actin cytoskeleton dynamics, we next assessed polymerized actin (F-actin) content by flow cytometry in platelets from the patient and healthy donors. The results were comparable between groups, indicating preserved actin polymerization in ARHGEF1-deficient platelets (Supplemental Figure S2).

One important function of the RhoA/ROCK pathway is the regulation of PI3K/AKT signaling through the control of PTEN activity[10,12]. We therefore compared AKT activation in platelets from the patient and healthy donors and assessed the levels of phosphorylated RPS6 (p-RPS6), a downstream target of the PI3K/AKT/mTOR pathway (Figure 2D). PTEN expression levels were overall similar between controls and the patient, with a trend toward increased phospho-PTEN in the patient cells. Total AKT levels were comparable, although a slight increase in phosphorylated AKT was observed in the patient, suggesting a modest alteration in AKT regulation. However, no difference in RPS6 phosphorylation was detected between groups, suggesting that this alteration did not result in a broader activation of downstream AKT/mTOR signaling. Taken together, these data indicate that AKT pathway activity is largely maintained in ARHGEF1-deficient platelets under the conditions tested, consistent with the functional profile observed in patient platelets.

The preservation of RhoA activation in ARHGEF1-deficient platelets indicates that RhoA signaling is not exclusively dependent on ARHGEF1 in humans. Given the functional

redundancy within the RhoGEF family and the existence of Gα13-independent pathways leading to RhoA activation, alternative mechanisms are likely to compensate for the loss of ARHGEF1. Consistent with these hypotheses, quantitative proteomic analyses have identified multiple RhoGEFs in human platelets[2], ranked by decreasing abundance as ARHGEF7 (~3,100 copies per platelet), ARHGEF6 (~1,600), ARHGEF1 and ARHGEF2 (~1,400 each), ARHGEF12 (~920), and ARHGEF10 (~560). Despite their relatively low abundance compared with highly expressed platelet proteins such as PF4 (~563,000 copies per platelet), these RhoGEFs may collectively provide sufficient activity to maintain RhoA signaling. Although ARHGEF2 and ARHGEF6 protein levels were unchanged in the patient's platelets (Figure S3), compensation may result in enhanced activation, rather than increased protein abundance or involve other platelet-expressed RhoGEFs. Reduced ARHGAP activity could also contribute to sustained RhoA signaling by limiting RhoA inactivation. Further studies will be required to identify these compensatory mechanisms and define their respective contributions, for example through targeted in vitro knockdown approaches.

In contrast to the severe platelet phenotype reported in Arhgef1$^{-/-}$ mice, loss of ARHGEF1 expression in human platelets was not associated with detectable functional abnormalities across multiple assays. These findings reveal substantial species-specific differences in platelet signaling and challenge the direct extrapolation of murine models to human platelet biology. More broadly, they highlight the importance of studying naturally occurring human genetic deficiencies to define the physiological relevance of candidate signaling pathways in platelets.

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Table 1: **Routine platelet count and function assessment.**

Methods for the different tests were described previously[13]. Analyses were performed at La Timone Hospital, Marseille. Reference ranges were defined as the minimum–maximum interval obtained from 30 healthy individuals in our laboratory. Abbreviations: MPV, mean platelet volume (measured using a fluorescence-based method on a Sysmex XN analyzer); ADP, adenosine diphosphate; TRAP6, thrombin receptor-activating peptide; PAI-1, plasminogen activator inhibitor-1; plt, platelet; MFI, mean fluorescence intensity; a.u., arbitrary units; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PS+, phosphatidylserine-positive cells.

	Reference ranges*	Patient
Platelet count (x10^9/l)	150-400	200-325
MPV (fL)	8.0-12.0	9.8
Platelet aggregation maximal intensity (%)		
ADP		
5μM	73-95	88
10μM	84-95	84-95
Collagen		
2μg/mL	85-96	85
10μg/mL	86-97	86
Epinephrine		
5μM	80-100	85
TRAP6		
10μM	88-97	81
20μM	80-98	86
50μM	86-97	87
Ristocetin		
1.25 mg/mL	90-99	95
Arachidonic acid		
1mM	86-96	90
Platelet glycoprotein MFI (a.u.)		
αIIbβ3 (CD41)		
Baseline	>12	11.9
TRAP6	>15	15.7
ADP	>15	15
PAC		
Baseline	>0.5	0.34
TRAP6	>0.7	3.03
ADP	>3.5	6.27
GP1b (CD42b)		
Baseline	1.7-6	2.5
TRAP6	0.9-4	1.4
ADP	0.8-3	1.4
Platelet granule content & secretion		
Mepacrine test MFI (a.u)		
Mepacrine 2μM uptake	>3.5	3.9
Mepacrine 2μM release ratio	1.6	1.6
CD63 MFI (a.u)		
Baseline	< 1	0.7
Baseline/TRAP6 ratio	>1.5	2.63
Baseline/ADP ratio	>1.2	1.71
CD62P MFI (a.u)		
Baseline	0.5	0.3
Baseline/TRAP6 ratio	>7	8.6
Baseline/ADP ratio	>4	6.67
ATP/ADP ratio	1.2-2.4	1.51
Serotonin *(*μg/10^9plt*)*	0.51-1.31	0.70
PAI-1 antigen (μg/10^9plt)	0.33-1.07	0.75
Extracellular vesicles (EV)		
PS^+-EV in PRP (events/μL)		
Baseline	100-700	210
Thrombin (1U/mL)/Collagen (10μg/mL)	>10000	22500
Total PS^+-EV in PPP (events/μL)	1500-3000	3020
Platelet $CD41^+$-EVs	500-3000	2100
Erythrocyte $CD235^+$-EVs	<400	280
Granulocyte $CD15^+$-EVs	<50	2
Monocyte HLA-DR^+-EVs	<200	40

*: Reference ranges are obtained from 30 healthy individuals.

Figure legends

Figure 1: ARHGEF1 deficiency in patient's platelets and analysis of GPIIb/IIIa outside-in signaling.

A- The compound heterozygous *ARHGEF1* variants in patient and their conservation across species: patient is carrying a paternal nonsense variant, c.853C>T (NM_004706.4), leading to p.Arg285Ter (frequency of 1.368×10^{-6} in the GnomAD Exome v4 database, CADD score: 37), and a maternal splice-site variant, c.1624-1G>T, absent from GnomAD Exome v4 database, predicted to disrupt canonical splicing (SpliceAI delta score (AL)= 1) and results in loss of protein function (p.?). Sequence alignment of ARHGEF1 across multiple species demonstrates the high conservation of the affected residues, with the variants reported in this study highlighted in yellow.

B- Western-blot analysis of ARHGEF1. ARHGEF1 expression was assessed in washed platelets from healthy controls and the patient. Platelet lysates were prepared simultaneously. GAPDH was used as a loading control. Briefly, platelet lysates prepared in RIPA buffer were separated on NuPAGE gels using MES SDS running buffer and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked and incubated overnight with mouse anti-ARHGEF1 (RhoGEFp115, Santa Cruz Biotechnology, sc-74565) or anti-GAPDH (Millipore, MAB374), followed by a 1-hour incubation with HRP-conjugated anti-mouse secondary antibody (Bio-Rad). Chemiluminescent signals were detected using Pierce ECL Western blotting substrate, and images were acquired using a CCD imager (iBright, Thermo Fisher Scientific). The presented image is representative of 3 independent experiments (n = 3).

C-D Platelet spreading. Whole blood was collected in citrate, and platelets were washed and resuspended at 4.10^8/mL in RPMI. Platelets were allowed to adhere to fibrinogen-coated coverslips (100 µg/mL, Sigma F3879), and analyzed under non-activated conditions (n=2) or after 45 minutes following activation (n=1 each condition) by TRAP6 (20 µM, Sigma S1820) or ADP (10 µM, Sigma A2754). Platelets were then fixed and permeabilized using 1% PFA and 0.3% Triton X-100, followed by staining with Alexa Fluor 488–conjugated phalloidin (Thermo Fisher Scientific, A12379) for 30 minutes to visualize F-actin. Scale bar, 10µm. The proportion of platelets classified as non-spread or displaying filopodia, lamellipodia, or actin nodules was quantified using Image J. The histogram represents the distribution of platelets across these different morphological states. For each condition, five fields per slide were quantified, with approximately 100 platelets analyzed per field.

E- Clot retraction. Whole blood was collected in CPDA and platelets were washed and resuspended at 4×10^8/mL in resuspension buffer (final concentrations: 2mg/mL glucose, NaCl 0.137 M, KCl 2.68 mM, $NaHCO_3$ 1.9 mM, $NaH_2PO_4 \cdot H_2O$ 0.417 mM; $MgCl_2$ 0.5 mM, HEPES 10 mM, pH 7.3). Clot retraction was initiated by adding thrombin (0.1 U/mL) and fibrinogen (0.4 mg/mL) to 60 million platelets (150 µL) in the presence of Ca^{2+} (2 mM). Photographs were taken at 3 different times. The presented images are representative of 2 experiments (n = 2).

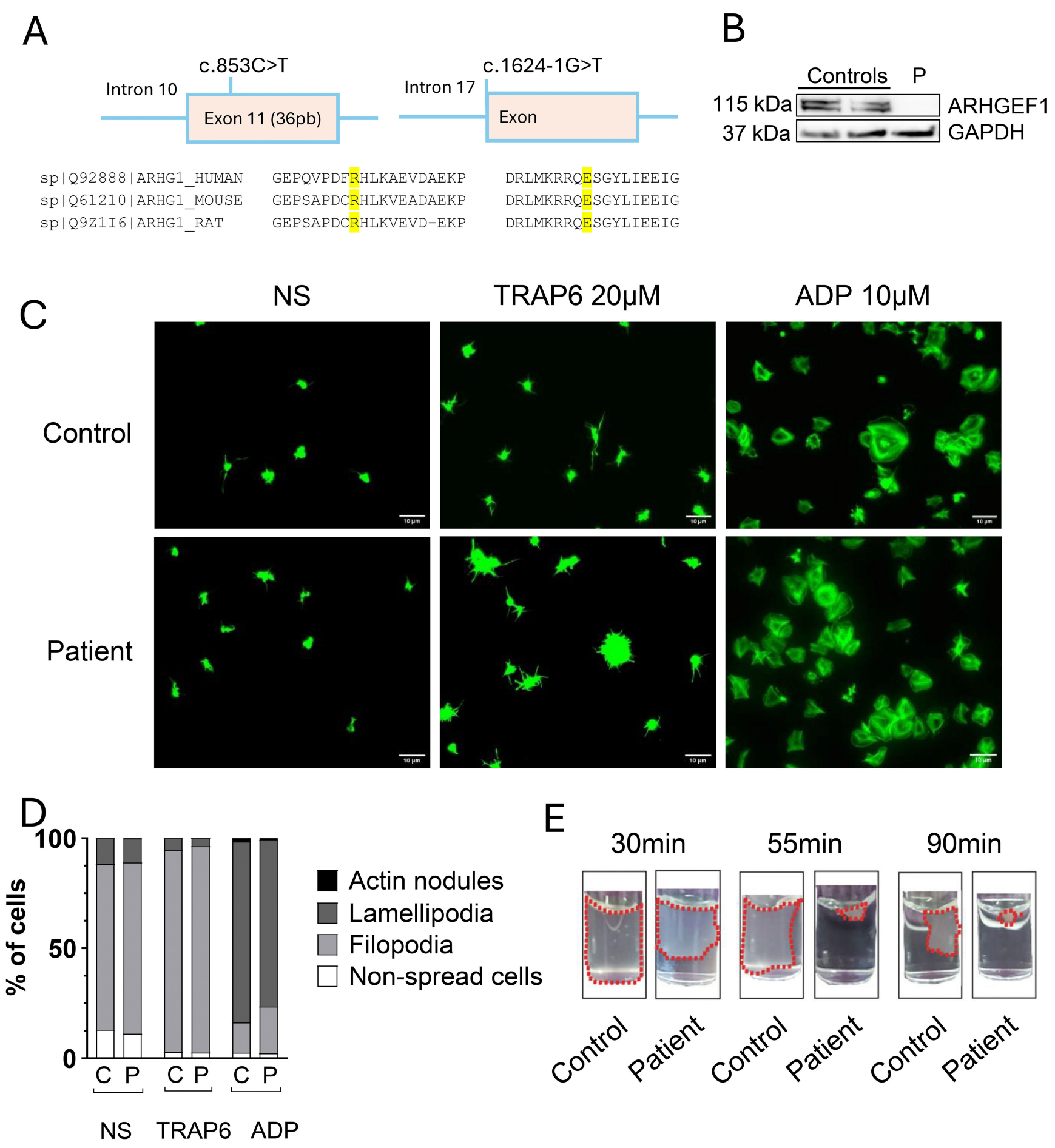
NS, non-stimulated; TRAP, Thrombin receptor-activating peptide; ADP, adenosine diphosphate.

Figure 2: Thrombin generation in platelet-rich and platelet-poor plasma and analysis of AKT signaling.

A-B **Thrombin generation test.** Thrombin generation testing was performed using the Calibrated Automated Thrombography method. Representative thrombin generation curves and thrombin generation parameters obtained from measurements performed in platelet-rich (A) and –poor (B) plasma at 1pM tissue factor are shown for a healthy control and the patient. The internal reference ranges used currently at the laboratory are also provided. The presented graphs are representative of two technical replicates (n = 2). PRP: platelet-rich plasma; PPP: platelet-poor plasma; TF: tissue factor; ETP: endogenous thrombin potential.

C- RhoA-GTP quantification. RhoA-GTP levels were measured using the G-LISA RhoA Activation Assay (Cytoskeleton Inc.), which specifically detects the active GTP-bound form of RhoA GTPase. Measurements were performed under basal conditions and after 50µM TRAP6 activation (1 and 5 min). Results are expressed as the ratio of TRAP6-activated to non-activated RhoA-GTP levels in two healthy controls and the patient (n=1).

D- **AKT signaling pathway analysis.** Western-blot analysis was performed to assess AKT signaling proteins in washed platelets from healthy controls and the patient. Platelets were analyzed under basal (non-activated) conditions and after activation with TRAP6 (50 µM, 5 min). Platelet lysates were prepared in parallel, and GAPDH was used as a loading control. Briefly, lysates in RIPA buffer were separated on NuPAGE gels using MES-SDS running buffer and transferred onto PVDF membranes. Membranes were blocked and incubated overnight with the following primary antibodies: mouse anti-AKT (cell signaling, # 4691), mouse anti-PTEN (cell signaling; #9559S), mouse anti-RPS6 (cell signaling; #2217, and anti-GAPDH (Millipore, MAB374) (left panel). For the panel on the right, phospho-specific antibodies were used: mouse anti-phospho-AKT (cell signaling; #4058), mouse anti-phospho-PTEN (cell signaling; #9549s), and rabbit anti-phospho-RPS6 (cell signaling; #4858T), followed by 1-hour incubation with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad). Chemiluminescent signals were detected using Pierce ECL substrate, and images were acquired using a CCD imager (iBright, Thermo Fisher Scientific). Densitometric analysis was normalized to GAPDH and expressed as mean ± SEM. Images were captured with a CCD imager (iBright, Thermo). The presented blots are representative of two independent experiments (n = 2). NS, non-simulated; TRAP, Thrombin receptor-activating peptide.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B
Controls
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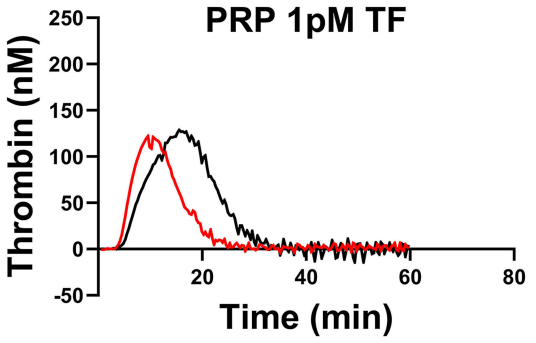
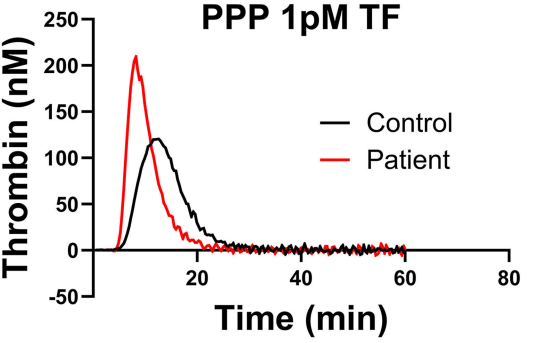
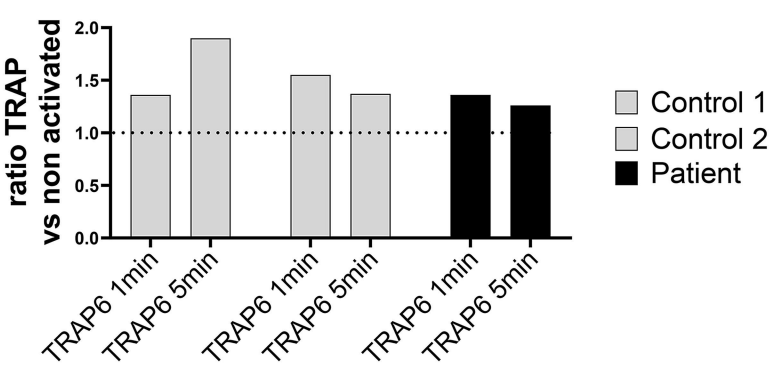




B

	PRP 1pM TF			PPP 1pM TF		
	Normal Pool	Patient	Reference range	Control	Patient	Reference range
Lagtime (min)	5.55	4.55	3.9-8.9	6.72	5.22	4.4-9
ETP (nM.min)	1869.5	1261	964-2430	1208	1251.5	1061-2378
Peak (nM)	125.96	119.8	26-222	120.13	203.37	128-438
Time to peak (min)	16.09	11.24	ND	12.41	8.23	6.7-12.9

C



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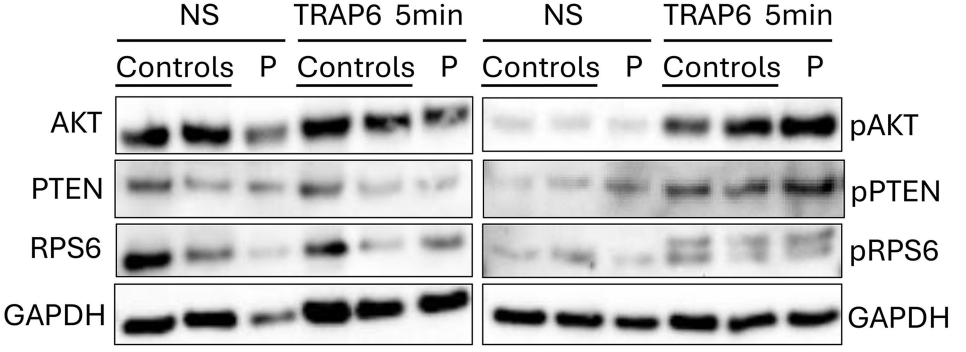


Supplemental Figure 1

Western blot analysis of ARGHEF1. ARHGEF1 expression was evaluated in washed platelets obtained from two healthy controls (C1 and C2) and the patient (P). Platelet lysates were prepared simultaneously. GAPDH served as the loading control.

Briefly, platelet lysates were prepared in RIPA buffer, separated on NuPAGE gels using MES SDS running buffer, and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked and incubated overnight with a mouse anti-ARHGEF1 (RhoGEF p115, Santa Cruz Biotechnology, sc-7565), or with an anti-GAPDH antibody (Millipore, MAB374). Membranes were then incubated for 1 hour with an HRP-conjugated anti-mouse secondary antibody (Bio-Rad). Chemiluminescent signals were detected using Pierce ECL Western Blotting Substrate, and images were acquired with an iBright CCD imaging system (Thermo Fisher Scientific). Two exposure times (8 s and 40 s) were acquired to illustrate the ARHGEF1 signal.

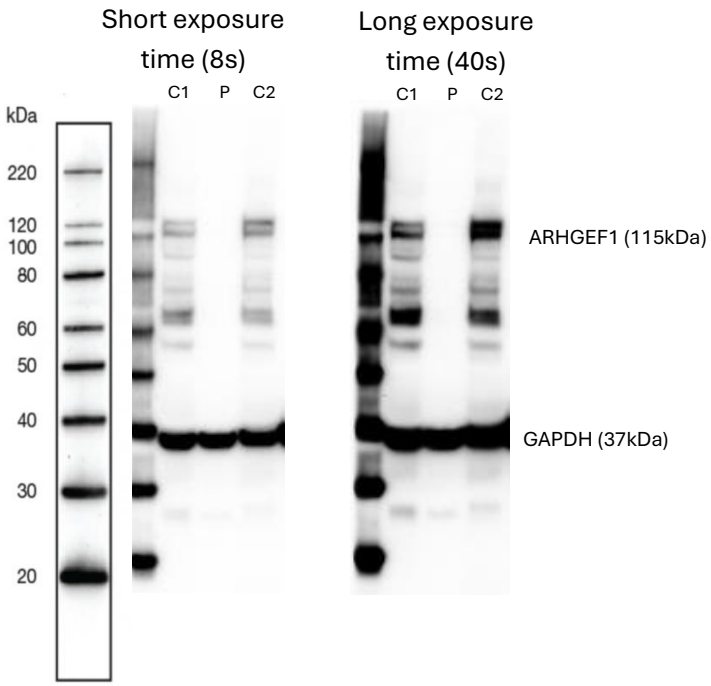
Supplemental Figure 2

Representative flow cytometry analysis showing actin polymerization in washed platelets under resting conditions or following stimulation with thrombin receptor-activating peptide (TRAP6 50 µM). Washed platelets were fixed with 2% paraformaldehyde in PBS for 5 min to stop stimulation, permeabilized with 0.5% Triton X-100, and stained with 1.5 µg/mL Alexa Fluor 488–conjugated phalloidin (Thermo Fisher Scientific, A12379) for 30 min to visualize F-actin.

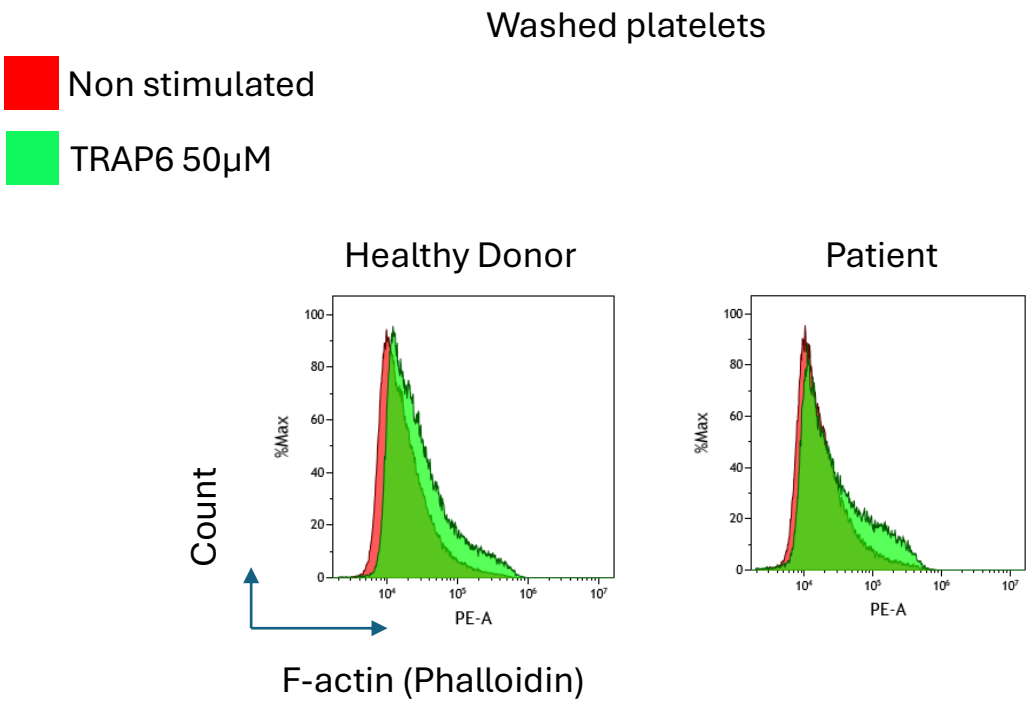
Supplemental Figure 3

Western blot analysis of ARHGEF2 and ARHGEF6. ARHGEF2 and ARHGEF6 expression was assessed in washed platelets from healthy controls and the patient. Platelet lysates were prepared simultaneously, and GAPDH was used as a loading control. Briefly, platelets were lysed in RIPA buffer, and proteins were separated on NuPAGE gels using MES SDS running buffer before being transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked and incubated overnight with primary antibodies against ARHGEF2 (GEF-H1; rabbit monoclonal antibody, clone 55B6, Cell signaling Technology, #4076) and ARHGEF6 (Cool2/α-PIX; rabbit monoclonal antibody, clone C23D2, Cell Signaling Technology, #4573) or GAPDH (mouse monoclonal antibody, Millipore, MAB374). Membranes were then incubated for 1 hour with the appropriate HRP-conjugated secondary antibody. Chemiluminescent signals were detected using Pierce ECL Western Blotting Substrate, and images were acquired using an iBright CCD imaging system (Thermo Fisher Scientific).

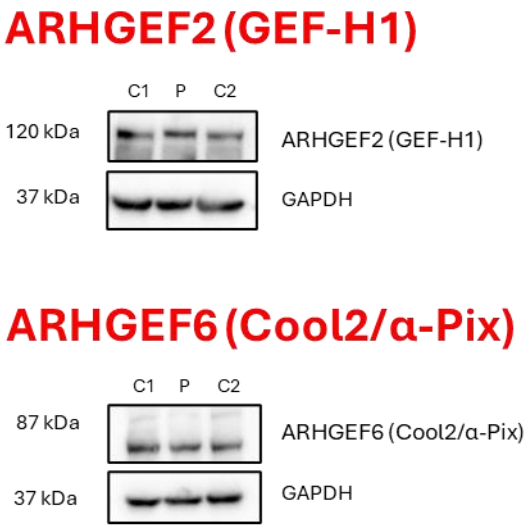
ARHGEF1 (RhoGEF P115)



Supplemental figure 1



Supplemental figure 2



Supplemental figure 3