REGULAR ARTICLE

blood advances

Protein kinase C signaling dysfunction in von Willebrand disease (p.V1316M) type 2B platelets

Caterina Casari,[1,2] David S. Paul,[1] Sophie Susen,[3] Cécile Lavenu-Bombled,[2,4] Annie Harroche,[5] Raymond Piatt,[1] Kathryn O. Poe,[1] Robert H. Lee,[1] Marijke Bryckaert,[2] Olivier D. Christophe,[2] Peter J. Lenting,[2] Cécile V. Denis,[2] and Wolfgang Bergmeier[1,6]

[1]McAllister Heart Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC; [2]INSERM Unité Mixte de Recherche Scientifique 1176, University Paris-Sud, University Paris-Saclay, Le Kremlin-Bicêtre, France; [3]Hematology Department, University Hospital, Lille-II University, Lille, France, [4]Service d'Hématologie Biologique, Assistance Publique–Hôpitaux de Paris, Le Kremlin-Bicêtre, France, [5]Department of Haematology, Haemophilia Care Centre, Hôpital Universitaire Necker-Enfants Malades, Université Paris Descartes, Sorbonne Paris Cité, Assistance Publique–Hôpitaux de Paris, Paris, France; and [6]Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Key Points

- In VWD (p.V1316M) type 2B, the VWF–GPIb interaction alters the sustained PKC-mediated pathway of Rap1 activation.
- VWF/p.V1316M expression in mice causes multiple platelet dysfunctions, including significant ADAM17-independent shedding of GPIbα.

von Willebrand disease (VWD) type 2B is characterized by gain-of-function mutations in von Willebrand factor (VWF), enhancing its binding affinity for the platelet receptor glycoprotein (GP)Ibα. VWD type 2B patients display a bleeding tendency associated with loss of high-molecular-weight VWF multimers and variable thrombocytopenia. We recently demonstrated that a marked defect in agonist-induced activation of the small GTPase, Rap1, and integrin αIIbβ3 in VWD (p.V1316M) type 2B platelets also contributes to the bleeding tendency. Here, we investigated the molecular mechanisms underlying impaired platelet Rap1 signaling in this disease. Two distinct pathways contribute to Rap1 activation in platelets: rapid activation mediated by the calcium-sensing guanine nucleotide exchange factor CalDAG–GEF-I (CDGI) and sustained activation that is dependent on signaling by protein kinase C (PKC) and the adenosine 5′-diphosphate receptor P2Y12. To investigate which Rap1 signaling pathway is affected, we expressed VWF/p.V1316M by hydrodynamic gene transfer in wild-type and *Caldaggef1*$^{-/-}$ mice. Using αIIbβ3 integrin activation as a read-out, we demonstrate that platelet dysfunction in VWD (p.V1316M) type 2B affects PKC-mediated, but not CDGI-mediated, activation of Rap1. Consistently, we observed decreased PKC substrate phosphorylation and impaired granule release in stimulated VWD type 2B platelets. Interestingly, the defect in PKC signaling was caused by a significant increase in baseline PKC substrate phosphorylation in circulating VWD (p.V1316M) type 2B platelets, suggesting that the VWF–GPIbα interaction leads to preactivation and exhaustion of the PKC pathway. Consistent with PKC preactivation, VWD (p.V1316M) type 2B mice also exhibited marked shedding of platelet GPIbα. In summary, our studies identify altered PKC signaling as the underlying cause of platelet hypofunction in p.V1316M-associated VWD type 2B.

Introduction

von Willebrand disease (VWD) type 2B is a paradoxical bleeding disorder resulting from gain-of-function mutations in the A1 domain of von Willebrand factor (VWF), which is responsible for the binding of the molecule to the platelet receptor glycoprotein (GP)Ibα. VWD type 2B is characterized by reduced VWF antigen levels, lack of high-molecular-weight VWF multimers,[1] circulating platelet aggregates, and variable thrombocytopenia, which are dependent on the causative mutation.[2,3] For many years, the

Submitted 17 November 2017; accepted 17 May 2018. DOI 10.1182/bloodadvances.2017014290.

The full-text version of this article contains a data supplement.

severity of the bleeding tendency in VWD type 2B patients has been associated with the low platelet count and the absence of high-molecular-weight VWF multimers.[2] We have recently demonstrated that a severe thrombopathy also aggravates this complex clinical picture. Indeed, we showed that VWF/p.V1316M alters platelet signaling by inhibiting the activation of the small GTPase Rap1B, which is critical for talin recruitment and subsequent integrin αIIbβ3 activation.[4]

The 2 Rap1 isoforms, Rap1A and Rap1B, are the most abundant small GTPases expressed in platelets.[5] Rap1 GTPases switch between a GTP-bound (active) and a GDP-bound (inactive) state. All known platelet agonists stimulate GTP loading of Rap1.[6-9] Our recent work identified key pathways regulating Rap1B activation in platelets: fast, but reversible, activation mediated by a calcium-sensing guanine nucleotide exchange factor (CalDAG–GEF-I) and slow, but sustained, activation mediated by protein kinase C (PKC) and the platelet receptor for adenosine 5′-diphosphate (ADP), P2Y12.[10-12]

PKC has a well-documented role in platelet signaling, where it controls the release of storage granules and, thus, the release of the second-wave mediator of platelet activation, ADP.[13] Moreover, PKC activation has been shown to induce the proteolytic cleavage (shedding) of various platelet surface receptors, including the GPIbα subunit of the VWF receptor complex (GPIb-V-IX). Shedding of GPIbα is a constitutive process in humans and mice, as confirmed by the presence of basal amounts of soluble GPIbα (glycocalicin) in plasma.[14,15] Constitutive and agonist-induced shedding of GPIbα are strongly dependent on the metalloproteinase ADAM17.[16] However, alternative sheddases may also contribute to the regulation of surface expression levels of GPIbα.[16-18] In cells other than platelets, distinct signaling pathways control shedding of receptors, such as epidermal growth factor receptor[19] and CD44.[20] Both PKC-dependent (mainly PKCα and PKCδ) and PKC-independent mechanisms have been described.

In the present study, we investigated the molecular mechanisms leading to the severe thrombopathy recently described in p.V1316M-associated VWD type 2B patients and mice.[4] We demonstrate that mutant VWF/p.V1316M engagement of the GPIbα receptor at the platelet surface leads to upregulated baseline PKC activity in human and murine VWD (p.V1316M) type 2B platelets and a defect in the PKC/P2Y12/Rap1 signaling response to agonist stimulation. These changes in PKC activity lead to increased shedding of GPIbα in mice, a marked reduction in platelet granule release, and impaired integrin activation in mice and humans. Together, these alterations protect the remaining circulating platelets from clearance, an adaptation that is critical to prevent thrombocytopenia and/or thrombosis.

Methods

Detailed information is provided in supplemental Methods.

Mouse strains

Eight- to 12-week-old C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

IL4R-Ibα–transgenic (Tg),[21] *Caldaggef1*$^{-/-}$,[22] *Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$,[23] *Caldaggef1*$^{-/-}$*P2Y12*$^{-/-}$,[24] *talin1*$^{fl/fl}$*PF4-Cre*$^{+}$,[25] and *Adam17*$^{fl/fl}$*PF4-Cre*$^{+}$ (*Adam17*$^{tm1.2Bbl}$/J mice were purchased from The Jackson Laboratory), and *Vwf*/p.V1316M[26] mice were bred and housed in the animal facility at the University of North Carolina at Chapel Hill.

Hydrodynamic gene transfer

Hydrodynamic gene transfer (50-100 μg of pLIVE-murineVWF/p.V1316M plasmid) was performed in mice expressing endogenous VWF/WT, as previously reported for *Vwf*$^{-/-}$ mice[1,27,28] (supplemental Figure 1). WT, *IL4R-IbαTg*, *Caldaggef1*$^{-/-}$, *Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$, *Caldaggef1*$^{-/-}$*P2Y12*$^{-/-}$, *talin1*$^{fl/fl}$*PF4-Cre*$^{+}$, *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$, and *Vwf*$^{-/-}$ mice (as control) were subjected to the procedure. VWF antigen plasma levels were measured before and after hydrodynamic injections to confirm VWF expression (supplemental Table 1). All experiments were conducted 3-7 days after gene transfer. Mice expressing mutant VWF due to hydrodynamic gene transfer, together with endogenous wild-type VWF (ie, *WT*-VWF/p.V1316M for [*WT*-] wild-type mice expressing mutant VWF and endogenous VWF/WT), were compared to mice with the same genotype expressing only endogenous VWF/WT (ie, *WT*-VWF/WT for [*WT*-] wild-type mice expressing endogenous VWF/WT only). Following hydrodynamic gene transfer, all mice (with the exception of *Vwf*$^{-/-}$) will express endogenous VWF/WT and high levels of mutant VWF (p.V1316M).

Immunoblotting

Proteins in mouse platelet lysates or plasma, prepared as described in supplemental Methods, were separated by sodium dodecyl sulfate gel electrophoresis (4%-20% gels; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane. Phosphorylated PKC substrates, β-actin, or glycocalicin were detected using appropriate secondary antibodies and an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification was performed with Image Studio Lite software (LI-COR Biosciences).

Phosphorylated PKC substrates in human platelet lysates from 3 VWD (p.V1316M) type 2B patients were detected as previously described.[4]

Saphenous vein laser injury

Mice anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) received Alexa Fluor 488–conjugated antibodies to GPIX (2.5 μg) to label circulating platelets. The saphenous vein was exposed and injured as described previously.[29] Platelet accumulation at the site of laser injury was assessed by intravital microscopy.

Study approval

All animal experiments and protocols were reviewed, approved by, and performed in accordance with the Animal Care and Use Committee of the University of North Carolina at Chapel Hill. All patients were recruited in France, informed about the anonymous use of their data, and gave written informed consent in accordance with the Declaration of Helsinki. The French VWD cohort (Centre de Référence Maladie de Willebrand) was reported to the French data protection authority.

Statistics

Statistical significance was assessed using Prism 7 software (GraphPad, La Jolla, CA). Two- or 3-way analysis of variance (Bonferroni post hoc analysis) was applied for multiple- and 3-group comparison, respectively. A 2-tailed Student *t* test was performed

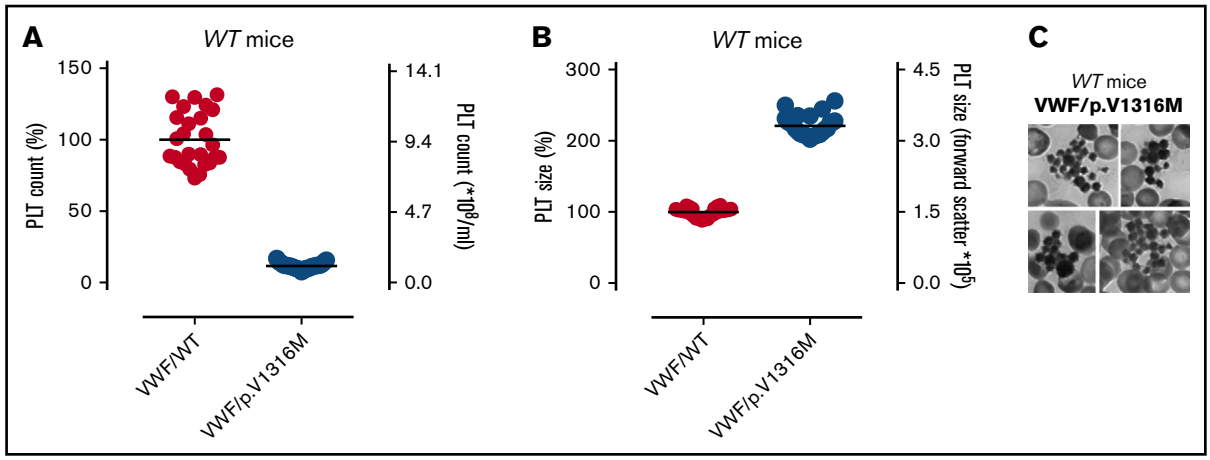

Figure 1. VWD type 2B phenotypes in WT mice overexpressing VWF/p.V1316M. (A) Platelet counts in WT mice expressing only VWF/WT (n = 26, red circles; mean platelet count set to 100%) and mice expressing VWF/p.V1316M (n = 25, blue circles) 4 days after hydrodynamic gene transfer. The right axis shows absolute platelet count. (B) Platelet size; the left axis represents relative values, and the right axis represents FSC values. (C) Representative images of platelet aggregates in blood smears from *WT*-VWF/p.V1316M mice, stained with eosin and methylene blue. Images were acquired with a Nikon TE300 microscope and 100×/1.4 NA oil objective (Nikon Instruments, Melville, NY) equipped with a QImaging Retiga EXi CCD camera (QImaging, Surrey, Canada) using SlideBook software version 5.0, Intelligent Imaging Innovation. Total magnification ×1000.

for 2-group comparison of parametric data. $P \leq .05$ was considered significant. All data are presented as mean ± standard deviation (SD).

Results

VWF/p.V1316M overexpression in WT mice results in VWD type 2B–like phenotype

We applied hydrodynamic gene transfer, which was previously used to express VWF/p.V1316M in *Vwf*$^{-/-}$ mice,[1,27] to induce VWD type 2B–like phenotypes in mice with normal levels of endogenous VWF. Expression of VWF/p.V1316M in WT mice resulted in an ~90% decrease in platelet count, reducing the peripheral platelet count (PPC) from 9.51 ± 1.76 × 10^8/mL to 1.11 ± 0.21 × 10^8/mL (Figure 1A). This reduction is consistent with that observed in *Vwf*$^{-/-}$ mice expressing VWF/p.V1316M, in which the PPC dropped from 12.01 ± 2.23 × 10^8/mL to 1.35 ± 0.55 × 10^8/mL (supplemental Figure 1A).

Flow cytometry forward scatter (FSC) values were determined as an estimation of platelet size. Compared with controls, platelet size was increased approximately twofold in WT mice expressing VWF/p.V1316M (Figure 1B). A similar twofold increase in platelet size was observed in *Vwf*$^{-/-}$-VWF/p.V1316M mice compared with their respective controls (supplemental Figure 1B). Furthermore, platelet aggregates were present in blood smears prepared from *WT*-VWF/p.V1316M mice (Figure 1C) similarly to *Vwf*$^{-/-}$-VWF/p.V1316M mice (supplemental Figure 1C). Thus, VWD type 2B phenotypes can be induced in WT mice expressing VWF/p.V1316M by hydrodynamic gene transfer.

Macrothrombocytopenia in VWD (p.V1316M) type 2B depends on the interaction between mutant VWF/p.V1316M and platelet GPIbα

We and other investigators have demonstrated that VWF is present at the platelet surface in VWD type 2B patients and mice.[27,30] VWD type 2B mutations alter VWF conformation and increase its binding affinity for platelet receptor GPIbα.[31,32] To test whether VWF/p.V1316M affects platelet count and size via binding to GPIbα, we performed hydrodynamic gene transfer in Tg mice expressing a chimeric human interleukin-4 (IL-4) receptor α/GPIbα protein (IL4R-IbαTg) (ie, mice that lack the extracellular domain of GPIbα required for binding of VWF).[21] At baseline, the platelet count in IL4R-IbαTg mice is ~35% lower than in WT controls (6.15 ± 0.30 × 10^8/mL and 9.51 ± 1.76 × 10^8/mL, respectively; Figures 1A and 2A). Importantly, platelet counts in IL4R-IbαTg mice were not significantly altered 4 days after VWF/p.V1316M overexpression (Figure 2A). Moreover, platelet size, which was increased twofold in WT mice expressing VWF/p.V1316M, did not change in IL4R-IbαTg mice after hydrodynamic gene transfer (Figure 2B). Finally, platelet aggregates were not detected in blood smears from *IL4R-IbαTg*-VWF/p.V1316M mice (Figure 2C). These results demonstrate that the onset of the VWD (p.V1316M) type 2B–associated macrothrombocytopenia is highly dependent on the physical interaction between VWF and the extracellular portion of GPIbα at the platelet surface.

We next investigated whether signaling events downstream of GPIbα engagement, which are important for αIIbβ3 activation, may contribute to abnormal platelet clearance and thrombocytopenia in VWD (p.V1316M) type 2B. To address this question, we expressed VWF/p.V1316M in mice with select signaling defects, such as mice defective in store-operated calcium entry (*Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$), calcium-dependent and -independent Rap1 GTPase signaling (*Caldaggef1*$^{-/-}$*P2Y12*$^{-/-}$), or talin-mediated integrin signaling (*talin1*$^{fl/fl}$*PF4-Cre*$^{+}$). GPIbα engagement was shown by different groups to cause calcium mobilization and integrin activation in platelets.[33-35] We and other investigators demonstrated a key role for Rap GTPase signaling in talin-dependent integrin activation in platelets.[8,22,36] Unexpectedly, platelet aggregate formation and macrothrombocytopenia induced by VWF/p.V1316M (80-90% reduction in platelet count and twofold increase in platelet size) were not different among WT (Figure 1), *Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$, *Caldaggef1*$^{-/-}$*P2Y12*$^{-/-}$, and *talin1*$^{fl/fl}$*PF4-Cre*$^{+}$ mice (Figure 2D-E). These data suggest that platelet aggregate formation and

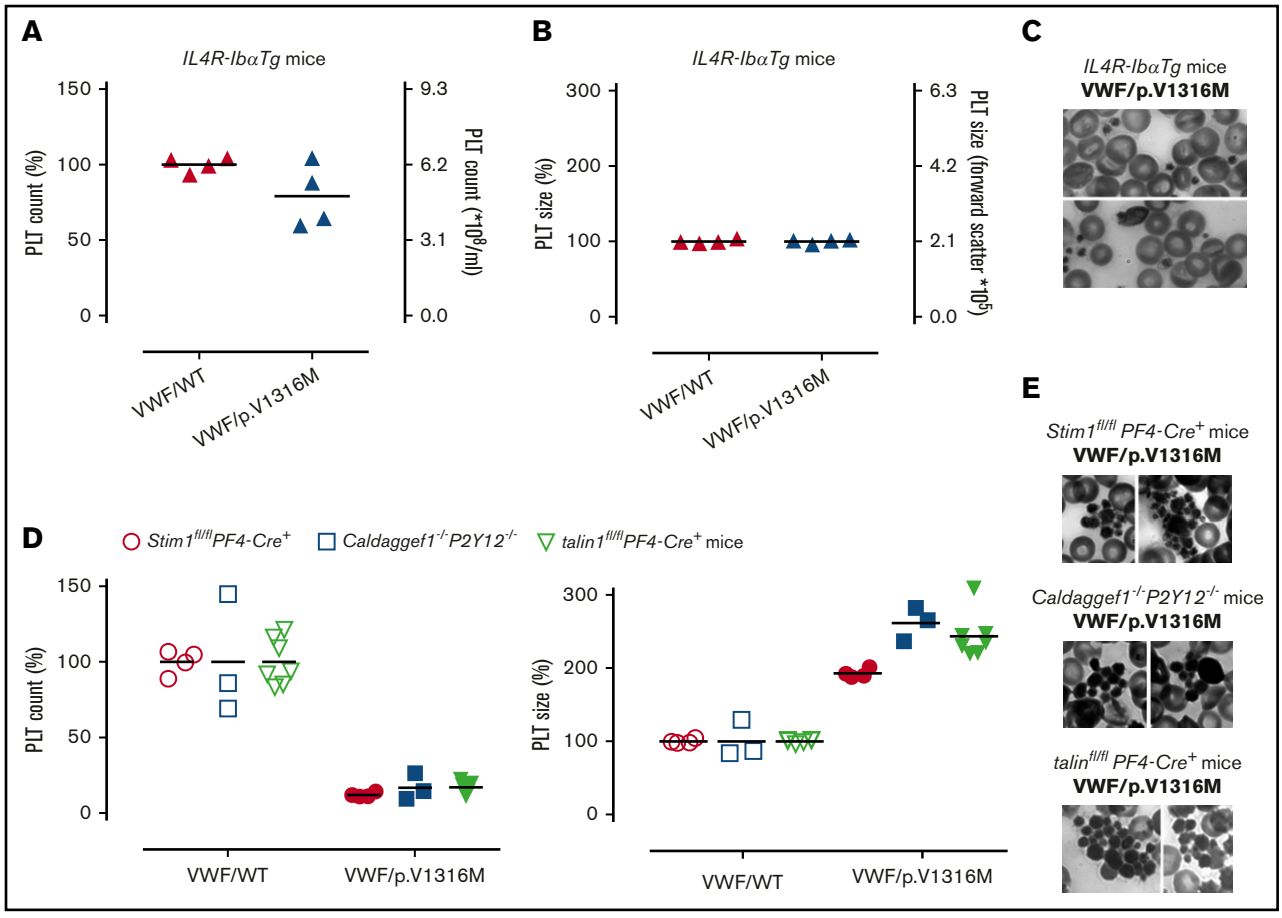

Figure 2. Platelets from IL4R-IbαTg mice are unaffected by the presence of VWF/p.V1316M. (A) Platelet counts in IL4R-IbαTg mice expressing only VWF/WT (100%, n = 4, red triangles) and mice overexpressing VWF/p.V1316M (n = 4, blue triangles) 4 days after hydrodynamic gene transfer. The left axis represents relative counts, and the right axis shows absolute platelet counts. (B) Platelet size in IL4R-IbαTg mice (100%, n = 4, red triangles) and *IL4R-IbαTg*-VWF/p.V1316M mice (n = 4, blue triangles). (C) Single platelets in blood smears from *IL4R-IbαTg*-VWF/p.V1316M mice. (D) Platelet counts and platelet size in *Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$ (red circles), *Caldaggef1*$^{-/-}$ *P2Y12*$^{-/-}$ (blue squares), and *talin1*$^{fl/fl}$*PF4-Cre*$^{+}$ (green triangles) mice in the presence of VWF/WT (100%, open symbols) and VWF/p.V1316M (filled symbols). n = 3-7 mice per group. (E) Platelet aggregates found in blood smears of *Stim1*$^{fl/fl}$*PF4-Cre*$^{+}$, *Caldaggef1*$^{-/-}$*P2Y12*$^{-/-}$, and *talin1*$^{fl/fl}$*PF4-Cre*$^{+}$ mice expressing VWF/p.V1316M. Images in panels C and E were acquired as described in Figure 1.

macrothrombocytopenia depend on the binding of VWF/p.V1316M to GPIbα but are independent of downstream platelet signaling events important for αIIbβ3 integrin activation.

Decreased integrin activation in VWD (p.V1316M) type 2B results from a defect in the PKC/P2Y12-dependent signaling pathway

We previously demonstrated that the activation of Rap1B was decreased in VWD (p.V1316M) type 2B platelets stimulated via G protein–coupled or immunoglobulin-like receptors.[4] We also demonstrated that Rap1B activation upon agonist stimulation is mediated by a fast, but reversible, pathway (CalDAG–GEF-I dependent) and a slow, but sustained, pathway (PKC/P2Y12 dependent)[10-12] (supplemental Figure 2A). To establish the molecular mechanism underlying this cellular activation defect, we first studied the kinetics of αIIbβ3 activation in platelets from WT and WT-VWF/p.V1316M mice [VWD2B(p.V1316M)-platelets]. To do so, we added JON/A-PE, a probe that irreversibly binds to activated αIIbβ3, to platelets before and 10 minutes after agonist stimulation (supplemental Figure 2B). As expected, JON/A-PE binding in PAR4p-activated WT platelets was similar, irrespective of when the antibody was added (Figure 3A-B, open bars), indicating that both pathways required for full integrin activation were active. In contrast, JON/A-PE binding in PAR4p-activated VWD2B(p.V1316M) platelets was comparable to controls when the antibody was added before the agonist (Figure 3A), but it was markedly reduced if the antibody was added 10 minutes after the agonist (Figure 3B). These results suggested that a defect in PKC/P2Y12 signaling, mediating sustained integrin activation, is responsible for the decreased αIIbβ3 activation response observed in VWD2B(p.V1316M) platelets.

To further test this conclusion, we expressed VWF/p.V1316M in *Caldaggef1*-deficient mice. Integrin αIIbβ3 activation in platelets collected from these mice depends primarily on the contribution of the PKC/P2Y12 signaling pathway. Expression of VWF/p.V1316M led to severe macrothrombocytopenia in *Caldaggef1*$^{-/-}$ mice (supplemental Figure 3). JON/A-PE binding in platelets isolated from *Caldaggef1*$^{-/-}$-VWF/p.V1316M mice did not significantly change upon agonist stimulation (Figure 3C), confirming that αIIbβ3 integrin dysfunction in VWD (p.V1316M) type 2B is a consequence of a defect in the PKC/P2Y12 signaling pathway.

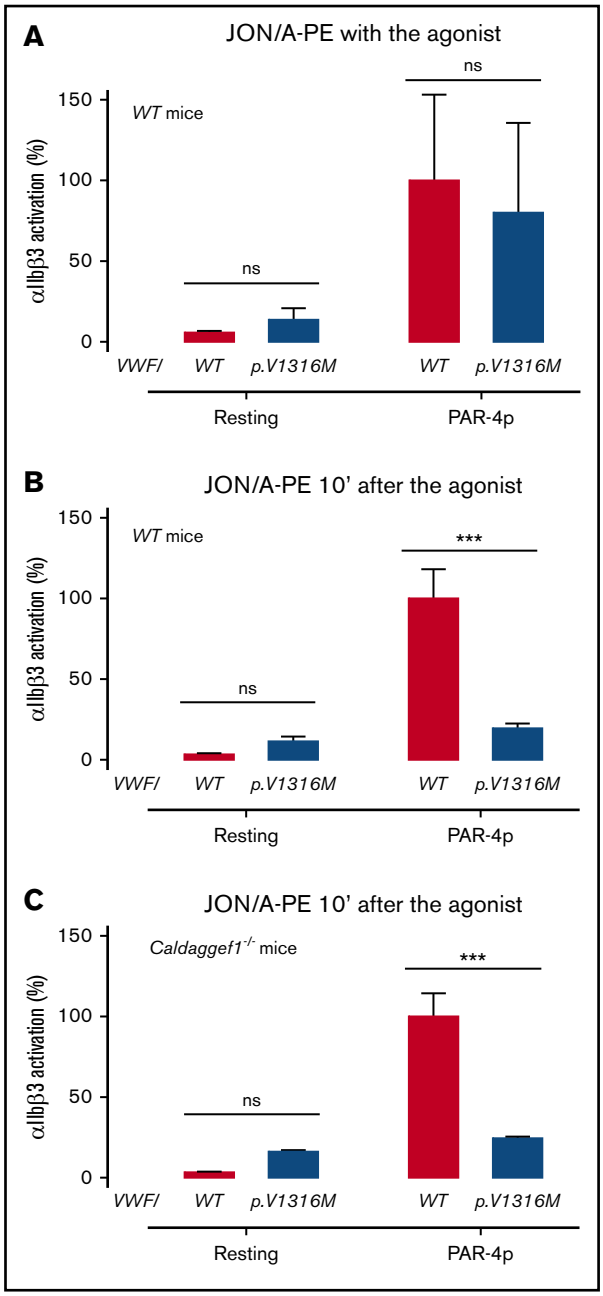

Figure 3. Sustained platelet activation is impaired in mice overexpressing VWF/p.V1316M. αIIbβ3 integrin activation measured by flow cytometry with JON/A-PE antibody (selectively binds to the activated form of the receptor[51]). JON/A-PE was added with the agonist (PAR4p, 200 μM) (A) or 10 minutes after the agonist (B-C). The mean fluorescence intensity measured in stimulated platelets from WT (A-B) or *Caldaggef1*$^{-/-}$ (C) mice expressing VWF/WT (red bars) was set to 100%. Red bars, platelets expressing VWF/WT only; blue bars, platelets expressing VWF/p.V1316M. Data are shown as mean ± SD; n = 3-13 mice per group. ***$P \leq .001$. ns, not significant.

Importantly, no difference in the kinetics of αIIbβ3 activation was observed between platelets from *IL4R-IbαTg*-VWF/WT and *IL4R-IbαTg-VWF/p.V1316M* mice (supplemental Figure 4), demonstrating that the abnormal interaction between GPIb and mutant VWF in VWD (p.V1316M) type 2B induces the change in PKC/P2Y12 signaling in these cells.

VWF/p.V1316M affects PKC and inhibits platelet granule release

To investigate whether the alteration in Rap1 signaling that we observed in VWD (p.V1316M) type 2B is due to an impaired signaling function of PKC or P2Y12, we next studied integrin activation under conditions of direct stimulation of P2Y12. Only a small, but significant, defect in JON/A-PE binding was observed between control and VWD2B(p.V1316M) platelets upon costimulation with the second-wave mediators (ADP and U46619) (Figure 4A). These results suggested that VWF/p.V1316M engagement of GPIbα primarily affects PKC function in platelets. Consistent with this conclusion, VWD2B(p.V1316M) platelets exhibited marked defects in α-granule and dense granule secretion, processes that are dependent on PKC signaling. To study dense granule release, platelets were loaded with radiolabeled [^{3}H]serotonin. Compared with controls (90% release), VWD2B (p.V1316M) platelets released only 40% of the incorporated serotonin upon PAR4p agonist stimulation (Figure 4B). As a marker for α-granule release, we quantified P-selectin at the platelet surface. Surface P-selectin levels were highly increased (30-50–fold) in PAR4p-activated WT platelets compared with nonstimulated cells (Figure 4C, red bars). In contrast, P-selectin surface expression only increased approximately fivefold in stimulated VWD2B(p.V1316M) platelets (Figure 4C, blue bars).

VWF/p.V1316M enhances PKC activity in circulating platelets

To directly monitor PKC activity, we determined the phosphorylation status of PKC substrates in resting and PAR4p-activated platelets. Although mouse WT platelets showed little PKC activity under resting conditions, a significant increase (6.4-fold) in PKC substrate phosphorylation was observed 10 minutes after agonist stimulation (Figure 5A-B). Compared with controls, PKC substrate phosphorylation was markedly elevated in resting VWD2B(p.V1316M) platelets (fourfold increase) (Figure 5A-B). However, upon cellular stimulation, PKC substrate phosphorylation increased only twofold in VWD2B(p.V1316M) platelets (Figure 5A-B). To exclude that the observed phenotype was due to the high circulating levels of mutant VWF associated with the gene-transfer procedure, we performed similar platelet studies in *Vwf*/p.V1316M–knock-in mice,[26] such as mice that express physiologic levels (≈100%) of VWF/p.V1316M. PKC substrate phosphorylation was also significantly increased in resting platelets from the knock-in mice compared with controls (supplemental Figure 5). Importantly, similar to our observations with murine platelets, PKC substrate phosphorylation was increased approximately twofold (1.6-, 2.3-, and 1.8-fold for P1, P2, and P3, respectively) in circulating platelets from 3 patients carrying the VWF/p.V1316M mutation (Figure 5C-D; supplemental Figure 6), despite their low plasmatic VWF antigen levels (supplemental Table 2).

VWF/p.V1316M expression induces GPIbα shedding

PKC activation provides a powerful signal for the proteolytic cleavage of cell surface receptors,[17,19] including GPIbα on platelets.[37] Given the markedly increased baseline levels of PKC substrate phosphorylation observed in VWD2B(p.V1316M) platelets (Figure 5), we evaluated platelet surface expression of GPIbα

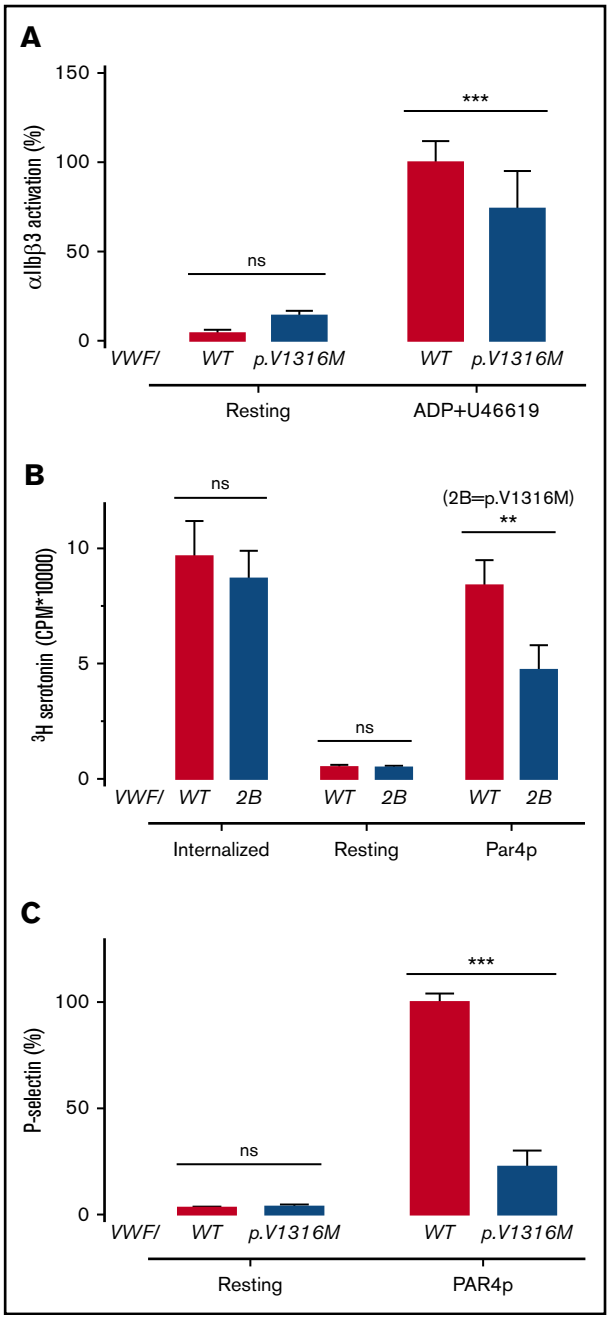

Figure 4. VWF/p.V1316M affects platelet granule release. (A) αIIbβ3 integrin activation measured with JON/A-PE, added 10 minutes after stimulation with ADP (10 μM) and U46619 (5 μM). MFI in stimulated WT platelets (red bars) set as 100%. Red bars, VWD2B(p.V1316M) platelets. (B) Quantification of secreted [^{3}H]serotonin from δ-granules of washed WT and VWD2B(p.V1316M) platelets (red and blue bars, respectively), stimulated or not with PAR4p (1 mM). (C) P-selectin surface expression on PAR4p-stimulated WT and VWD2B(p.V1316M) platelets (red and blue bars, respectively). Data are shown as mean ± SD of 3-17 mice per group. $^{**}P \leq .01$; $^{***}P \leq .001$.

and GPIX by flow cytometry, before and after expression of the mutant VWF/p.V1316M in mice. Although GPIbα expression was almost completely abolished following expression of VWF/p.V1316M (Figure 6A), no significant difference in expression was observed for GPIX (Figure 6B). Reduced expression of GPIbα was confirmed with a second antibody recognizing a different epitope on its extracellular domain (data not shown).

The main platelet sheddase for GPIbα is ADAM17.[16] We next expressed VWF/p.V1316M in *ADAM17$^{fl/fl}$PF4-Cre$^+$* mice. Similar to what we observed in *WT*-VWF/p.V1316M mice (Figure 1), the PPC was reduced by 84% compared with controls (Figure 6C), platelet size was increased approximately twofold (Figure 6D), and platelet aggregates were found in the blood smears of *ADAM17$^{fl/fl}$PF4-Cre$^+$* mice expressing VWF/p.V1316M (Figure 6E). Unexpectedly, GPIbα surface expression was not retained in platelets from *ADAM17$^{fl/fl}$PF4-Cre$^+$*-VWF/p.V1316M mice (Figure 6F), suggesting that ADAM17 is not critical for GPIbα shedding under these conditions. Consistent with this conclusion, plasma levels of glycocalicin were significantly increased in *ADAM17$^{fl/fl}$PF4-Cre$^+$*-VWF/p.V1316M and *WT*-VWF/p.V1316M mice compared with control mice expressing only VWF/WT (Figure 6G).

VWF/p.V1316M impairs hemostatic plug formation

We next evaluated the impact of these platelet function defects on hemostasis using a recently developed model of laser ablation injury to the saphenous vein.[29] In this model, repeated laser ablations to the same site can be performed to test the hemostatic response to injuries of different severity. Compared with controls, platelet adhesion was significantly reduced at sites of laser damage in WT-VWF/p.V1316M mice (Figure 7A-B). Consistent with the platelet-adhesion defect, *WT*-VWF/p.V1316M mice also exhibited markedly prolonged blood loss from the injury site (Figure 7C). We next determined whether the low platelet count was responsible for the hemostasis defect observed in *WT*-VWF/p.V1316M mice. Using our recently established method for adoptive platelet transfer,[38,39] we generated mice with a peripheral count $<1 \times 10^8$ WT platelets per mL of blood, comparable to the PPC of *WT*-VWF/p.V1316M mice. In agreement with a recent study,[40] a platelet count of ~10% of normal was sufficient to prevent prolonged bleeding in our model (Figure 7D). In summary, these studies confirm that defects other than the low platelet count contribute to the hemostasis defect in p.V1316M-associated VWD type 2B.

Discussion

Our study is the first to provide genetic evidence that the macrothrombocytopenia and platelet aggregates observed in p.V1316M-associated VWD type 2B are due to the direct interaction of mutant VWF/p.V1316M and GPIbα. We did not observe evidence for a role of integrin activation downstream of engagement of GPIbα in these processes. However, we did detect a marked upregulation of PKC activity in platelets from mice and 3 patients expressing VWF/p.V1316M. This latter finding is consistent with previous work showing increased PKC activity in platelets stimulated with VWF/ristocetin[41] and more recent work showing limited granule secretion, a process controlled by PKC signaling, immediately after unfolding of the mechanosensory domain of GPIbα.[42] Importantly, in the studies by Deng et al,[42] limited granule secretion was also observed in platelets incubated with plasma obtained from a patient carrying the VWF/p.V1316M mutation. Thus, the binding of mutant VWF/p.V1316M to GPIbα induces the activation of PKC in mice and in humans. Consistent with this alteration in PKC signaling, VWD (p.V1316M) type 2B platelets exhibit a desensitization of PKC-mediated granule release and integrin activation and proteolytic degradation of GPIbα

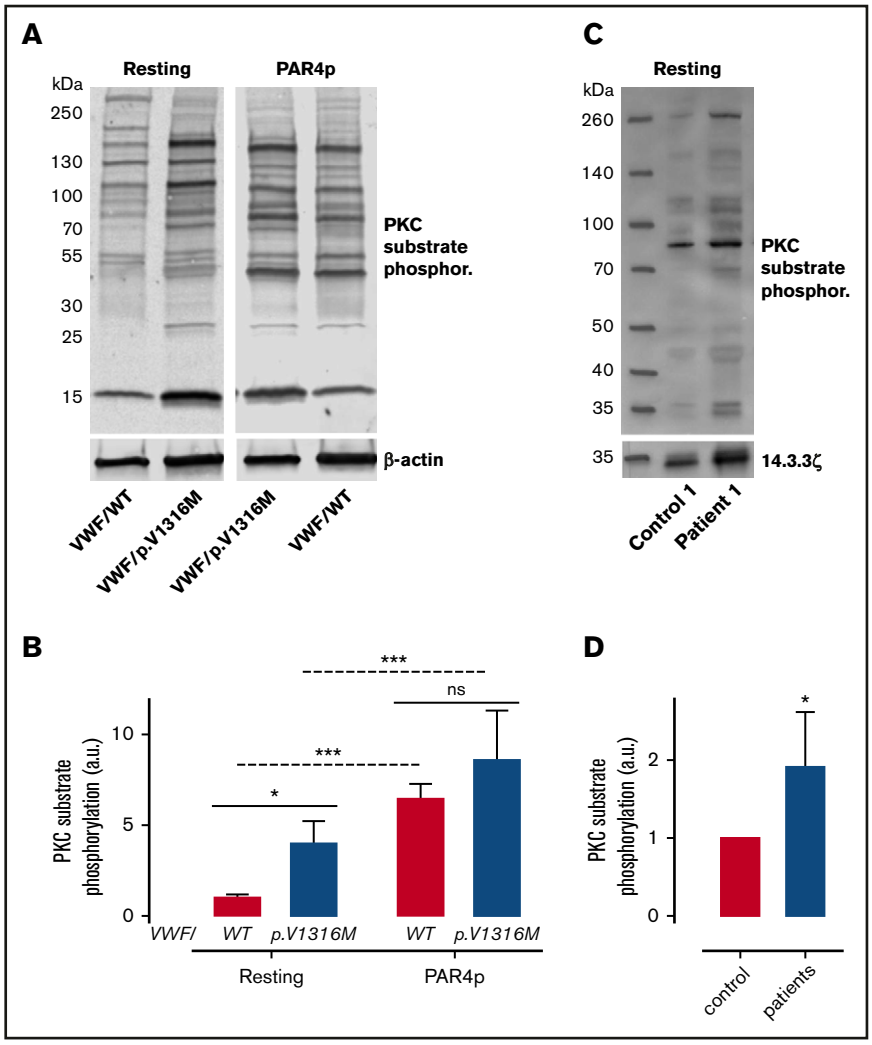

Figure 5. Phosphorylation of PKC substrates is dysregulated in VWD2B(p.V1316M) platelets. (A) Immunoblotting for phosphorylated PKC substrates following sodium dodecyl sulfate–polyacrylamide gel electrophoresis of platelet lysates from *WT*-VWF/WT and *WT*-VWF/p.V1316M mice (resting and PAR4p activated [200 μM]). β-actin was detected in every sample as loading control. (B) Quantification of PKC substrate phosphorylation (whole lane), normalized to β-actin content, using Image Studio Lite software (LI-COR Biosciences). Data are shown as arbitrary units (a.u.); PKC substrate phosphorylation in resting *WT*-VWF/WT platelets was set at 1. Data are mean ± SD, n = 5-8 mice per group. (C) Immunoblotting for phosphorylated PKC substrates following sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human platelet lysates (resting) from 1 patient affected by VWD (p.V1316M) type 2B and a healthy donor (control). 14.3.3ζ (anti-14.3.3 ζ 4.; Santa Cruz Biotechnology) was detected as loading control. Horseradish peroxidase–conjugated secondary antibodies were applied, and immunoreactive bands were revealed using Pierce ECL Western Blotting Substrate. (D) Quantification of PKC substrate phosphorylation, normalized to loading control, performed with ImageJ software. Data are shown as a.u.; PKC substrate phosphorylation in resting control platelets was set at 1. Data are mean ± SD of 3 patients and 3 healthy controls. Immunoblots for patients 2 and 3 are reported in supplemental Figure 7. *$P \leq .05$; ***$P \leq .001$.

(at least in mice). Together with the marked thrombocytopenia and changes in VWF multimer composition, these platelet defects lead to marked bleeding complications in VWD (p.V1316M) type 2B.

Most of our studies were performed in mice expressing the VWF/p.V1316M variant by hydrodynamic gene transfer.[1,4,27,28] This model is very powerful, because it allows for the very rapid induction of a severe VWD type 2B phenotype in *Vwf*$^{-/-}$ mice, as previously reported, and in WT or genetically modified mice, such as mice with endogenous WT/VWF, as demonstrated here. However, it is important to remember that hydrodynamic gene transfer leads to overexpression of VWF in plasma[26,27] and that mutant VWF generated following hydrodynamic gene transfer is mainly produced in hepatocytes, whereas endogenous VWF/WT is produced in endothelial cells and megakaryocytes. As a result, no heteromultimers composed of mutant and WT monomers are expected to circulate in plasma of these mice. In contrast, heteromultimers would be expected for patients and knock-in mice heterozygous for the VWD type 2B mutation. These differences may explain, at least in part, why the observed platelet phenotype in this murine model of VWD (p.V1316M) type 2B was stronger than that reported for platelets from patients carrying the same mutation. For example, shedding of GPIbα, induced by the VWF–GPIb interaction, is a striking observation in mice expressing VWF/p.V1316M due to hydrodynamic gene transfer. In contrast, shedding of GPIbα is rarely described for VWD type 2B patients,[43] even though there is strong evidence that human platelet GPIbα undergoes increased proteolysis in situations of increased platelet turnover.[14,44] Unfortunately, large studies to determine the clinical relevance of GPIbα shedding in VWD type 2B patients are missing. Compared with mice expressing VWF/p.V1316M due to hydrodynamic gene transfer,[27] plasma VWF levels are markedly lower in type 2B patients.[2,26] In our studies, we observed an inverse correlation between mutant VWF/p.V1316M levels in plasma and GPIbα surface expression in *WT*-VWF/p.V1316M mice (supplemental Figure 7). Furthermore, GPIbα levels were only reduced approximately twofold in platelets from knock-in mice carrying the VWF/p.V1316M mutation (data not shown) (ie, mice in which plasma VWF/p.V1316M levels are lower than in our hydrodynamic gene-transfer model).[26]

Given the well-documented effect of PKC signaling on sheddase activity,[16,17,19] it is likely that GPIbα shedding is a result of the above-described PKC activation in VWD2B(p.V1316M) platelets. Surprisingly, VWF/p.V1316M-induced cleavage of GPIbα was not inhibited in mice deficient in ADAM17, an enzyme that we previously identified as the main sheddase for GPIbα in the circulation of healthy mice and immediately following PKC stimulation in vitro.[16]

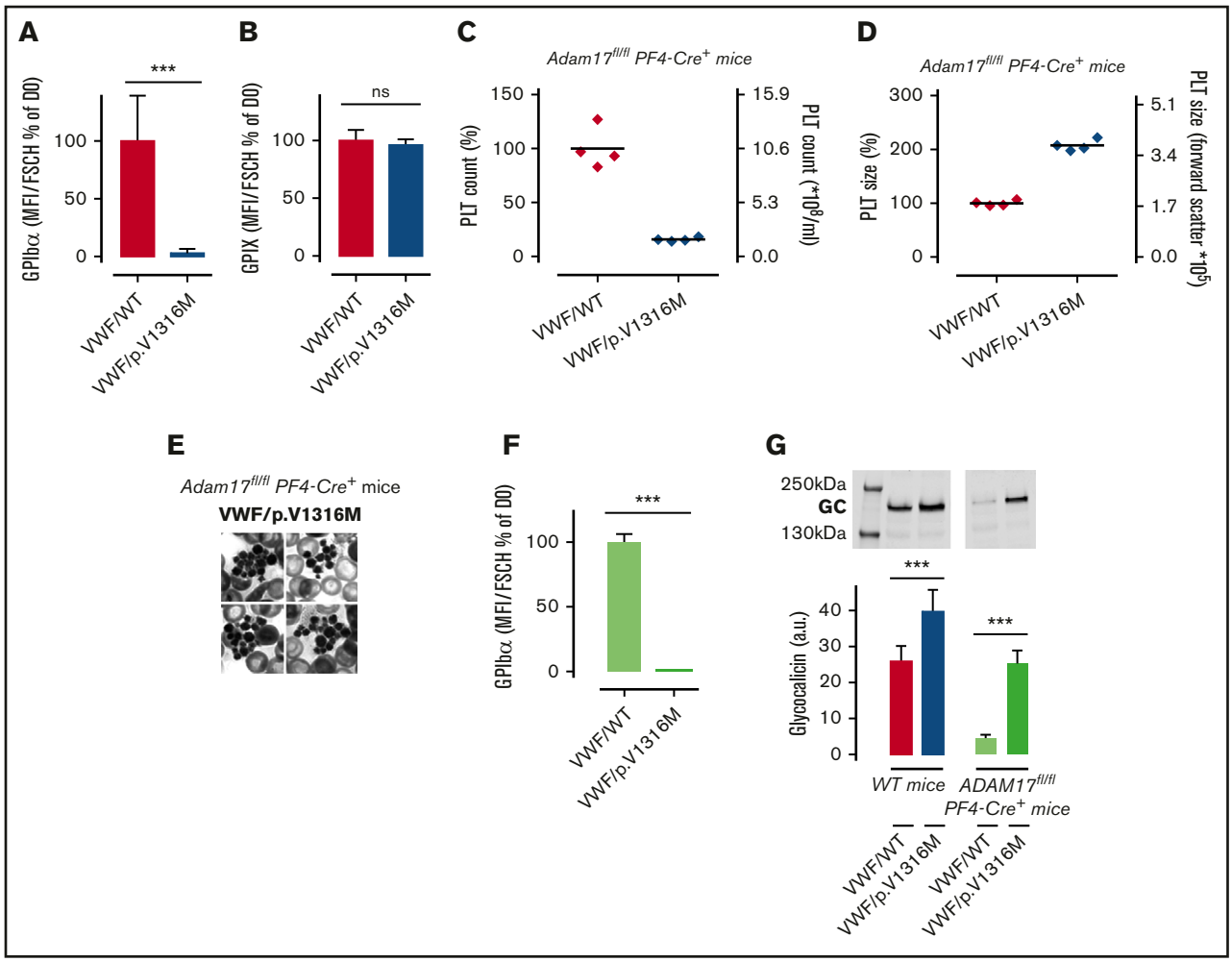

Figure 6. VWF/p.V1316M induces ADAM17-independent GPIbα shedding. Quantification of GPIbα (A) and GPIX (B) receptor levels by flow cytometry at the surface of circulating platelets collected from *WT*-VWF/WT (red bars, n = 26) or *WT*-VWF/p.V1316M (blue bars, n = 26) mice. (C) Platelet count in *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$ mice expressing VWF/WT (100%, n = 4, red diamonds) or VWF/p.V1316M (n = 4, blue diamonds) 4 days after hydrodynamic gene transfer. The right axis shows the absolute platelet count. (D) Platelet size in *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$ mice (100%, n = 4, red diamonds) and *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$ -VWF/p.V1316M mice (n = 4, blue diamonds). The right axis shows FSC values. (E) Platelet aggregates found in blood smears from *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$-VWF/p.V1316M mice. Images were acquired as described in Figure 1. (F) Quantification of GPIbα receptor levels by flow cytometry at the surface of circulating platelets collected from *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$-VWF/WT mice (n = 4, lime green bar) and *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$-VWF/p.V1316M mice (n = 4, light green bar). (G) Representative immunoblot for glycocalicin (GC; detected with anti-GPIbα antibody Xia.G7 from EMFRET Analytics, Würzburg, Germany) in plasma from WT and *ADAM17*$^{fl/fl}$*PF4-Cre*$^{+}$ mice expressing VWF/WT and VWF/p.V1316M (upper panel). Quantification of data in upper panel using Image Studio Lite software (lower panel); data are mean ± SD. n = 3-9 mice per group. ***$P \leq .001$.

Our findings do not exclude the possibility that VWF/p.V1316M–GPIbα engagement induces ADAM17 activity. However, a long-term VWF/p.V1316M–GPIbα engagement, as seen after hydrodynamic gene transfer, may activate GPIbα sheddases other than ADAM17, such as ADAM10[17] or the cysteine protease calpain.[45] Similar ADAM17-dependent and -independent shedding events were shown for other receptors, such as CD44 on leukocytes.[20] Of note, defective calpain activity has been reported in platelets obtained from patients affected by Montreal platelet syndrome[46] (subsequently identified as p.V1316M-associated VWD type 2B[47]), but it was never studied again in patients carrying the aforementioned or other type 2B mutations. Future studies should be directed toward a better understanding of the molecular mechanisms by which engagement of GPIbα in VWD type 2B leads to altered PKC signaling in platelets. Information derived from these studies could then be used to develop strategies to prevent the thrombopathy and, thus, to improve hemostasis in these patients.

Although alterations in PKC signaling and GPIbα surface expression are less pronounced in platelets from VWD type 2B patients carrying the VWF/p.V1316M mutation, there is good phenotypic similarity between the patients and the mouse model with regard to impaired activation of Rap1B and αIIbβ3 integrin in response to agonist stimulation.[4] Our studies strongly suggest that the intracellular signaling defect also occurs at the level of PKC activity regulation. In mice, compared with the sixfold increase in PKC substrate phosphorylation observed in activated control platelets, only a twofold increase in PKC activity over baseline was observed in activated VWF/p.V1316M platelets. Consistent with this defect, platelets from VWF/p.V1316M mice exhibited markedly impaired granule secretion in response to agonist stimulation, similar to what

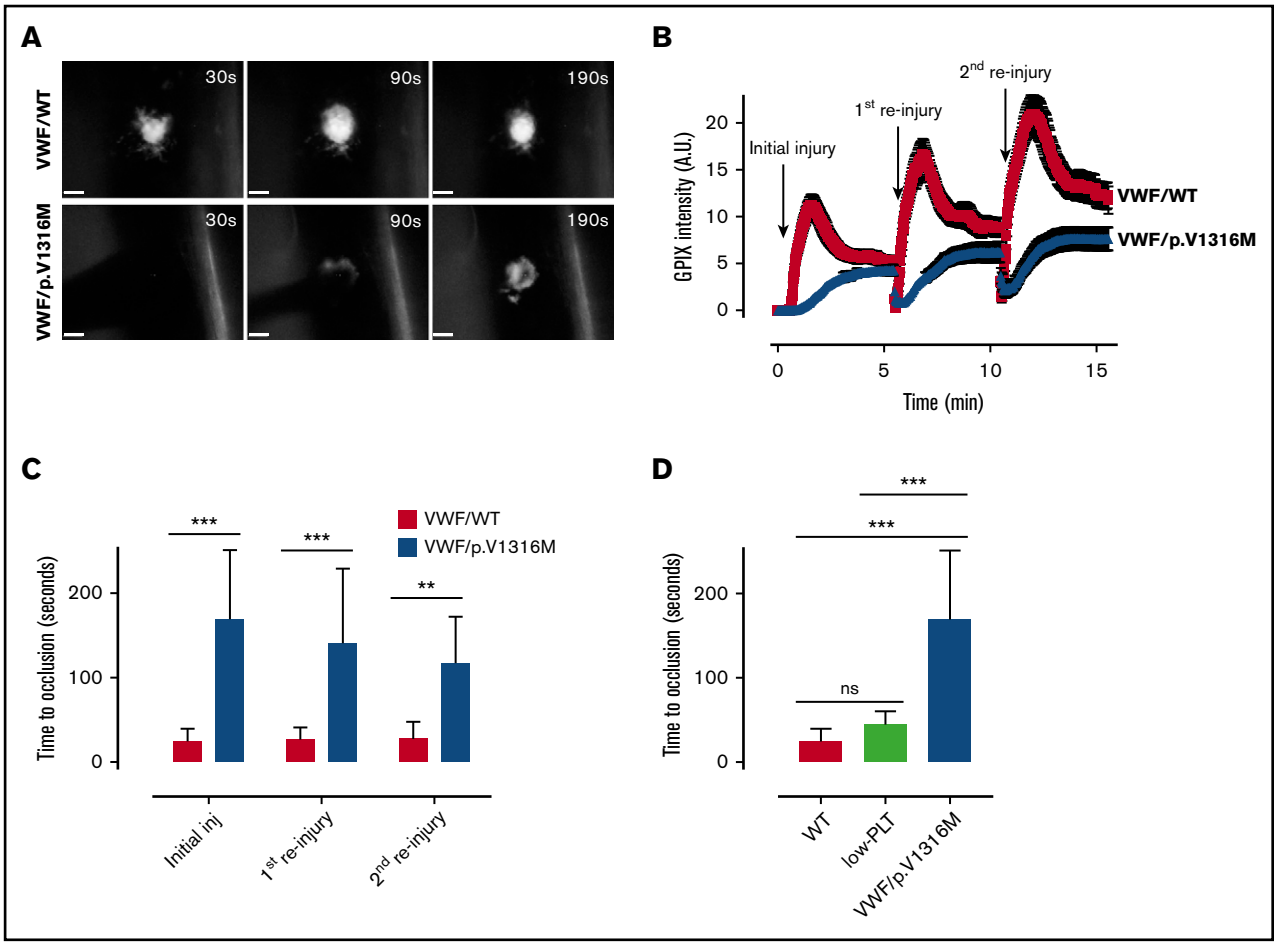

Figure 7. VWF/p.V1316M decreases hemostasis in a laser-injury model. Quantitative analysis (SlideBook software) of platelet accumulation and bleeding time after laser injury to the saphenous vein of *WT*-VWF/WT (red) and *WT*-VWF/p.V1316M (blue) mice. Mice were injected with an Alexa Fluor 488–labeled antibody against GPIX to monitor platelet accumulation. Repeated vascular injury was induced by laser ablation. (A) Representative images at the indicated times after the first injury. Intravital microscopy was performed with a Zeiss Examiner Z1 microscope (Zeiss, Oberkochen, Germany) equipped with a Hamamatsu Orca Flash 4.0 camera (Hamamatsu Photonics, Hamamtsu City, Japan) and a 20× water immersion objective (numerical aperture 1, working distance 1.8 mm) (Zeiss, Jena, Germany). Scale bars, 50 μm. (B) Accumulation of platelets at the site of injury, quantified as sum fluorescence intensity (± SEM). (C) Bleeding time. (D) Bleeding time after the first laser injury to the saphenous vein of *WT*-VWF/WT mice (red bar), control mice with low peripheral platelet counts (low-PLT; light green bar), and *WT*-VWF/p.V1316M mice (blue bar). Low-PLT mice were generated by adoptive transfer of WT platelets into thrombocytopenic IL4R-IbαTg mice, yielding a PPC ~0.7 × 10^8/mL of blood. Data for *WT*-VWF/WT and *WT*-VWF/p.V1316M mice are the same as in (C). Total number of injuries at distinct locations: n = 30 (obtained in 3 *WT*-VWF/WT mice), n = 38 (obtained in 5 *WT*-VWF/p.V1316M mice), and n = 15 (obtained in 4 low-PLT mice). Data in panels C-D are mean ± SD. $^{**}P \leq .01$; $^{***}P \leq .001$.

we described for human platelets.[4] Considering that integrin activation was only mildly impaired in VWF/p.V1316M platelets stimulated with the second-wave mediators, thromboxane A_2 and ADP, and only the second phase of integrin activation was altered in the mutant platelets, it can be concluded that the defect in Rap1B signaling in VWD (p.V1316M) type 2B is the result of impaired ADP secretion and feedback signaling via P2Y12. Consistent with these results, Akt phosphorylation was unaffected in resting conditions and markedly reduced in PAR4p-stimulated platelets from mice expressing the mutant VWF/p.V1316M (supplemental Figure 8). It is unclear whether the defective PKC signaling response to agonist stimulation in VWF/p.V1316M platelets reflects a desensitization of this pathway or whether engagement of GPIbα by mutant VWF/p.V1316M specifically activates PKC isoforms that inhibit, rather than activate, platelets. The latter was demonstrated for collagen-activated platelets, in which activation of PKC(δ) downstream of the collagen receptor antagonizes signaling by classical PKC isoforms required for granule secretion and platelet adhesion.[48] Irrespective of the underlying mechanism, however, we provide the first evidence that an inherited alteration in a plasma protein can lead to an acquired platelet function disorder due to a defect in PKC/P2Y12/Rap1 signaling. It will be interesting to see whether similar defects in Rap1 signaling can be detected in VWD patients carrying mutations other than p.V1316M and in patients affected by other diseases, especially those that are caused by nonphysiological engagement of GPIbα, as seen in immune thrombocytopenia purpura or thrombotic thrombocytopenia purpura.

It is reasonable to speculate that the observed downmodulation of platelet reactivity toward mutant VWF/p.V1316M is intended to limit thrombosis. Another reason could be the prevention of near-complete thrombocytopenia, a condition that, in the mouse model,

leads to increased embryonic lethality and marked blood–lymphatic mixing.[11,38] Interestingly, the molecular mechanisms by which platelets secure vascular integrity during development differ significantly from those established for classical hemostatic plug formation.[38] Adhesion receptors, such as GPIb-V-IX or αIIbβ3 integrin, and signaling pathways, such as the Rap1 GTPase pathway, are critical for plug formation but not vascular integrity during development. Thus, downmodulation of GPIbα expression and PKC/P2Y12/Rap1 signaling would provide an elegant mechanism to increase the peripheral platelet count to levels required for vascular integrity in development.

In summary, we applied the hydrodynamic gene-transfer technique in mice with specific platelet signaling defects to demonstrate that the thrombopathy in VWD (p.V1316M) type 2B is due to impaired PKC/P2Y12/Rap1 signaling, a pathway critical for sustained integrin activation in platelets. We show that engagement of GPIbα by mutant VWF/p.V1316M leads to increased PKC activity in murine platelets, which affects cellular function in at least 2 ways: (1) the increased baseline activation makes platelets refractory to agonist-induced PKC signaling, leading to impaired granule release and reduced Rap1-mediated integrin activation and (2) shedding of the GPIbα receptor, which limits the interaction with mutant VWF/p.V1316M. Both alterations protect platelets from incorporation into intravascular thrombi and clearance due to the interaction with mutant VWF/p.V1316M. Of particular relevance, disturbed PKC signaling was also observed in 3 patients affected by VWD (p.V1316M) type 2B, providing evidence that a PKC-dependent hypofunction may contribute to the severe bleeding phenotype of these patients. Indeed, our data indicate that VWD type 2B patients carrying the VWF/p.V1316M variant suffer from a congenital VWD and an acquired platelet defect, suggesting that treatments should address the 2 deficiencies. Replacement therapy, the mainstay of VWD treatment, does not correct platelet counts, and it is unlikely that it can impact platelet functions. Thus, platelet transfusions should be considered as adjunctive treatment in VWD type 2B patients presenting with platelet defects.[49,50]

Acknowledgments

The authors thank Jerry Ware (University of Arkansas), Dougald M. Monroe III (University of North Carolina at Chapel Hill), Brian Petrich (Emory University), and Pamela B. Conley (Portola Pharmaceuticals Inc.) for providing mice deficient in extracellular GPIbα, VWF, talin1, and P2Y12, respectively. The authors also thank Delphine Borgel (INSERM, UMR-S1176, Université Paris-Sud, Université Paris-Saclay, Le Kremlin-Bicêtre, France and Assistance Publique–Hôpitaux de Paris, Laboratoire d'Hématologie, Hôpital Universitaire Necker-Enfants Malades) and Jenny Goudemand (Département d'Hématologie et de Transfusion, Centre Hospitalier Universitaire de Lille, Université Lille 2, EA 2693, Faculté de Médecine, Lille, France, and Institut Pasteur de Lille, European Genomic Institute for Diabetes, INSERM UMR 1011, Université Lille 2, Lille, France) for valuable help with enrolling patients and providing clinical laboratory information.

This work was supported by a Research Fellowship award granted by the European Hematology Association (C.C.) and by National Institutes of Health, National Heart, Lung, and Blood Institute grants R01 HL121650 and R01 HL130404 (W.B.).

Authorship

Contribution: C.C., O.D.C., P.J.L., C.V.D., and W.B. designed the study; C.C. and D.S.P. performed most of the experiments and analyzed data; S.S., A.H., and C.L.-B. enrolled the VWD type 2B patients; R.P. performed and analyzed laser-injury experiments; R.H.L. assisted with experiments; K.O.P. was responsible for mouse colony maintenance and genotyping; M.B. helped to prepare human platelet samples; C.C. and W.B. wrote the manuscript; and authors contributed to the editing of the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Caterina Casari, INSERM UMR-S1176, 80 rue du General Leclerc, 94276 Le Kremlin-Bicêtre, France; e-mail: caterina.casari@inserm.fr; and Wolfgang Bergmeier, University of North Carolina, 120 Mason Farm Rd, Campus Box 7260, Chapel Hill, NC 27599; e-mail: bergmeie@e-mail.unc.edu.

References

1. Rayes J, Hollestelle MJ, Legendre P, et al. Mutation and ADAMTS13-dependent modulation of disease severity in a mouse model for von Willebrand disease type 2B. *Blood*. 2010;115(23):4870-4877.
2. Federici AB, Mannucci PM, Castaman G, et al. Clinical and molecular predictors of thrombocytopenia and risk of bleeding in patients with von Willebrand disease type 2B: a cohort study of 67 patients. *Blood*. 2009;113(3):526-534.
3. Castaman G, Federici AB. Type 2B von Willebrand disease: a matter of plasma plus platelet abnormality. *Semin Thromb Hemost*. 2016;42(5):478-482.
4. Casari C, Berrou E, Lebret M, et al. von Willebrand factor mutation promotes thrombocytopathy by inhibiting integrin αIIbβ3. *J Clin Invest*. 2013;123(12):5071-5081.
5. Burkhart JM, Vaudel M, Gambaryan S, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120(15):e73-e82.
6. Franke B, Akkerman JW, Bos JL. Rapid Ca2+-mediated activation of Rap1 in human platelets. *EMBO J*. 1997;16(2):252-259.
7. Guidetti GF, Torti M. The small GTPase Rap1b: a bidirectional regulator of platelet adhesion receptors. *J Signal Transduct*. 2012;2012:412089.
8. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC II. Rap1b is required for normal platelet function and hemostasis in mice. *J Clin Invest*. 2005;115(3):680-687.
9. Bertoni A, Tadokoro S, Eto K, et al. Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *J Biol Chem*. 2002;277(28):25715-25721.
10. Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood*. 2009;114(12):2506-2514.

11. Stefanini L, Paul DS, Robledo RF, et al. RASA3 is a critical inhibitor of RAP1-dependent platelet activation. *J Clin Invest*. 2015;125(4):1419-1432.

12. Cifuni SM, Wagner DD, Bergmeier W. CalDAG-GEFI and protein kinase C represent alternative pathways leading to activation of integrin alphaIIbbeta3 in platelets. *Blood*. 2008;112(5):1696-1703.

13. Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost*. 2010;8(3): 454-462.

14. Beer JH, Büchi L, Steiner B. Glycocalicin: a new assay–the normal plasma levels and its potential usefulness in selected diseases. *Blood*. 1994;83(3): 691-702.

15. Bergmeier W, Rackebrandt K, Schröder W, Zirngibl H, Nieswandt B. Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies. *Blood*. 2000;95(3):886-893.

16. Bergmeier W, Piffath CL, Cheng G, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates GPIbalpha shedding from platelets in vitro and in vivo. *Circ Res*. 2004;95(7):677-683.

17. Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Berndt MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. *J Thromb Haemost*. 2007;5(7):1530-1537.

18. Cheng H, Yan R, Li S, et al. Shear-induced interaction of platelets with von Willebrand factor results in glycoprotein Ibalpha shedding. *Am J Physiol Heart Circ Physiol*. 2009;297(6):H2128-H2135.

19. Kveiborg M, Instrell R, Rowlands C, Howell M, Parker PJ. PKCα and PKCδ regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. *PLoS One*. 2011;6(2):e17168.

20. Nagano O, Murakami D, Hartmann D, et al. Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. *J Cell Biol*. 2004;165(6):893-902.

21. Kanaji T, Russell S, Ware J. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. *Blood*. 2002;100(6): 2102-2107.

22. Crittenden JR, Bergmeier W, Zhang Y, et al. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation [published correction appears in *Nat Med*. 2004;10(10):1139]. *Nat Med*. 2004;10(9):982-986.

23. Ahmad F, Boulaftali Y, Greene TK, et al. Relative contributions of stromal interaction molecule 1 and CalDAG-GEFI to calcium-dependent platelet activation and thrombosis. *J Thromb Haemost*. 2011;9(10):2077-2086.

24. Stefanini L, Boulaftali Y, Ouellette TD, et al. Rap1-Rac1 circuits potentiate platelet activation. *Arterioscler Thromb Vasc Biol*. 2012;32(2):434-441.

25. Petrich BG, Marchese P, Ruggeri ZM, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. *J Exp Med*. 2007; 204(13):3103-3111.

26. Adam F, Casari C, Prévost N, et al. A genetically-engineered von Willebrand disease type 2B mouse model displays defects in hemostasis and inflammation. *Sci Rep*. 2016;6(1):26306.

27. Casari C, Du V, Wu YP, et al. Accelerated uptake of VWF/platelet complexes in macrophages contributes to VWD type 2B-associated thrombocytopenia. *Blood*. 2013;122(16):2893-2902.

28. Golder M, Pruss CM, Hegadorn C, et al. Mutation-specific hemostatic variability in mice expressing common type 2B von Willebrand disease substitutions. *Blood*. 2010;115(23):4862-4869.

29. Getz TM, Piatt R, Petrich BG, Monroe D, Mackman N, Bergmeier W. Novel mouse hemostasis model for real-time determination of bleeding time and hemostatic plug composition. *J Thromb Haemost*. 2015;13(3):417-425.

30. Nurden P, Debili N, Vainchenker W, et al. Impaired megakaryocytopoiesis in type 2B von Willebrand disease with severe thrombocytopenia. *Blood*. 2006; 108(8):2587-2595.

31. Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS. Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. *N Engl J Med*. 1980;302(19):1047-1051.

32. De Marco L, Mazzucato M, De Roia D, et al. Distinct abnormalities in the interaction of purified types IIA and IIB von Willebrand factor with the two platelet binding sites, glycoprotein complexes Ib-IX and IIb-IIIa. *J Clin Invest*. 1990;86(3):785-792.

33. 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(9):3403-3411.

34. Mangin P, Yuan Y, Goncalves I, et al. Signaling role for phospholipase C gamma 2 in platelet glycoprotein Ib alpha calcium flux and cytoskeletal reorganization. Involvement of a pathway distinct from FcR gamma chain and Fc gamma RIIA. *J Biol Chem*. 2003;278(35):32880-32891.

35. Chow TW, Hellums JD, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood*. 1992;80(1):113-120.

36. Stefanini L, Bergmeier W. RAP1-GTPase signaling and platelet function. *J Mol Med (Berl)*. 2016;94(1):13-19.

37. Bergmeier W, Burger PC, Piffath CL, et al. Metalloproteinase inhibitors improve the recovery and hemostatic function of in vitro-aged or -injured mouse platelets. *Blood*. 2003;102(12):4229-4235.

38. Boulaftali Y, Hess PR, Getz TM, et al. Platelet ITAM signaling is critical for vascular integrity in inflammation. *J Clin Invest*. 2013;123(2):908-916.

39. Bergmeier W, Boulaftali Y. Adoptive transfer method to study platelet function in mouse models of disease. *Thromb Res*. 2014;133(suppl 1):S3-S5.

40. Morowski M, Vögtle T, Kraft P, Kleinschnitz C, Stoll G, Nieswandt B. Only severe thrombocytopenia results in bleeding and defective thrombus formation in mice. *Blood*. 2013;121(24):4938-4947.

41. 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(5):1568-1573.

42. Deng W, Xu Y, Chen W, et al. Platelet clearance via shear-induced unfolding of a membrane mechanoreceptor. *Nat Commun*. 2016;7:12863.

43. Casonato A, Steffan A, Pontara E, et al. Post-DDAVP thrombocytopenia in type 2B von Willebrand disease is not associated with platelet consumption: failure to demonstrate glycocalicin increase or platelet activation. *Thromb Haemost*. 1999;81(2):224-228.

44. Steinberg MH, Kelton JG, Coller BS. Plasma glycocalicin. An aid in the classification of thrombocytopenic disorders. *N Engl J Med*. 1987;317(17): 1037-1042.

45. Wang Z, Shi Q, Yan R, Liu G, Zhang W, Dai K. The role of calpain in the regulation of ADAM17-dependent GPIbalpha ectodomain shedding. *Arch Biochem Biophys*. 2010;495(2):136-143.

46. Okita JR, Frojmovic MM, Kristopeit S, Wong T, Kunicki TJ. Montreal platelet syndrome: a defect in calcium-activated neutral proteinase (calpain). *Blood*. 1989;74(2):715-721.

47. Jackson SC, Sinclair GD, Cloutier S, Duan Z, Rand ML, Poon MC. The Montreal platelet syndrome kindred has type 2B von Willebrand disease with the VWF V1316M mutation. *Blood*. 2009;113(14):3348-3351.

48. Murugappan S, Tuluc F, Dorsam RT, Shankar H, Kunapuli SP. Differential role of protein kinase C delta isoform in agonist-induced dense granule secretion in human platelets. *J Biol Chem*. 2004;279(4):2360-2367.

49. Kruse-Jarres R, Johnsen JM. How I treat type 2B von Willebrand disease. *Blood*. 2018;131(12):1292-1300.

50. Proud L, Ritchey AK. Management of type 2b von Willebrand disease in the neonatal period. *Pediatr Blood Cancer*. 2017;64(1):103-105.

51. Bergmeier W, Schulte V, Brockhoff G, Bier U, Zirngibl H, Nieswandt B. Flow cytometric detection of activated mouse integrin alphaIIbbeta3 with a novel monoclonal antibody. *Cytometry*. 2002;48(2):80-86.