Regular Article

PLATELETS AND THROMBOPOIESIS

Twinfilin 2a regulates platelet reactivity and turnover in mice

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Key Points

- Deficiency in twinfilin 2a causes macrothrombocytopenia and hyperreactivity of platelets in mice.
- We provide the first in vivo evidence for an inhibitory function of twinfilin 2a in platelet actin dynamics.

Regulated reorganization of the actin cytoskeleton is a prerequisite for proper platelet production and function. Consequently, defects in proteins controlling actin dynamics have been associated with platelet disorders in humans and mice. Twinfilin 2a (Twf2a) is a small actin-binding protein that inhibits actin filament assembly by sequestering actin monomers and capping filament barbed ends. Moreover, Twf2a binds heterodimeric capping proteins, but the role of this interaction in cytoskeletal dynamics has remained elusive. Even though Twf2a has pronounced effects on actin dynamics in vitro, only little is known about its function in vivo. Here, we report that constitutive Twf2a-deficient mice ($Twf2a^{-/-}$) display mild macrothrombocytopenia due to a markedly accelerated platelet clearance in the spleen. $Twf2a^{-/-}$ platelets showed enhanced integrin activation and α-granule release in response to stimulation of (hem) immunoreceptor tyrosine-based activation motif (ITAM) and G-protein–coupled receptors, increased adhesion and aggregate formation on collagen I under flow, and accelerated clot retraction and spreading on fibrinogen. In vivo, Twf2a deficiency resulted in shortened tail bleeding times and faster occlusive arterial thrombus formation. The hyperreactivity of $Twf2a^{-/-}$ platelets was attributed to enhanced actin dynamics, characterized by an increased activity of n-cofilin and profilin 1, leading to a thickened cortical cytoskeleton and hence sustained integrin activation by limiting calpain-mediated integrin inactivation. In summary, our results reveal the first in vivo functions of mammalian Twf2a and demonstrate that Twf2a-controlled actin rearrangements dampen platelet activation responses in a n-cofilin– and profilin 1–dependent manner, thereby indirectly regulating platelet reactivity and half-life in mice. (*Blood*. 2017;130(15):1746-1756)

Introduction

Platelets are small anucleate cell fragments that are continuously produced from megakaryocytes (MKs) in the bone marrow by a cytoskeleton-driven process. Proplatelet formation is characterized by the extension of long cytoplasmic protrusions into bone marrow sinusoids, where larger fragments, so-called preplatelets, are shed and further mature within the circulation. However, the molecular mechanisms orchestrating platelet biogenesis are incompletely understood.[1] While microtubule sliding enables proplatelet elongation and transport of organelles, the actin cytoskeleton regulates branching of extending proplatelets, thereby increasing the number of available proplatelet tips.[2,3] In line with this model, we previously demonstrated that the small actin-binding proteins ADF/cofilin and profilin 1 (Pfn1) are critical determinants of platelet formation and sizing.[4,5]

In circulating platelets, the actin cytoskeleton is essential to maintain cell morphology and exert key functions, such as the transduction of mechanical forces, granule release, and filopodia and lamellipodia formation.[6] The critical role of the actin cytoskeleton for platelet production and function is further evidenced by the association of genetic variants in actin cytoskeleton-related genes such as *DIAPH1*,[7] *FLNA*,[8] *WASP*,[9,10] *ACTN1*,[9] *MYH9*,[11] or *TRPM7*[12] with platelet disorders in humans and mice. In addition, orchestrated rearrangements of the actin cytoskeleton also control the affinity of αIIbβ3- and α2β1-

integrins to their ligands, and deficiencies in actin-related proteins result in altered platelet and MK integrin function.[4,13-18] Nonetheless, the precise molecular mechanisms linking actin dynamics and platelet integrin function have remained ill-defined.

Twinfilins (Twfs) are evolutionarily conserved (40 kDa) actin-binding proteins that are composed of 2 actin depolymerizing factor (ADF)-homology domains connected by a short linker region and followed by a C-terminal tail.[19,20] Despite their pronounced effects on both actin filament assembly and disassembly in vitro, Twfs have so far received only limited attention, and their in vivo function is largely unknown. Previous studies showed that inactivation of Twf in budding yeast and fruit fly results in defects in actin-dependent processes and reduced viability.[21-23] In mammals, the ubiquitously expressed Twf1 and Twf2a, as well as the muscle-specific Twf2b, have been identified.[24-26] However, physiological functions of the mammalian Twf isoforms have not been reported to date.

Twfs inhibit actin polymerization on barbed ends by capping filament ends with a preference toward adenosine 5′-diphosphate (ADP)–bound actin. Both barbed and pointed end growth are additionally inhibited through Twf-mediated sequestration of ADP–G-actin monomers.[21,27] Moreover, Twfs interact with heterodimeric capping proteins, which inhibits both actin filament depolymerization and the addition of new

monomers to filament barbed ends. However, the mechanisms of this interaction, as well as its role in cytoskeletal dynamics, have remained unclear.[25,28,29] Upon ADF/cofilin–mediated dissociation of G-actin monomers from actin filaments, Twf may compete with ADF/cofilin in binding to ADP–G-actin, thus favoring the release of these monomers from ADF/cofilin.[24,30,31] Surprisingly, despite the central role of Twfs in actin dynamics, Twf2a-deficient mice ($Twf2a^{-/-}$) are viable and apparently healthy. Since Twf2a is dispensable for mouse development, it was speculated that its lack might be compensated by the functionally redundant Twf1 isoform.[26]

Here, we report that $Twf2a^{-/-}$ mice display mild macrothrombocytopenia associated with a decreased platelet half-life and marked hyperreactivity of these cells in vitro and in vivo. In summary, these studies identify Twf2a as an important negative regulator of platelet reactivity and demonstrate for the first time an in vivo function of Twf-family proteins in mammals.

Materials and methods

Animals

Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken). Conditional Twf1-deficient mice were generated by intercrossing $Twf1^{fl/fl}$ mice (exon 3 flanked by loxP sites) with mice carrying the Cre-recombinase under the $Pf4$ promoter.[32] $Twf1^{fl/fl}$ mice were obtained from EUCOMM (The European Conditional Mouse Mutagenesis Program; strain ID EM:05232). Constitutive $Twf2a^{-/-}$ mice have been described earlier.[26] For all experiments, 12- to 16-week-old $Twf2a^{-/-}$ mice and the respective $Twf2a^{+/+}$ littermate controls were used. All mice were derived from the breeding strategy $Twf2a^{+/-} \times Twf2a^{+/-}$, resulting in 25% $Twf2a^{+/+}$, 50% $Twf2a^{+/-}$, and 25% $Twf2a^{-/-}$ mice.

Preparation of platelets, determination of platelet lifespan, count, and size,[12,33] immunostaining of resting or spread platelets, flow cytometry, platelet adhesion under flow,[33] immunoblotting, macrophage depletion and splenectomy,[4] platelet terminal galactose levels,[34] reticulated platelets, actin polymerization,[12] in vivo thrombus formation, and tail bleeding time[33] were performed as described previously, and detailed information can be found in supplemental Methods (available on the *Blood* Web site).

Data analysis

The presented results are mean ± standard deviation (SD) from at least 3 independent experiments per group, if not stated otherwise. Data distribution was analyzed using the Shapiro-Wilk test, and differences between control and $Twf2a^{-/-}$ mice were statistically analyzed using a Student t test or Wilcoxon-Mann-Whitney test. P values <.05 were considered statistically significant (*$P < .05$; **$P < .01$; ***$P < .001$). Results with a P value >.05 were considered not significant (NS).

Results

$Twf2a^{-/-}$ mice display macrothrombocytopenia

Expression profiling revealed that both $Twf1/TWF1$ and $Twf2a/TWF2$ are prominently expressed in murine and human platelets with a distinct subcellular localization, while the murine $Twf2b$ isoform could not be detected (supplemental Figure 1). We generated MK- and platelet-specific Twf1-deficient ($Twf1^{fl/fl-Pf4Cre}$) mice (supplemental Figure 2A) and found that platelet count, size, lifetime, and functionality were unaltered compared with controls, excluding an essential role of Twf1 for platelet production and function (supplemental Figure 2B-J). To test whether Twf2a may compensate for the lack of Twf1 in MKs and

platelets, we capitalized on constitutive $Twf2a^{-/-}$ mice.[26] Immunoblot analysis on platelet lysates confirmed ablation of Twf2a protein expression, whereas no compensatory upregulation of Twf1 was detectable (supplemental Figure 3A). $Twf2a^{-/-}$ mice displayed macrothrombocytopenia (Figure 1A-B) and, in line with the increased platelet size, a slightly increased surface abundance of major platelet glycoproteins (supplemental Figure 3B). Ultrastructurally, $Twf2a^{-/-}$ platelets showed an enlarged and spherical morphology, with a normal number of α-, δ-granules as well as mitochondria, but an increased presence of vacuoles (supplemental Figure 4). In contrast, leukocyte populations in blood, spleen, lymph nodes, and thymus (supplemental Figure 5) were grossly unaltered in mutant mice as compared with controls. These results suggested a critical and specific role of Twf2a in the regulation of circulating platelet numbers that is nonredundant to that of the closely related Twf1 isoform.

### Markedly reduced platelet lifespan in $Twf2a^{-/-}$ mice

Next, we analyzed the cause of the observed thrombocytopenia and found that $Twf2a^{-/-}$ mice had a markedly decreased platelet lifespan ($T_{1/2}$ of control platelets: 48.6 ± 2.0 hours vs $T_{1/2}$ of $Twf2a^{-/-}$ platelets: 22.3 ± 2.11 hours; ***$P < .001$) in vivo (Figure 1C). Assessment of surface-bound antibodies revealed no significant differences between platelets of control and $Twf2a^{-/-}$ mice (supplemental Figure 6A), suggesting that the accelerated clearance of $Twf2a^{-/-}$ platelets is not caused by autoantibodies. In agreement, depletion of macrophages using clodronate-encapsulated liposomes increased platelet counts in control and similarly, although to a lesser extent, $Twf2a^{-/-}$ mice, demonstrating that $Twf2a^{-/-}$ platelets were only in part removed from the circulation by macrophages (Figure 1D; supplemental Figure 6B). Interestingly, however, splenectomy restored platelet counts in $Twf2a^{-/-}$ mice to those seen in controls (Figure 1E; supplemental Figure 6C), suggesting that macrophage-independent clearance of $Twf2a^{-/-}$ platelets by cells of the reticuloendothelial system also contributes to the thrombocytopenia in these animals. In support of this, we found an expansion of the red pulp (54.42% ± 5.73% of the cross-sectional area in control spleens vs 66.42% ± 2.92% of the cross-sectional area in $Twf2a^{-/-}$ spleens; ***$P < .001$; supplemental Figure 7) and an increased fraction of platelets in the spleen of $Twf2a^{-/-}$ mice (Figure 1F-G). The accelerated platelet clearance in $Twf2a^{-/-}$ mice was associated with an increased number of young platelets in the circulation, as evidenced by a significantly reduced percentage of desialylated platelets and an increased prevalence of reticulated (RNA-rich) platelets (Figure 1H-I),[35] indicating that increased platelet production may partially compensate for the accelerated platelet clearance. In conclusion, these results indicated that the thrombocytopenia in $Twf2a^{-/-}$ mice is, to a large extent, caused by premature clearance of platelets in the spleen.

Twf2a is a negative regulator of platelet reactivity

We next hypothesized that the reduced platelet half-life and hence the resulting thrombocytopenia might be a result of premature platelet activation or consumption and assessed agonist-induced platelet integrin inside-out activation and degranulation (P-selectin exposure) by flow cytometry. Interestingly, $Twf2a^{-/-}$ platelets displayed a pronounced hyperreactivity toward all tested agonists with both increased integrin activation and α-granule release (Figure 2A-B). Of note, $Twf2a^{-/-}$ platelets did not display a reduced sensitivity toward inhibitors of platelet activity, since similar to controls, pretreatment with prostacyclin (PGI$_2$) resulted in reduced platelet integrin activation and degranulation in response to all tested agonists (supplemental Figure 8).
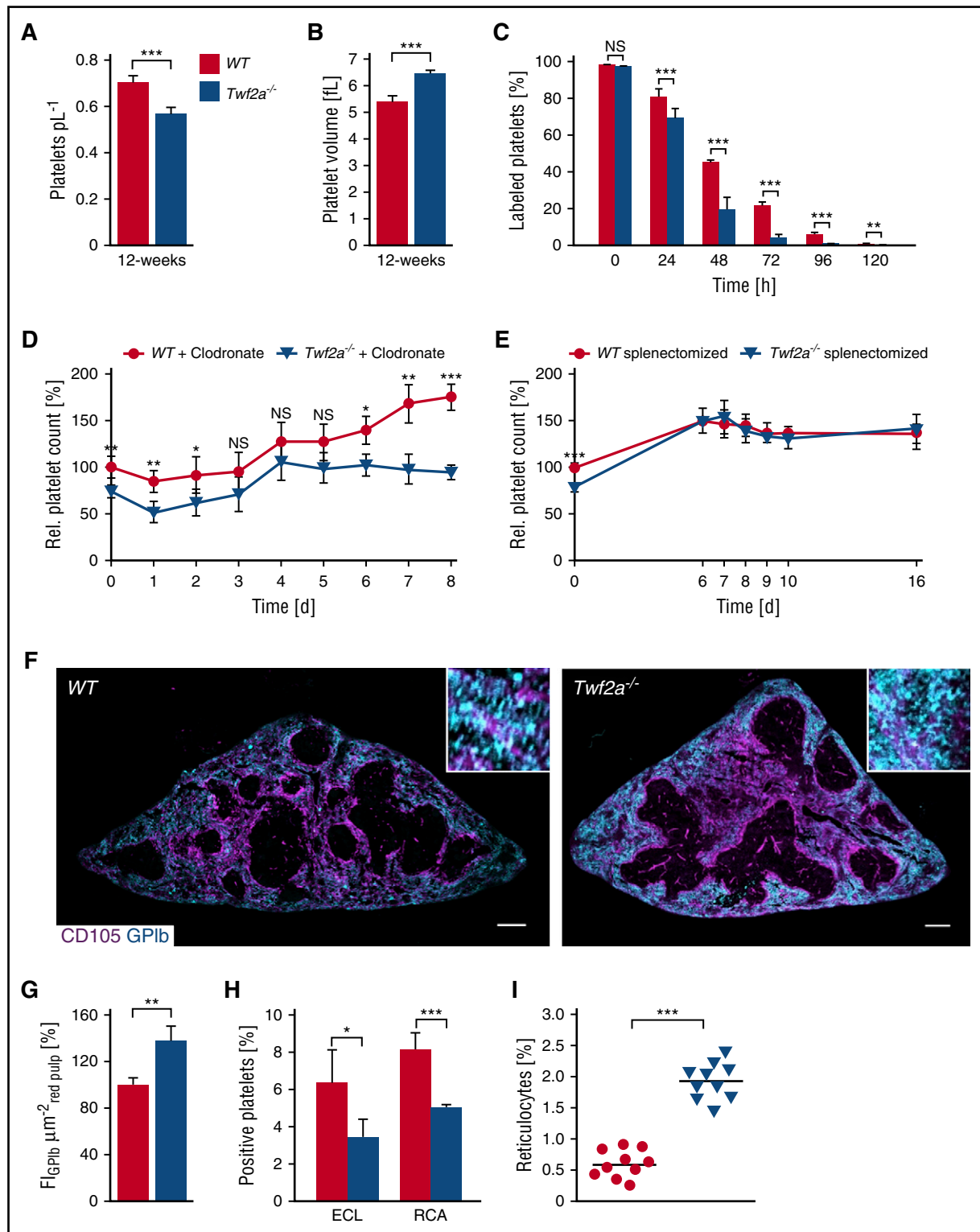
Figure 1. Accelerated platelet clearance accounts for macrothrombocytopenia in *Twf2a*^{−/−} mice. (A-B) Platelet count (A) and size (B) were determined with an automated cell analyzer (Sysmex). (C) Platelet lifespan was assessed by flow cytometric measurement of the fluorescence-positive platelet population at the indicated time points after injection of a fluorophore-conjugated anti-GPIX antibody derivative. (D-E) Platelet counts were monitored over time after clodronate-encapsulated liposome-mediated macrophage depletion (D) and splenectomy (E). (F-G) The relative platelet content in control and *Twf2a*^{−/−} spleens was determined by immunostaining (platelet GPIb, cyan; endothelial CD105, magenta) and confocal microscopy of cryosections. Scale bars, 250 μm. Images were acquired with a TCS SP8 confocal microscope (25×/0.95 FLUOTAR VISIR water objective, Leica Microsystems) and are representative of at least 4 individuals. (H-I) The fraction of aged platelets was assessed by platelet lectin-binding (*Erythrina crista-galli* lectin [ECL] and *Ricinus communis* agglutinin [RCA]) and the fraction of young (RNA-rich) platelets by thiazole orange (TO) staining and flow cytometry. The overall gated platelet population (based on forward sideward characteristics and GPIX labeling) was set as 100%. Neuraminidase-treated platelets were used as a positive control (H) and RNase-treated platelets as a negative control (I). Values represent mean ± SD (n = 6). Each symbol represents 1 individual. Horizontal lines represent mean (I). ***$P < .001$, **$P < .01$, and *$P < .05$, unpaired Student t test. NS, not significant; *WT*, wild-type.
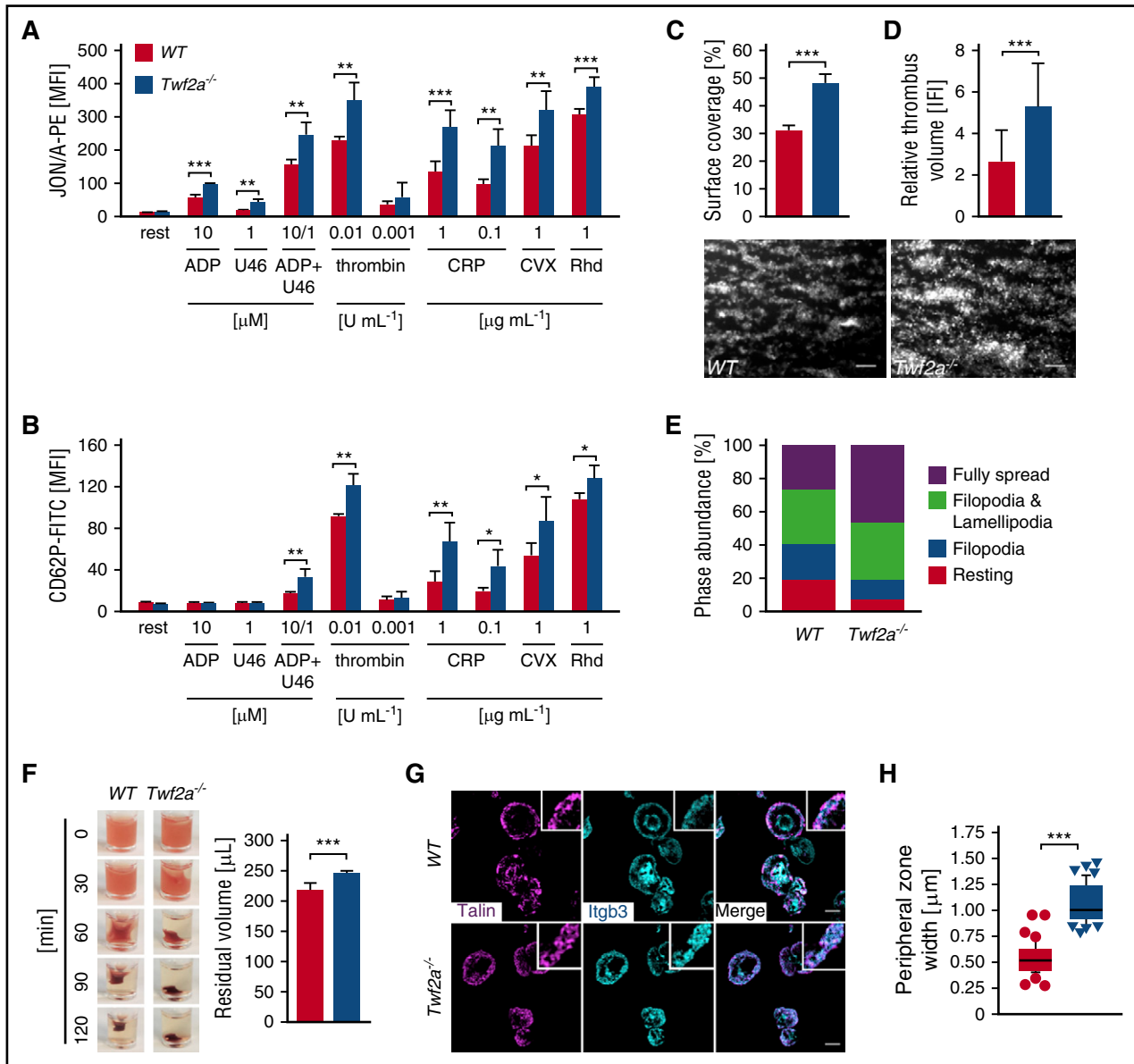
Figure 2. Increased integrin activation in *Twf2a*$^{-/-}$ platelets. (A-B) Platelet αIIbβ3-integrin (JON/A-PE) activation (A) and P-selectin (anti-CD62P-FITC) exposure (B), serving as a measure for α-granule release, were determined by flow cytometry. (C-D) Platelet adhesion (C) and thrombus formation (D) under flow (1000 s^{-1}) were analyzed in a flow chamber system. Scale bars, 25 μm. Images were acquired with a Zeiss Axiovert 200 inverted microscope (40×/0.6 oil objective). (E) Washed platelets were allowed to spread (15 min) on fibrinogen (100 μg/mL), and phase abundance was determined. (F) Platelet clot retraction in response to stimulation with thrombin (5 U/mL) over time. Residual plasma volume was determined 120 minutes after the addition of thrombin. Images are representative of 4 individuals. (G) Tln recruitment to β3-integrin tails was assessed by immunostaining (talin, magenta; Itgb3, cyan) and confocal microscopy. Scale bars, 3 μm. Images were acquired with a TCS SP8 confocal microscope (100×/1.4 STED WHITE oil objective, Leica Microsystems). (H) Quantification of the peripheral zone (talin and β3-integrin co-localization) width. Values are mean ± SD (n = 6). Images are representative of at least 6 individuals. ***P < .001, **P < .01, and *P < .05, unpaired Student t test (A-G) and Wilcoxon-Mann-Whitney test (H). CRP, collagen-related peptide; CVX, convulxin; rest, resting; Rhd, rhodocytin; U46, U46619, a stable thromboxane A2 analog.

The enhanced activation response to agonist stimulation translated into markedly increased adhesion and aggregate formation on collagen I under flow (Figure 2C-D). Furthermore, *Twf2a*$^{-/-}$ platelets displayed enhanced integrin outside-in signaling as detected by accelerated spreading on fibrinogen (Figure 2E; supplemental Figure 9; supplemental Videos 1 and 2) and clot retraction (Figure 2F).

Talin (Tln) recruitment to β-integrin tails represents a key step in integrin activation. In agreement with the enhanced integrin activation, we found increased Tln and β3-integrin colocalization on the leading edge of spread *Twf2a*$^{-/-}$ platelets compared with controls, as evidenced by a doubling of the width (0.54 ± 0.13 μm for controls vs 1.08 ± 0.18 μm in *Twf2a*$^{-/-}$ platelets; ***P < .001) of the cortical zone (Figure 2G-H).

Accelerated arterial thrombus formation in *Twf2a*$^{-/-}$ mice

To investigate whether the platelet hyperreactivity observed in vitro can similarly be observed in vivo, we next analyzed arterial thrombus formation and hemostatic function in *Twf2a*$^{-/-}$ mice. Upon FeCl$_3$-induced damage of the endothelium in mesenteric arterioles, the time to initiation of thrombus formation (7.4 ± 2.9 minutes in control vs 4.8 ± 3.1 minutes in *Twf2a*$^{-/-}$ mice; **P < .01) and vessel occlusion (15.6 ± 6.3 minutes in control vs 10.6 ± 2.4 minutes in *Twf2a*$^{-/-}$ mice; ***P < .001) was significantly lower in *Twf2a*$^{-/-}$ mice than in controls (Figure 3A-B). Similarly, hemostatic plug formation and cessation of bleeding (5.4 ± 1.8 minutes in control vs
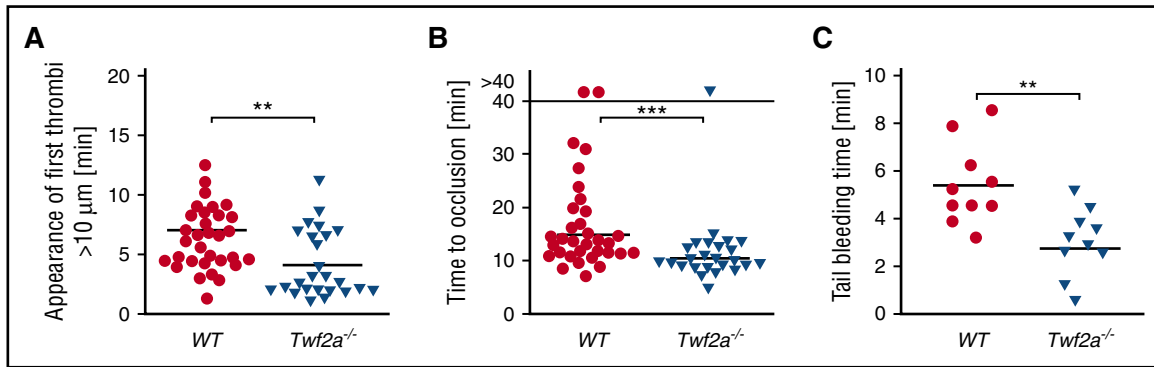
Figure 3. Twf2a is a critical regulator of platelet reactivity in vivo. (A-B) Accelerated adhesion of platelets (A) and occlusion of mesenteric arterioles (B) in $Twf2a^{-/-}$ mice upon FeCl$_3$-induced injury of the endothelial barrier. Each symbol represents 1 mesenteric arteriole (n = 12 individuals). (C) Accelerated hemostatic plug formation in $Twf2a^{-/-}$ mice. Each symbol represents 1 individual (n = 10 individuals). Horizontal lines represent mean. ***P < .001 and **P < .01, Wilcoxon-Mann-Whitney test (A-B) and unpaired Student t test (C).

2.8 ± 1.5 minutes in $Twf2a^{-/-}$ mice; **P < .01) in a tail bleeding time model was markedly accelerated in $Twf2a^{-/-}$ mice (Figure 3C). These results further corroborated the notion that Twf2a acts as negative regulator of platelet reactivity.

Sustained integrin activation contributes to the hyperreactivity of $Twf2a^{-/-}$ platelets

We next sought to identify the cause of the sustained integrin activation in $Twf2a^{-/-}$ platelets. It has previously been reported that sustained activation of platelet αIIbβ3-integrins and the exposure of phosphatidylserine (PS) on the outer platelet membrane leaflet are critically linked. Upon sustained Ca^{2+} signaling, αIIbβ3-integrins can be switched back to their low-affinity state through Ca^{2+}-dependent activation of the protease calpain and cleavage of the cytoplasmic tails of β3-integrins.[36-38]

Strikingly, whereas Anxa5 fluorescein isothiocyanate (FITC) binding to individual $Twf2a^{-/-}$ platelets (mean fluorescence intensity of the PS-positive population) was unaltered, fewer mutant platelets exposed PS on their surface (Figure 4A). Moreover, in line with the reduced percentage of PS exposing platelets, activation-induced mitochondrial depolarization was less pronounced in $Twf2a^{-/-}$ platelets (Figure 4B-C). Time-course experiments assessing the distribution of platelets with activated αIIbβ3-integrins (quadrant Q1), PS exposure (Q3), or both (Q2) revealed an increased percentage of $Twf2a^{-/-}$ platelets with activated integrins (events in Q1 and Q2) after both 5 minutes (39.1% ± 3.7% for controls and 43.9% ± 2.9% for

$Twf2a^{-/-}$ platelets; *P < .05) and 30 minutes (31.8% ± 1.5% for controls and 43.3% ± 3.6% for $Twf2a^{-/-}$ platelets; ***P < .001) upon simultaneous stimulation with thrombin and collagen-related peptide (Figure 5A-B; supplemental Figure 10). Assessment of calpain-mediated β3-integrin cleavage by immunoblotting revealed a decreased loss of full-length β3-integrin in $Twf2a^{-/-}$ platelets as compared with control (Figure 5C-D). These results strongly suggested altered Ca^{2+} signaling or calpain activity in $Twf2a^{-/-}$ platelets; however, we did not observe differences in store-operated Ca^{2+} entry or alterations in the cleavage of further calpain targets such as filamin A or Tln upon stimulation with different agonists (Figure 5E-F; supplemental Figure 11).

Together, these results reveal sustained integrin activation due to reduced calpain-mediated integrin closure as cause of the pronounced hyperreactivity of $Twf2a^{-/-}$ platelets in different in vitro and in vivo settings.

Twf2a is a negative regulator of actin filament assembly in platelets

Given the important role of Twfs in actin dynamics and the central role of actin rearrangements for integrin activation, we hypothesized that altered cytoskeletal dynamics may contribute to the observed hyperreactivity of $Twf2a^{-/-}$ platelets by restricting the accessibility of β3-integrin tails to calpain.[19,22,30,39] Morphological analyses, however, revealed no gross cytoskeletal alterations but did reveal a mildly enlarged size of resting $Twf2a^{-/-}$ platelets (Figure 6A;
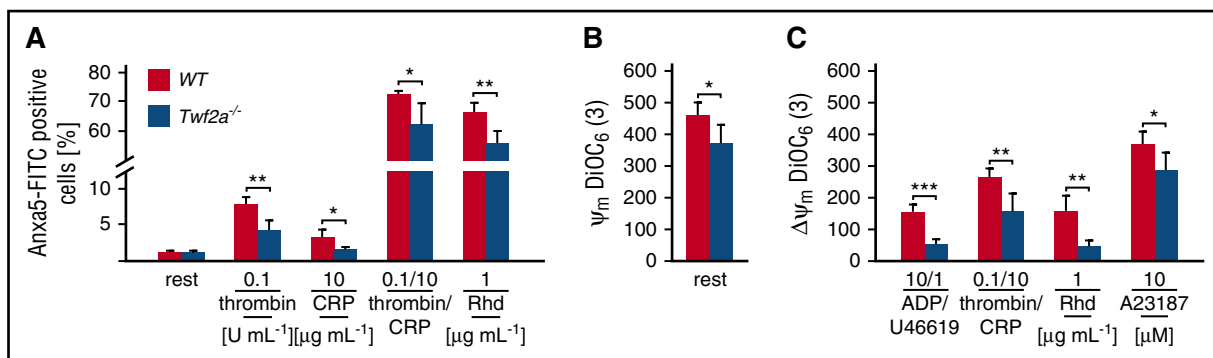
Figure 4. $Twf2a^{-/-}$ platelets are less prone to apoptosis. Platelet apoptosis was assessed using Anxa5-FITC binding to exposed phosphatidylserine (A) or the mitochondrial membrane potential sensor 3,3′-dihexyloxacarbocyanine iodide (DiOC$_6$(3); B-C). A23187, Ca^{2+} ionophore; CRP, collagen-related peptide; rest, resting; Rhd, rhodocytin; U46, U46619, a stable thromboxane A$_2$ analog. Values are mean ± SD (n = 6). ***P < .001, **P < .01, and *P < .05, unpaired Student t test.
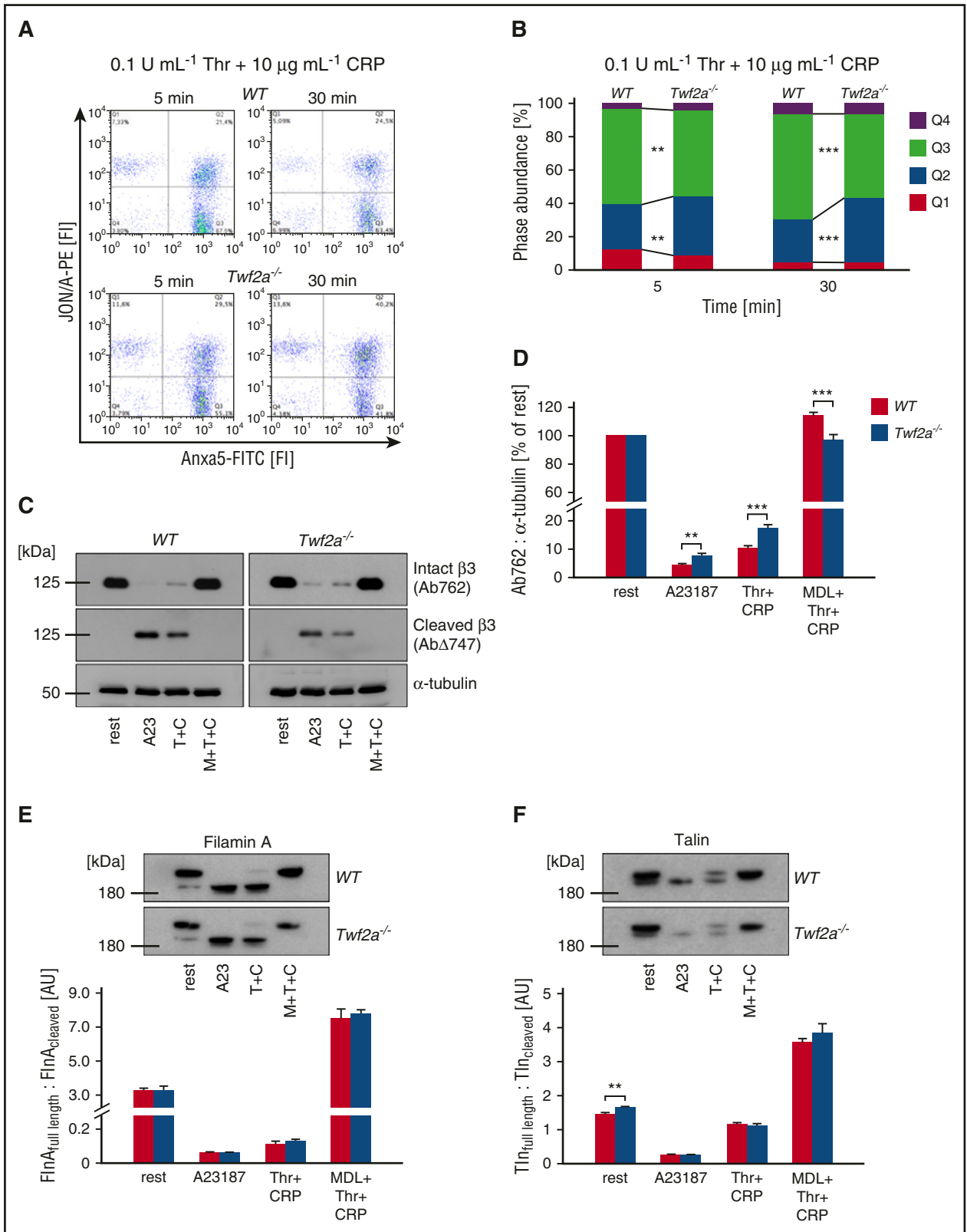
Figure 5. Delayed integrin closure accounts for the hyperresponsiveness of *Twf2a$^{-/-}$* platelets. (A) Washed platelets were simultaneously stimulated with thrombin (Thr/T) and collagen-related peptide (CRP/C) for the indicated periods of time. Activation of αIIbβ3-integrins (JON/A-PE) and phosphatidylserine exposure (Anxa5-FITC) on the outer leaflet of the platelet membrane was determined by flow cytometry. Flow cytometry plots are representative of at least 6 individuals. (B) Percentage of cells per quadrant. Q1, JON/A$^+$ Anxa5$^-$ (top left); Q2, JON/A$^+$ Anxa5$^+$ (top right); Q3, JON/A$^-$ Anxa5$^+$ (bottom right); Q4, JON/A$^-$ Anxa5$^-$ (bottom left). Values are mean (n = 6). (C-F) Platelets were left untreated or preincubated for 10 minutes in the presence of the calpain inhibitor MDL-28170 (MDL/M, 200 μM). Subsequently samples were stimulated with the calcium ionophore A23187 (A23, 10 μM) or thrombin (0.1 U/mL) and CRP (10 μg/mL), lysed, and processed for immunoblotting. Full-length (Ab762) and calpain-cleaved (AbΔ747) β3-integrin (C), as well as filamin A (E), talin (F), and α-tubulin, were probed with the respective antibodies and analyzed by densitometry. Values are mean ± SD (n = at least 4). ***$P < .001$, **$P < .01$, and *$P < .05$, unpaired Student t test.
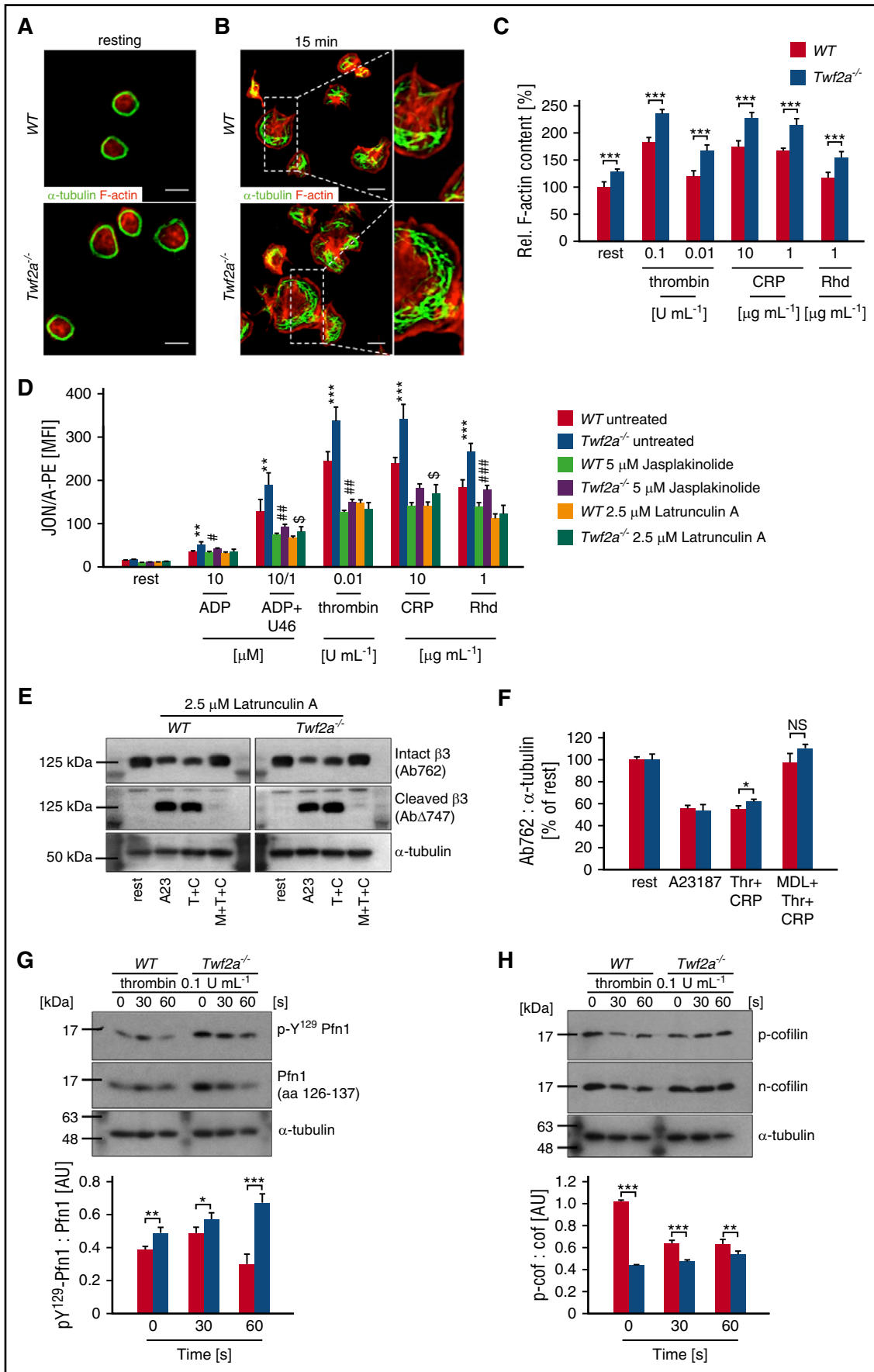
Figure 6.

supplemental Figure 4). Strikingly, upon spreading, $Twf2a^{-/-}$ platelets displayed an increased size and thickened cortical actin cytoskeleton (Figure 6B) correlating with the enlarged colocalization area of Tln and β3-integrins (Figure 2G-H). This was further supported by an increased content of filamentous actin in resting and activated $Twf2a^{-/-}$ platelets (Figure 6C), which is in agreement with previous reports showing an inhibitory function of fly and yeast Twfs in actin dynamics.[19,22,40]

To test whether increased actin polymerization could contribute to the hyperreactivity, platelets of control and $Twf2a^{-/-}$ mice were pretreated with vehicle (dimethyl sulfoxide) or toxins interfering with actin dynamics (jasplakinolide or latrunculin A). Pretreatment with the actin assembly–promoting toxin jasplakinolide impaired integrin activation overall in control and $Twf2a^{-/-}$ platelets as compared with vehicle-treated samples, but it did not revert the hyperresponsiveness of $Twf2a^{-/-}$ platelets (Figure 6D; supplemental Figure 12). In contrast, pretreatment with the F-actin–destabilizing toxin latrunculin A efficiently inhibited actin polymerization and similarly impaired αIIbβ3-integrin activation and, to a lesser extent, degranulation in control and $Twf2a^{-/-}$ platelets (Figure 6D; supplemental Figures 12 and 13). In addition, latrunculin A pretreatment restored calpain-mediated β3-integrin cleavage in stimulated $Twf2a^{-/-}$ platelets to the levels observed in controls (Figure 6E-F).

Given the central role of Twf2a in actin dynamics,[19-23] we next studied the molecular mechanisms leading to increased actin polymerization and hence restriction of integrin inactivation in $Twf2a^{-/-}$ platelets. Assessment of the activation-dependent phosphorylation of Pfn1 on tyrosine 129, which is known to increase Pfn1's affinity toward G-actin monomers and its actin polymerization activity,[41] revealed a significantly higher activity of Pfn1 in $Twf2a^{-/-}$ platelets (Figure 6G). Similarly, $Twf2a^{-/-}$ platelets showed increased levels of dephosphorylated, active n-cofilin that acts in concert with Pfn1 in enhancing actin dynamics by providing free barbed ends and thereby promoting actin polymerization (Figure 6H).[42]

These data provide the first in vivo evidence for an inhibitory function of mammalian Twf2a in F-actin assembly during platelet activation by fine-tuning Pfn1 as well as n-cofilin function and thereby limiting platelet integrin activation and reactivity.

In conclusion, our results support a critical role of Twf2a and the actin cytoskeleton in integrin activation and strongly suggest that altered cytoskeletal dynamics account for the sustained integrin activation in $Twf2a^{-/-}$ platelets, presumably by sterically limiting calpain-mediated integrin closure.

Discussion

In the present study, we demonstrate for the first time an inhibitory function of mammalian Twf2a in actin dynamics in vivo by limiting Pfn1 and n-cofilin activity, with critical implications for platelet

biogenesis, reactivity, and turnover. Our findings point to specific functions of Twf2a in these processes, since the Twf1 isoform could not compensate for the lack of Twf2a (supplemental Figure 3A). In support of this, $Twf1^{fl/fl-Pf4Cre}$ MKs and platelets did not display any overt phenotype in the tested experimental settings (supplemental Figures 2B-J and 14).

Although TWF1 and TWF2 have recently been implicated in human disorders,[43-46] so far, TWFs have not been associated with platelet disorders. However, given the relatively moderate macro-thrombocytopenia in $Twf2a^{-/-}$ mice, we speculate that humans carrying variants in TWF2 might not show overt clinical manifestations of a platelet disorder. Hence, TWF2-related abnormalities of platelet function could be overseen in routine diagnostic checkups. However, based on our data, it is tempting to speculate that genetic variants in TWF2 could be associated with an increased risk of platelet-dependent thrombotic events, but this will need further investigation.

Our results revealed accelerated clearance of hyperresponsive $Twf2a^{-/-}$ platelets from the circulation by the reticuloendothelial system in the spleen as the cause of macrothrombocytopenia, which might be partially compensated by increased platelet production (Figure 1C-I; supplemental Figures 6 and 7). Based on our findings, we speculate that platelets in the circulation are frequently exposed to trace amounts of agonist such as ADP/adenosine triphosphate released from damaged cells, very small amounts of locally produced thrombin, and, importantly, shear gradients in the vascular system, which may not be sufficient to fully activate them in healthy conditions.[47] However, if platelets are hyperreactive, these factors may induce an activation state that leads to their rapid clearance by the reticuloendothelial system. In support of this, we found an increased fraction of platelets in the spleen of $Twf2a^{-/-}$ mice as compared with controls, which was independent of macrophages, since splenectomy, but not clodronate-mediated macrophage depletion, could normalize platelet counts (Figure 1C-G; supplemental Figure 6). Furthermore, we found an elevated fraction of young, reticulated, and sialylated platelets (Figure 1H-I), which have previously been implicated as a marker of increased thrombopoiesis.[35,48]

The pronounced hyperreactivity of $Twf2a^{-/-}$ platelets in vitro as well as in vivo was characterized by sustained agonist-induced recruitment of Tln to β3-integrin tails (Figures 2 and 3) that coincided with a reduced fraction of PS-exposing platelets (Figure 4A). In addition, despite the strong dependency of PS exposure on sustained Ca^{2+} signaling, we could not detect altered Ca^{2+} entry into $Twf2a^{-/-}$ platelets in response to thrombin or collagen-related peptide (supplemental Figure 11). This finding is also of particular interest with regards to the enhanced degranulation, a process strongly dependent on Ca^{2+} signaling. However, besides Ca^{2+}, the actin cytoskeleton is also critically involved in regulating platelet granule release,[49] suggesting that altered actin dynamics may account for the increased α-granule release in $Twf2a^{-/-}$ platelets.

The hyperresponsiveness of $Twf2a^{-/-}$ platelets is different from the described platelet phenotypes of mice lacking immunoreceptor

Figure 6. Disinhibition of actin assembly prevents integrin closure in $Twf2a^{-/-}$ platelets. (A-B) Resting (A) or fibrinogen-spread (B) platelets were stained for F-actin (red) and α-tubulin (green) after the indicated time periods. Images were acquired with a TCS SP8 confocal microscope (100×/1.4 STED WHITE oil objective, Leica Microsystems). Scale bars, 3 μm. Images are representative of at least 6 individuals. (C) Relative F-actin content of resting and activated platelets was determined by flow cytometry. (D) Control (dimethyl sulfoxide) or toxin-treated (5 μM jasplakinolide, 2.5 μM latrunculin A) platelets remained resting or were activated with the indicated agonists and concentrations. αIIbβ3-integrin activation (JON/A-PE) was determined by flow cytometry. (E-F) Latrunculin A–treated (2.5 μM for 10 minutes) platelets were left untreated (rest) or preincubated for 10 minutes in the presence of the calpain inhibitor MDL-28170 (200 μM). Subsequently, samples were stimulated with the Ca^{2+} ionophore A23187 (10 μM) or thrombin (0.1 U/mL) and collagen-related peptide (10 μg/mL), lysed, and processed for immunoblotting. Full-length (Ab762) and calpain-cleaved (AbΔ747) β3-integrin, as well as α-tubulin, were probed with the respective antibodies and analyzed by densitometry. (G-H) Platelets were left untreated or stimulated for the indicated time points with 0.1 U/mL thrombin, lysed, and processed for immunoblotting. Total Pfn1 (aa 126-137), phospho-Y^{129} Pfn1 (G), total n-cofilin, phospho-cofilin (H), and α-tubulin were probed with the respective antibodies and analyzed by densitometry. Values are mean ± SD (n = at least 4). A23, A23187 (Ca^{2+} ionophore); CRP/C, collagen-related peptide; MDL/M, MDL28170 (calpain inhibitor); Rhd, rhodocytin; Thr/T, thrombin; U46, U46619 (synthetic thromboxane A_2 analog). ***,###$P < .001$, **,##$P < .01$, and #,$$P < .05$, unpaired Student t test.

tyrosine-based inhibition motif signaling components such as the leukocyte-associated immunoglobulin-like receptor-1 or the associated signaling molecules, Src-like adapter proteins SLAP and SLAP2, in which platelets showed a (hem) immunoreceptor tyrosine-based activation motif (ITAM)-specific hyperreactivity.[50,51] Moreover, hyperreactive $Stim1^{+/Sax}$ platelets (due to a constitutive store-operated Ca^{2+} entry) showed selectively impaired (hem)ITAM signaling.[52] Finally, Ras GTPase-activating protein 3 mutant mice showed increased platelet activation and a markedly accelerated platelet turnover that was independent of splenic clearance.[53] Our findings on the first in vivo function of mammalian Twf2a are thus clearly distinct from previously identified negative regulators of platelet activity and highlight the central role of the actin cytoskeleton in regulating platelet function and turnover.

The precise molecular function of Twfs in vivo is still a matter of debate. Twfs were shown to inhibit barbed end growth by capping of actin filaments and pointed end growth by sequestration of G-actin monomers, as well as acceleration of filament depolymerization.[27,40] Twfs also interact with capping protein, but whether this interaction contributes to filament assembly, disassembly, or proper subcellular localization of Twfs has remained elusive.[25,27,29,31] We found increased actin polymerization in the absence of Twf2a, thus revealing for the first time an inhibitory function of mammalian Twf2a on actin assembly in vivo (Figure 6C). The increase in filamentous actin was particularly prominent at the cell cortex, coinciding with the localization of Twf2a in spread murine and human platelets (Figure 6B; supplemental Figure 1B,D).[24] Based on this, we speculate that Twf2a specifically controls actin filament assembly and disassembly in platelets to regulate the width of lamellipodia and thereby regulates integrin activation, cell adhesion, and spreading. This function appears to be specific to Twf2a, since $Twf1^{fl/fl-Pf4Cre}$ platelets showed normal platelet reactivity, cytoskeletal architecture, and dynamics (supplemental Figures 2 and 14).

In $Twf2a^{-/-}$ platelets, the lack of Twf2a's G-actin sequestering function may increase the pool of free G-actin in mutant platelets, thereby enhancing n-cofilin activity, which generates free barbed ends, favoring actin polymerization, as well as promote Pfn1- and cyclase-associated protein–mediated nucleotide exchange and actin polymerization, particularly at the cell cortex (Figure 6B-C,G-H). In line with this, we have previously observed a defective cortical F-actin meshwork in spread Pfn1-deficient platelets.[4] The accelerated spreading and lamellipodia formation of $Twf2a^{-/-}$ platelets further support this hypothesis (Figures 2E,G and 6B; supplemental Figure 9; supplemental Videos 1 and 2). In addition, the importance of Twf2a's inhibitory function on actin assembly has previously been shown in vitro by Twf2a-mediated limitation of stereocilia elongation in the cochlea, which is a prerequisite for normal audition.[54]

In agreement with the critical role of the cytoskeleton for integrin inactivation, cleavage of β3-integrin tails and hence dissociation of the integrin–cytoskeleton linkage was reduced in stimulated $Twf2a^{-/-}$ platelets even when using the A23187 ionophore (Figure 5; supplemental Figure 10).[37,55] Strikingly, toxin-induced depolymerization of the actin cytoskeleton could restore the hyperreactivity and calpain-mediated cleavage of β3-integrin tails in $Twf2a^{-/-}$ platelets similar to that of controls (Figure 6D-F; supplemental Figures 12 and 13). In addition, our results suggest normal calpain activity in $Twf2a^{-/-}$ platelets, since the cleavage of FlnA and Tln in resting and stimulated $Twf2a^{-/-}$ platelets was unaltered as compared with controls (Figure 5E-F). Moreover, chemical inhibition of calpain activity as well as genetic deletion of calpain-1 resulted in decreased platelet degranulation, aggregation, and spreading, the latter of which was

characterized by limited filopodia and lamellipodia formation, which stands in stark contrast to our findings in $Twf2a^{-/-}$ platelets.[56-58] Based on these results, we hypothesize that in the absence of $Twf2a^{-/-}$, enhanced actin dynamics via increased activity of Pfn1 and n-cofilin results in a thickened cortical cytoskeleton, which may sterically limit the accessibility of β3-integrins, hence restricting calpain-mediated cleavage of β3-integrin tails and leading to the unremitting recruitment and association of Tln. Ultimately, the resulting sustained integrin activation manifests as pronounced hyperresponsiveness of $Twf2a^{-/-}$ platelets.

In summary, our study provides the first direct in vivo evidence for an inhibitory function of mammalian Twf2a, but not the closely related Twf1 isoform, in actin dynamics, with implications for thrombopoiesis, platelet function, and turnover. These results point to a novel cytoskeleton-related cause of macrothrombocytopenia that is independent of platelet production and highlight the critical need to monitor platelet function in patients with variants in actin cytoskeleton–associated genes.

Acknowledgments

Authorship

Contribution: S.S. designed research, performed experiments, analyzed data, and wrote the manuscript; S.B., I.C.B., T.V., M.B., and A.B. performed experiments, analyzed data, and commented on the manuscript; M.H., X.D., and P.L. provided vital reagents and commented on the manuscript; K.G.H. provided technical support with imaging and commented on the manuscript; and B.N. supervised research, analyzed data, and wrote the manuscript.

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References

1. Machlus KR, Thon JN, Italiano JE Jr. Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. *Br J Haematol.* 2014; 165(2):227-236.

2. Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol.* 1999; 147(6):1299-1312.

3. Bender M, Thon JN, Ehrlicher AJ, et al. Microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein. *Blood.* 2015;125(5):860-868.

4. Bender M, Stritt S, Nurden P, et al. Megakaryocyte-specific Profilin1-deficiency alters microtubule stability and causes a Wiskott-Aldrich syndrome-like platelet defect. *Nat Commun.* 2014; 5:4746.

5. Bender M, Eckly A, Hartwig JH, et al. ADF/n-cofilin-dependent actin turnover determines platelet formation and sizing. *Blood.* 2010; 116(10):1767-1775.

6. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol.* 1992; 118(6):1421-1442.

7. Stritt S, Nurden P, Turro E, et al; BRIDGE-BPD Consortium. A gain-of-function variant in DIAPH1 causes dominant macrothrombocytopenia and hearing loss. *Blood.* 2016;127(23):2903-2914.

8. Nurden P, Debili N, Coupry I, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. *Blood.* 2011;118(22):5928-5937.

9. Thrasher AJ, Burns SO. WASP: a key immunological multitasker. *Nat Rev Immunol.* 2010;10(3):182-192.

10. Kunishima S, Okuno Y, Yoshida K, et al. ACTN1 mutations cause congenital macrothrombocytopenia. *Am J Hum Genet.* 2013; 92(3):431-438.

11. Pecci A, Klersy C, Gresele P, et al. MYH9-related disease: a novel prognostic model to predict the clinical evolution of the disease based on genotype-phenotype correlations. *Hum Mutat.* 2014;35(2):236-247.

12. Stritt S, Nurden P, Favier R, et al. Defects in TRPM7 channel function deregulate thrombopoiesis through altered cellular Mg(2+) homeostasis and cytoskeletal architecture. *Nat Commun.* 2016;7:11097.

13. Pula G, Poole AW. Critical roles for the actin cytoskeleton and cdc42 in regulating platelet integrin alpha2beta1. *Platelets.* 2008;19(3): 199-210.

14. Bennett JS, Zigmond S, Vilaire G, Cunningham ME, Bednar B. The platelet cytoskeleton regulates the affinity of the integrin alpha(IIb)beta (3) for fibrinogen. *J Biol Chem.* 1999;274(36): 25301-25307.

15. Leng L, Kashiwagi H, Ren XD, Shattil SJ. RhoA and the function of platelet integrin alphaIIbbeta3. *Blood.* 1998;91(11):4206-4215.

16. Obergfell A, Judd BA, del Pozo MA, Schwartz MA, Koretzky GA, Shattil SJ. The molecular adapter SLP-76 relays signals from platelet integrin alphaIIbbeta3 to the actin cytoskeleton. *J Biol Chem.* 2001;276(8):5916-5923.

17. Obergfell A, Eto K, Mocsai A, et al. Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol.* 2002;157(2):265-275.

18. Shcherbina A, Cooley J, Lutskiy MI, Benarafa C, Gilbert GE, Remold-O'Donnell E. WASP plays a novel role in regulating platelet responses

19. Palmgren S, Vartiainen M, Lappalainen P. Twinfilin, a molecular mailman for actin monomers. *J Cell Sci.* 2002;115(Pt 5):881-886.

20. Poukkula M, Kremneva E, Serlachius M, Lappalainen P. Actin-depolymerizing factor homology domain: a conserved fold performing diverse roles in cytoskeletal dynamics. *Cytoskeleton.* 2011;68(9):471-490.

21. Goode BL, Drubin DG, Lappalainen P. Regulation of the cortical actin cytoskeleton in budding yeast by twinfilin, a ubiquitous actin monomer-sequestering protein. *J Cell Biol.* 1998;142(3): 723-733.

22. Wahlström G, Vartiainen M, Yamamoto L, Mattila PK, Lappalainen P, Heino TI. Twinfilin is required for actin-dependent developmental processes in Drosophila. *J Cell Biol.* 2001;155(5):787-796.

23. Wang D, Zhang L, Zhao G, et al. Drosophila twinfilin is required for cell migration and synaptic endocytosis. *J Cell Sci.* 2010;123(Pt 9): 1546-1556.

24. Vartiainen MK, Sarkkinen EM, Matilainen T, Salminen M, Lappalainen P. Mammals have two twinfilin isoforms whose subcellular localizations and tissue distributions are differentially regulated. *J Biol Chem.* 2003;278(36): 34347-34355.

25. Nevalainen EM, Skwarek-Maruszewska A, Braun A, Moser M, Lappalainen P. Two biochemically distinct and tissue-specific twinfilin isoforms are generated from the mouse Twf2 gene by alternative promoter usage. *Biochem J.* 2009; 417(2):593-600.

26. Nevalainen EM, Braun A, Vartiainen MK, et al. Twinfilin-2a is dispensable for mouse development. *PLoS One.* 2011;6(8):e22894.

27. Helfer E, Nevalainen EM, Naumanen P, et al. Mammalian twinfilin sequesters ADP-G-actin and caps filament barbed ends: implications in motility. *EMBO J.* 2006;25(6):1184-1195.

28. Carlier MF, Laurent V, Santolini J, et al. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J Cell Biol.* 1997;136(6): 1307-1322.

29. Falck S, Paavilainen VO, Wear MA, Grossmann JG, Cooper JA, Lappalainen P. Biological role and structural mechanism of twinfilin-capping protein interaction. *EMBO J.* 2004;23(15):3010-3019.

30. Ojala PJ, Paavilainen VO, Vartiainen MK, Tuma R, Weeds AG, Lappalainen P. The two ADF-H domains of twinfilin play functionally distinct roles in interactions with actin monomers. *Mol Biol Cell.* 2002;13(11):3811-3821.

31. Moseley JB, Okada K, Balcer HI, Kovar DR, Pollard TD, Goode BL. Twinfilin is an actin-filament-severing protein and promotes rapid turnover of actin structures in vivo. *J Cell Sci.* 2006;119(Pt 8):1547-1557.

32. 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(4):1503-1506.

33. Stritt S, Wolf K, Lorenz V, et al. Rap1-GTP-interacting adaptor molecule (RIAM) is dispensable for platelet integrin activation and function in mice. *Blood.* 2015;125(2):219-222.

34. Rumjantseva V, Grewal PK, Wandall HH, et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat Med.* 2009; 15(11):1273-1280.

35. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic

thrombopoietin production via JAK2-STAT3 signaling. *Nat Med.* 2015;21(1):47-54.

36. Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J Thromb Haemost.* 2008; 6(8):1253-1261.

37. Mattheij NJ, Gilio K, van Kruchten R, et al. Dual mechanism of integrin αIIbβ3 closure in procoagulant platelets. *J Biol Chem.* 2013; 288(19):13325-13336.

38. Munnix IC, Kuijpers MJ, Auger J, et al. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol.* 2007; 27(11):2484-2490.

39. Palmgren S, Ojala PJ, Wear MA, Cooper JA, Lappalainen P. Interactions with PIP2, ADP-actin monomers, and capping protein regulate the activity and localization of yeast twinfilin. *J Cell Biol.* 2001;155(2):251-260.

40. Johnston AB, Collins A, Goode BL. High-speed depolymerization at actin filament ends jointly catalysed by Twinfilin and Srv2/CAP. *Nat Cell Biol.* 2015;17(11):1504-1511.

41. Fan Y, Arif A, Gong Y, et al. Stimulus-dependent phosphorylation of profilin-1 in angiogenesis. *Nat Cell Biol.* 2012;14(10):1046-1056.

42. Ghosh M, Song X, Mouneimne G, Sidani M, Lawrence DS, Condeelis JS. Cofilin promotes actin polymerization and defines the direction of cell motility. *Science.* 2004;304(5671):743-746.

43. Wei JH, Haddad A, Wu KJ, et al. A CpG-methylation-based assay to predict survival in clear cell renal cell carcinoma. *Nat Commun.* 2015;6:8699.

44. Samaeekia R, Adorno-Cruz V, Bockhorn J, et al. miR-206 Inhibits Stemness and Metastasis of Breast Cancer by Targeting MKL1/IL11 Pathway. *Clin Cancer Res.* 2017;23(4):1091-1103.

45. Bockhorn J, Dalton R, Nwachukwu C, et al. MicroRNA-30c inhibits human breast tumour chemotherapy resistance by regulating TWF1 and IL-11. *Nat Commun.* 2013;4:1393.

46. Li Q, Song XW, Zou J, et al. Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. *J Cell Sci.* 2010;123(Pt 14): 2444-2452.

47. Shankaran H, Alexandridis P, Neelamegham S. Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension. *Blood.* 2003; 101(7):2637-2645.

48. Harrison P, Robinson MS, Mackie IJ, Machin SJ. Reticulated platelets. *Platelets.* 1997;8(6): 379-383.

49. Flaumenhaft R, Dilks JR, Rozenvayn N, Monahan-Earley RA, Feng D, Dvorak AM. The actin cytoskeleton differentially regulates platelet alpha-granule and dense-granule secretion. *Blood.* 2005;105(10):3879-3887.

50. Smith CW, Thomas SG, Raslan Z, et al. mice lacking the inhibitory collagen receptor LAIR-1 exhibit a mild thrombocytosis and hyperactive platelets. *Arterioscler Thromb Vasc Biol.* 2017; 37(5):823-835.

51. Cherpokova D, Bender M, Morowski M, et al. SLAP/SLAP2 prevent excessive platelet (hem) ITAM signaling in thrombosis and ischemic stroke in mice. *Blood.* 2015;125(1):185-194.

52. Grosse J, Braun A, Varga-Szabo D, et al. An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. *J Clin Invest.* 2007;117(11):3540-3550.

53. 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.

54. Peng AW, Belyantseva IA, Hsu PD, Friedman TB, Heller S. Twinfilin 2 regulates actin filament lengths in cochlear stereocilia. *J Neurosci*. 2009; 29(48):15083-15088.

55. Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH. Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. *J Biol Chem*. 1995;270(44): 26146-26151.

56. Croce K, Flaumenhaft R, Rivers M, et al. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem*. 1999;274(51): 36321-36327.

57. Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol*. 2001;21(6): 2213-2220.

58. Schoenwaelder SM, Yuan Y, Cooray P, Salem HH, Jackson SP. Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin alphaIIbbeta3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. *J Biol Chem*. 1997;272(3):1694-1702.