Direct Interaction of Kindlin-3 With Integrin αIIbβ3 in Platelets Is Required for Supporting Arterial Thrombosis in Mice

Zhen Xu, Xue Chen, Huiying Zhi, Juan Gao, Katarzyna Bialkowska, Tatiana V. Byzova, Elzbieta Pluskota, Gilbert C. White, Junling Liu, Edward F. Plow, Yan-Qing Ma

Objective—Kindlin-3 is a critical supporter of integrin function in platelets. Lack of expression of kindlin-3 protein in patients impairs integrin αIIbβ3–mediated platelet aggregation. Although kindlin-3 has been categorized as an integrin-binding partner, the functional significance of the direct interaction of kindlin-3 with integrin αIIbβ3 in platelets has not been established. Here, we evaluated the significance of the binding of kindlin-3 to integrin αIIbβ3 in platelets in supporting integrin αIIbβ3–mediated platelet functions.

Approach and Results—We generated a strain of kindlin-3 knockin (K3KI) mice that express a kindlin-3 mutant that carries an integrin-interaction defective substitution. K3KI mice could survive normally and express integrin αIIbβ3 on platelets similar to their wild-type counterparts. Functional analysis revealed that K3KI mice exhibited defective platelet function, including impaired integrin αIIbβ3 activation, suppressed platelet spreading and platelet aggregation, prolonged tail bleeding time, and absence of platelet-mediated clot retraction. In addition, whole blood drawn from K3KI mice showed resistance to in vitro thrombus formation and, as a consequence, K3KI mice were protected from in vivo arterial thrombosis.

Conclusions—These observations demonstrate that the direct binding of kindlin-3 to integrin αIIbβ3 is involved in supporting integrin αIIbβ3 activation and integrin αIIbβ3-dependent responses of platelets and consequently contributes significantly to arterial thrombus formation. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: blood platelets ■ kindlin-3 protein, mouse ■ thrombosis

Integrin αIIbβ3 on platelets plays a major role in vascular homeostasis by mediating platelet aggregation.1,2 As expressed on circulating platelets, the integrin αIIbβ3 receptors exist in a quiescent state where they exhibit low affinity for their cognate ligands. However, on encounter with stimulatory agonists, the integrin receptors undergo a transformation to a state in which they can orchestrate productive functional responses, including formation of platelet aggregates leading to arterial thrombus formation. Such integrin activation depends on 2 intracellular proteins: talin and kindlin.3–6

Talin is involved in integrin activation by virtue of its binding to the cytoplasmic tail (CT) of the integrin β subunit.7 In the absence of agonists, talin exists in the cytosol in an autoinhibited state in which its rod domain interacts with and shields the integrin-binding site within the F3 subdomain of the talin head domain.8,9 This autoinhibition is relieved by multiple mechanisms that encourage talin to interact with membrane and the integrin β CT, thereby disrupting the integrin α/β CT complex.10 The kindlins, consisting of 3 members in mammals (kindlin-1, kindlin-2, and kindlin-3), are also capable of binding to the integrin β CT through their own F3 subdomains, but they bind primarily to a different site than talin.11–13 Although the essential role of kindlins in supporting integrin activation has been established by in vivo deficits in integrin function associated with their deficiencies,14,15 their mechanism of action is unclear. Kindlins do not dissociate the integrin α/β CT complex and do not enhance the capacity of talin to dissociate the CT complex,16 events that are necessary for integrin activation.

Kindlin-3 is preferentially expressed in and particularly important for the function of integrins on hematopoietic cells.13,17 Deficiency of kindlin-3 expression in humans causes type III leukocyte adhesion deficiency (LAD-III), which is associated with an inability to activate integrins on platelets and leukocytes and manifests as susceptibility to bleeding and infections.18–21 To date, multiple distinct mutations in
kindlin-3 gene have been identified in patients with LAD-III, which all lead to the absence of kindlin-3 protein expression in blood cells, but the severity of symptoms in the patients has been variable.\textsuperscript{22,23} For example, only 1 patient with LAD-III was reported to have abnormally shaped red blood cells.\textsuperscript{24} Genetically modified mice with deficiency of kindlin-3 have been described and do exhibit the defects in platelet and leukocyte functions observed in patients with LAD-III\textsuperscript{11,25} that were attributable to an inability to activate multiple integrin subclasses. However, these mice only survived 1 week,\textsuperscript{26} and, although their erythrocytes were misshaped, their shape was unlike that seen in the 1 human patient.\textsuperscript{24,26} Also, the kindlin-3 knockout mice displayed altered expression of multiple genes in hematopoietic cells,\textsuperscript{26} including integrins, suggesting that the integrin dysfunction in kindlin-3-deficient mice might arise from effects unrelated from the direct interaction of kindlin-3 with integrin.

For kindlin-1 and kindlin-2, reconstitution experiments have clearly shown that the integrin-binding site in their F3 subdomain is important for integrin activation in model cells.\textsuperscript{12,27} Even though kindlin-3 is similar to the other 2 family members, to assume a similar mechanism of action might be premature. A recent finding showed that ADAP, an adaptor protein restricted to hematopoietic cells, recruits talin and kindlin-3 to integrin \( \alpha I b \beta 3 \) in platelets and raises the possibility that kindlin-3 activates integrin independent of direct binding.\textsuperscript{28} Adding further to this uncertainty are the recent observations that kindlin-3 had no effect on integrin activation in nanodiscs in which talin head induced a measurable effect\textsuperscript{29} and the in vivo observation that kindlin-2 could exert integrin-independent functions.\textsuperscript{30} These findings bring into question the premise that direct interaction of kindlin-3 and integrin is essential for its regulation of hematopoietic cell responses that are blunted in kindlin-3 deficient mice and human patients.

In this study, we have generated kindlin-3 gene knockin (K3KI) mice that carry a mutation that disrupts the interaction of kindlin-3 with integrin \( \alpha I b \beta 3 \). Using this animal model, we demonstrate that the direct contact between kindlin-3 and integrin \( \alpha I b \beta 3 \) is indeed required to support integrin function in platelets in arterial thrombosis.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Identification of an Integrin Interaction–Defective Substitution in Kindlin-3 protein**

The inability of kindlin-3 to influence \( \alpha I b \beta 3 \) activation in nanodiscs\textsuperscript{30}; the ability of kindlin-3 to be recruited to integrin together with talin by adaptor proteins\textsuperscript{30}; and the effects of kindlin-3 deficiency on integrin and many other protein expression levels in mice\textsuperscript{26} are among the data that bring into question the role of the direct interaction between kindlin-3 and integrins in controlling the responses of hematopoietic cells. These uncertainties prompted us to test initially whether kindlin-3 does indeed share the same integrin-binding properties as kindlin-1 and kindlin-2. A double-substitution (QW/AA) in the F3 subdomains of kindlin-1 and kindlin-2 has been shown to disrupt the interaction of integrin \( \beta 3 \) CTs with kindlin-1 and kindlin-2.\textsuperscript{27} The corresponding substitutions were introduced into kindlin-3 (Figure 1A). Using a previously established FACS-based protein–peptide interaction assay,\textsuperscript{31} we attached EGFP-kindlin-3 and EGFP-kindlin-3 mutant from transfected cell lysates to microbeads and measured their interaction with the biotinylated integrin \( \beta 3 \) CT.
peptides by flow cytometry. To restrict the involvement of any unrelated proteins in this assay, the EGFP-kindlin-3–coupled microbeads were extensively washed and no unexpected protein band was detected after SDS-PAGE and coomassie blue staining (data not shown). As shown in Figure 1B, when compared with EGFP control, EGFP-kindlin-3 on the microbeads displayed a significant interaction with the integrin β3 CT peptides. However, kindlin-3 with the QW/AA substitution exhibited a dramatically reduced interaction with the integrin β3 CT peptides. These results demonstrate that the QW/AA substitution in kindlin-3 significantly perturbs the interaction between kindlin-3 and integrin β3 CT. To consider whether the QW/AA substitution affected the structural stability of the kindlin-3 protein, EGFP-kindlin-3 and the QW/AA mutant expressed in CHO cells were loaded on a native gel and their mobilities on nondenaturing gels were assessed by Western blotting. As shown in Figure 1C, kindlin-3 protein and the QW/AA mutant migrated identically, suggesting that the kindlin-3 mutant exhibits a similar structural stability with its wild-type (WT) counterpart. We also noted that the expression levels of EGFP-kindlin-3 and the EGFP-fused QW/AA mutant form were similar in several mammalian cell lines, HEL, K562 and RAW cells (data not shown), which is also consistent with similar folding of the 2 kindlin-3 molecules. Therefore, our results suggest that kindlin-3 shares the same recognition mechanism as kindlin-1 and kindlin-2 where binding to β3 CT and the QW/AA substitutions can be used to perturb the interaction between kindlins and integrin specifically.

**Generation of K3KI Mice That Carry the QW/AA Substitution**

To evaluate the functional significance of the direct interaction between kindlin-3 and integrin in vivo, a K3KI allele in which a double-mutation (QW/AA) was introduced into the kindlin-3 gene locus (Figure 2A and 2B) was used to generate K3KI mice. K3KI mice were born in expected Mendelian ratios and were fertile. Significantly, K3KI mice showed normal survival (mice of >6 months with no overt signs of ill-health), which is different from kindlin-3−/− mice, which only survive 1 week postnatally.13 Spontaneous bleeding was not observed in K3KI mice, and platelet counts in peripheral blood of K3KI mice were comparable with those in WT mice (Figure 2C). As with kindlin-3−/− mice,25 K3KI mice exhibited significant leukocytosis (Figure 2D). In contrast to the kindlin-3 knockout mice, which were severely anemic,25 red blood cells in peripheral blood of K3KI mice were only slightly suppressed (Figure 2E). We did note some acanthocytes in blood smears of some of the K3KI mice as was reported in the kindlin-3−/− mice,26 but this varied among the individual K3KI mice and is being further explored.

We examined the expression of integrin αIIbβ3 on platelets by flow cytometry and found that integrin αIIbβ3 expression levels were identical on WT and K3KI platelets (Figure 2F). The expression of kindlin-3 protein in washed platelets from WT and K3KI mice was similar as assessed by Western blots (Figure 2G). In addition, 2 other proteins with important roles in integrin activation, talin, and Rap1 (Rap1a+Rap1b) were also expressed at similar levels in K3KI and WT platelets (Figure 2G). Significantly, the results of communoprecipitation experiments showed that integrin αIIbβ3 markedly reduced its association with the kindlin-3 mutant in K3KI platelets (≈85% reduction) when compared with the association between integrin αIIbβ3 and kindlin-3 in WT platelets under the same conditions (Figure 2H). Thus, we successfully generated a strain of mice expressing a kindlin-3 mutant with a significant reduction in integrin-binding activity, which can

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**Figure 2.** Generation of kindlin-3 gene knockin (K3KI) mice that carry the QW/AA mutation. A, A diagram illustrating the gene-targeting strategy for generating K3KI mice. The numbered rectangles represent kindlin-3 gene exons and the mutated nucleotides (CAATGG to GCCGCC) locate in exon14 (*). Neo represents the neomycin cassette, which was used for selecting positive ES cell clones. The 5′ and 3′ arms for homologous recombination are highlighted. B, Targeted genomic DNA fragment was amplified by polymerase chain reaction from the selected ES clones and sequenced, and the mutated sites in the kindlin-3 gene were verified. C, Platelet counts in peripheral blood of WT and K3KI mice were measured, and no significant difference was found between them. D, The counts of leukocytes in peripheral blood of K3KI mice were significantly elevated when compared with wild-type (WT) mice (*P<0.01). E, Red blood cell counts in peripheral blood of WT and K3KI mice (*P<0.05). F, Cell surface expression of integrin αIIbβ3 on K3KI platelets and WT counterparts was measured by flow cytometry using a PE-conjugated antimouse CD41 antibody. G, Washed platelets from WT and K3KI mice were lysed, and the expression of kindlin-3 (Kind-3), talin, and Rap1 (Rap1a and Rap1b) proteins in platelets was evaluated by Western blotting. Actin was also measured as a loading control. H, Integrin αIIbβ3 was immunoprecipitated from the platelet lysates, and kindlin-3 and integrin β3 in the precipitates were measured by Western blotting.
be used to evaluate the functional significance of the interaction between kindlin-3 and integrin in hematopoietic cells.

**Interaction Between Kindlin-3 and Integrin αIIbβ3 in Platelets Facilitates αIIbβ3 Activation and Is Essential for αIIbβ3-Mediated Platelet Spreading**

To evaluate whether the direct interaction between the kindlin-3 QW sequence and integrin affects hemostasis, tail bleeding assays were performed on WT and K3KI mice. After a small portion of tail-tip was removed, the bleeding time was measured. The results revealed that K3KI mice displayed a significantly prolonged bleeding time when compared with their WT counterparts (Figure 3A). In WT mice, the mean bleeding time was 211±126 s (n=8), whereas the bleeding time of all K3KI mice (n=8) exceeded 600 s. Thus, the QW/AA substitution in kindlin-3 led to a severe hemostatic defect in K3KI mice. The extended bleeding time from K3KI mice led us to analyze integrin αIIbβ3 signaling in K3KI platelets. Integrin inside-out signaling was evaluated by agonist-induced soluble fibrinogen binding to αIIbβ3 on platelets. As shown in Figure 3B, integrin αIIbβ3 activation on K3KI platelets was completely inhibited on stimulation with ADP (20 μmol/L) and partially but significantly inhibited in response to PAR-4 agonist peptide (AYPGKF, 150 μmol/L) or collagen-related peptide (2 μg/mL). As assessed by flow cytometry, the inhibition was ≈50% in mean fluorescence intensity. The residual fibrinogen binding to K3KI platelets in the presence of PAR-4 agonist peptide or collagen-related peptide suggests that K3KI platelets still retain some ability to support integrin αIIbβ3 inside-out signaling in response to strong stimuli.

Next, we measured the spreading of K3KI platelets on immobilized fibrinogen and found that integrin αIIbβ3-mediated K3KI platelet spreading was significantly compromised (Figure 3C), demonstrating that interaction with kindlin-3 is essential for integrin αIIbβ3 to mediate outside-in signaling. In spite of the impaired bidirectional signaling of integrin αIIbβ3 on K3KI platelets, degranulation was unaffected; P-selectin translocation to the platelet surface on WT

![Figure 3](http://atvb.ahajournals.org/DownloadedFrom/826619)
and K3KI platelets in response to stimulation with PAR-4 agonist peptide or collagen-related peptide was identical (data not shown). In addition, the fibrinogen levels in WT and K3KI platelets were similar as evaluated by Western blots (data not shown).

**Interaction Between Kindlin-3 and Integrin αIIbβ3 in Platelets Supports Platelet Aggregation and Clot Retraction**

Next, ex vivo platelet aggregation studies were performed in response to different agonists. As shown in Figure 3D, K3KI platelets showed negligible aggregation in response to ADP and only a weak response to U46619 when compared with WT platelets. Notably, partial aggregation of K3KI platelets was observed in response to collagen and thrombin. The capacity of K3KI platelets to retain an ability to aggregate in response to higher doses of strong agonists differs from previous observations on kindlin-3−/− platelets, which had negligible responses to all agonists tested.

We also evaluated clot retraction induced by platelets from WT and K3KI mice as a response driven by αIIbβ3-mediated outside-in signaling. Using high doses of thrombin, sufficient to induce aggregation, K3KI platelets failed to support clot retraction (Figure 3E), whereas WT platelets retracted well. Taken together, these findings demonstrate that the QW sequence-mediated interaction of kindlin-3 with integrin αIIbβ3 on platelets is important for supporting platelet aggregation and essential for clot retraction.

**Interaction Between Kindlin-3 and Integrin Is Essential for Thrombus Formation In Vivo**

To evaluate the functional significance of the direct interaction between kindlin-3 and integrin in platelets further, an in vitro thrombus formation assay was performed under flow conditions using a whole blood microfluidic perfusion system as previously described. Platelets in whole blood were labeled with mepacrine and allowed to flow over a collagen-coated surface at 80 dynes/cm². The adhesion and aggregation of platelets were visualized as the accumulation of fluorescence on the collagen-coated surface. As shown in Figure 3A, platelet adhesion was markedly diminished and thrombus formation was severely hampered in blood from K3KI mice in contrast with the strong signal for both platelet adhesion and thrombus formation obtained with blood from WT mice at the same conditions. This experiment was repeated twice and similar results were obtained. The calculated percentage of surface fluorescence coverage or total integrated fluorescence intensity per square micrometer area at different time points was increased ≈30-fold for blood from WT mice when compared with that from K3KI mice, suggesting that a direct interaction between kindlin-3 and integrin in platelets is essential for efficient platelet-mediated thrombus formation in this in vitro assay.

**Crosstalk Between Kindlin-3 and Integrin in Platelets Is Essential for Arterial Thrombus Formation In Vivo**

The function of K3KI platelets in vivo was measured in a FeCl₃-induced arterial thrombosis model. A transit-time thrombus formation assay was performed under flow conditions. This experiment was repeated twice and similar results were obtained. The calculated percentage of surface fluorescence coverage or total integrated fluorescence intensity per square micrometer area at different time points was increased ≈30-fold for blood from WT mice when compared with that from K3KI mice, suggesting that a direct interaction between kindlin-3 and integrin in platelets is essential for efficient platelet-mediated thrombus formation in this in vitro assay.

**Discussion**

The pathogenesis of human LAD-III has been attributed to the absence of kindlin-3 protein expression in hematopoietic cells because of heritable mutations in kindlin-3 gene in patients. It has been well documented that kindlin-3 is required to support integrin activation in hematopoietic cells. However, the molecular mechanisms involved are still poorly understood. Proteomic analysis disclosed that the expression levels of multiple proteins in kindlin-3−/− deficient murine cells could be either up or downregulated, bringing some uncertainty to the cause of integrin dysfunction in hematopoietic cells. Although kindlin-3 has been categorized as an integrin-binding protein, the functional significance of the direct interaction between kindlin-3 and integrin αIIbβ3 in platelets still remains unknown. By mutagenesis, it has been demonstrated that the kindlin-3–binding sites in the integrin β3 CT (NITY759 motif) is critical for supporting integrin αIIbβ3 activation. However, the NITY759 motif in the β3 CT is in a region that can...
mediate interaction with multiple proteins, so that mutations in this region could possibly produce off-target effects. In our present study, we generated a K3KI mouse model harboring a QW/AA substitution in kindlin-3 protein to disconnect kindlin-3 from integrins in hematopoietic cells. The value of the K3KI mouse model include (1) K3KI mice have normal expression levels of kindlin-3 protein in platelets, which limits possible indirect effects resulting from the absence of kindlin-3 protein; (2) Theoretically, the QW/AA substitution in kindlin-3 protein should be able to disconnect kindlin-3 from multiple integrin members in hematopoietic cells, but these limited and specific point mutations should not perturb nonintegrin-dependent kindlin functions; (3) The interaction of integrin with other integrin CT-binding partners also should not be directly affected in K3KI cells. These features suggest that K3KI mouse provides a unique opportunity to evaluate the integrin-kindlin-3 signaling in vivo. Using this model, we demonstrate that the direct interaction between kindlin-3 and integrin αIIbβ3 in platelets is essential for supporting certain platelet function and arterial thrombus formation, which could not be concluded from the previous studies on patients with both LAD-III and kindlin-3 null mice, thus representing an important step forward in our understanding kindlin-3 functions in platelets.

Unlike kindlin-3-deficient platelets, which display minimal integrin αIIbβ3 activation and aggregation in response to all agonists, we found that high concentrations of agonists, such as thrombin and collagen still, could induce significant integrin αIIbβ3 activation (Figure 3B) and aggregation of K3KI platelets (Figure 3D). Indeed, at a higher thrombin concentration, the extent of aggregation of K3KI platelets nearly approached that of WT platelets. Possible interpretations include (1) the interaction of the kindlin-3 QW/AA mutant with integrins in K3KI platelets is compromised but not eliminated; a weak interaction could still occur, which might be sufficient, together with talin, to induce platelet aggregation in the face of a strong stimulus; (2) the kindlin-3 QW/AA mutant might use a mechanism involving the third molecule, such as ADAP, or a signaling pathway to support integrin function, which is independent of the direct interaction between kindlin-3 and integrins and which is operative at high doses of strong agonists. Additional studies will be required to address these possibilities. Nonetheless, in vivo platelet aggregation in K3KI mice under pathological conditions was significantly suppressed, suggesting that the ex vivo K3KI platelet aggregation under strong stimulations may not have the opportunity to occur in vivo.

Although significant ex vivo platelet aggregation could be induced by thrombin (0.1–0.2 U/mL) for K3KI platelets (Figure 3D), the aggregates formed at a high thrombin concentration (0.8 U/mL) failed to support K3KI platelet-mediated clot retraction (Figure 3E), raising a possibility that kindlin-3 may use distinct mechanisms in supporting integrin αIIbβ3-mediated platelet aggregation (inside-out signaling dependent) and clot retraction (outside-in signaling dependent). The profound effect of kindlin-3 QW/AA mutation on platelet spreading is consistent with the major importance of kindlin-3 in outside-in signaling across integrin αIIbβ3.

Hypothetically, the absence of or a weak interaction between integrin αIIbβ3 and kindlin-3 in K3KI platelets may fail to sustain the high mechanical forces between platelet integrin αIIbβ3 and fibrinogen/fibrin, thus leading to clot retraction defects for K3KI platelets. Alternatively, kindlin-3, such as talin, may play distinct roles in orchestrating different integrin-dependent functions.

In summary, our findings in this study demonstrate the importance of the direct interaction between kindlin-3 and integrin αIIbβ3 in platelets for supporting integrin αIIbβ3-mediated platelet responses and arterial thrombosis, thus furthering our understanding of the molecular regulation of kindlin-3 signaling in platelets.

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Disclosures
None.

References

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The direct interaction of kindlin-3 with integrin αIIbβ3 in platelets is a key conduit in arterial thrombus formation and represents a potential therapeutic target for developing novel antithrombotic strategies.
Direct Interaction of Kindlin-3 With Integrin αIIbβ3 in Platelets Is Required for Supporting Arterial Thrombosis in Mice

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Materials and Methods

Generation of kindlin-3 knock-in (K3KI) mice

K3KI mice were generated by homologous recombinant technology. A BAC fragment containing a partial mouse kindlin-3 gene was introduced into a cloning vector. The target nucleotides CAATGG in exon 14 of the mouse kindlin-3 gene were mutated into GCCGCC by site-directed mutagenesis, which led to a substitution of Q:\textsuperscript{597}W:\textsuperscript{598}/AA in kindlin-3 protein. A neomycin cassette (Neo\textsuperscript{r}) was inserted into intron 13 of the mouse kindlin-3 gene. The fragment of kindlin-3 gene including 5' and 3' arms and the inserted Neo\textsuperscript{r} cassette were fully sequenced to ensure optimal targeting. Subsequently, the vector was electroporated into ES cells. The positive Neo\textsuperscript{r} clones were first selected with G418 and screened for correct gene targeting by PCR analysis. In addition, the specific integration of targeted DNA fragment was further verified by Southern blot and quantitative PCR with a probe for the inserted Neo\textsuperscript{r} gene. Correctly targeted ES cells were used to inject C57BL6 blastocysts, which were then implanted into pseudopregnant females. Chimeric male founders were mated with wild type C57BL/6 mice. Genotyped progeny that were positive for germline transmission were bred to generate K3KI mice.

Complete blood count and red blood cell morphology

Mouse blood samples were collected into heparinized tubes (BD Microtainer) and analyzed on an automated hematology analyzer (Sysmex XT-2000i). The results were presented as mean ± SD for \( n = 4-5 \) mice.

Platelet integrin αIIbβ3 expression and activation

To measure integrin αIIbβ3 expression on platelets, the washed platelets from WT and K3KI mice were incubated with a PE-conjugated anti-CD41 antibody (BD Biosciences, San Jose, CA). To measure platelet integrin αIIbβ3 activation, the washed platelets were incubated with soluble fibrinogen conjugated with Alexa Fluor 647 (Life
Technologies, Grand Island, NY) in the presence or absence of the indicated agonists. After 30 min at 22 °C without stirring, platelets were directly fixed using an equal volume of 4% paraformaldehyde and then washed twice with PBS. Binding of anti-CD41 antibody or soluble fibrinogen to platelets were analyzed on a BD LSRII flow cytometer.

**Platelet spreading and aggregation**

The washed platelets were allowed to incubate with immobilized fibrinogen on a chamber slide (Lab-Tek™ II) for 120 min at 37 °C. After incubation, the adhered platelets were fixed with 4% paraformaldehyde and permeabilized with 0.2% triton X-100 solution. To observe platelet spreading, the adherent platelets were stained with Rhodamine-conjugated phalloidin and the images were captured under a fluorescence microscope (100× objective). The spreading areas of adherent platelets were quantified using Metamorph software (Universal Imaging, Downingtown, PA). For measuring platelet aggregation, the washed platelets were analyzed on a whole-blood lumia-aggregometer (Chrono-log, Havertown, PA) in the presence of the indicated agonists.

**Tail-bleeding assay**

Mice were anesthetized and their tails were pre-warmed in sterile PBS buffer (37°C) for 5 min. A short portion of tail tip (~3 mm) was amputated and the tail was immediately returned into the warm buffer. Bleeding time was calculated from the start of bleeding to the first cessation of the bleeding. The time point for terminating the prolonged bleeding was 10 min and then the wounded tails was cauterized to stop the bleeding.

**FeCl₃ injury carotid artery thrombosis model**

Mice were anesthetized and the common carotid arteries were exposed and detached from surrounding tissues. A microvascular flow probe attached to a transit-time perivascular flowmeter (Transonic System Inc., New York) was positioned on the common carotid artery to monitor blood flow. Vascular injury was induced by applying a 1 × 2 mm piece of Whatmann filter paper saturated with 10% FeCl₃ to the top of the
vessel for 3 min. The blood flow was recorded immediately after removing the filter paper and the first occlusion was defined by blood flow less than 0.05 ml/min.

**In vitro thrombus formation under flow**

*In vitro* thrombus formation was performed by a whole-blood perfusion assay over fibrillar collagen as described previously. In brief, Vena8 FLUORO+ Biochips (Cellix Ltd, Dublin, Ireland) were coated with collagen (50μg/ml) overnight at 4°C and then postcoated with 0.1% BSA in HBSS buffer. Whole blood from WT and K3KI mice was collected from the inferior vena cava in the presence of heparin and PPACK as anticoagulants and then labeled with mepacrine (CalBiochem, La Jolla, CA). Afterwards, the labeled whole blood was perfused into collagen-coated microchannels at a flow rate of 80 dyne/cm². Platelet adhesion and aggregation were observed under an epifluorescence microscope and real-time images of platelet adhesion and thrombus formation were recorded at a frame rate of one frame per second.

**Clot retraction**

For clot retraction, human platelet-depleted plasma was mixed with the washed platelets from WT and K3KI mice that were adjusted to a final concentration of 4 × 10⁹/ml. Clot retraction was induced by thrombin at different concentrations. Each clot was allowed to retract at 37°C for 120 min and then photographed.

**Flow cytometry-based protein-peptide binding assays**

EGFP-kindlin-3 or empty EGFP vector were transiently transfected into CHO cells. The expressed EGFP-kindlin-3 or EGFP proteins in the cell lysates were enriched onto protein-G conjugated microbeads (Spherotech, Lake Forest, IL) via an anti-GFP antibody (Sigma-Aldrich Co. LLC). The amount of EGFP-kindlin-3 protein loaded on the microbeads was evaluated by both Western blotting and flow cytometry. Then, the EGFP-fusion loaded microbeads were incubated with biotinylated full length β₃ CT
peptides followed by probing with Alexa Fluor 633 conjugated streptavidin. The binding of Alexa Fluor 633 to the EGFP-fusions on the microbeads were analyzed on a BD LSRII Flow Cytometer to evaluate the interaction of the β₃ CT peptide with kindlin-3 protein.

**Immunoprecipitation, pull-down assays and Western blot analysis**

Washed platelets from WT and K3KI mice were lysed by Cellytic cell lysis reagent (Sigma-Aldrich Co. LLC). To test the interaction of integrin αIIBβ3 and kindlin-3 in platelets, an anti-CD41 antibody (BD Biosciences, San Jose, CA) was used to immunoprecipitate integrin αIIBβ3 from cell lysates. Kindlin-3 protein in the precipitates was measured by SDS-PAGE and Western blotting.

To evaluate if the QW/AA substitution affects the structural stability of kindlin-3 protein, native PAGE was performed. In brief, EGFP-kindlin-3 protein and the QW/AA mutant expressed in CHO cells were subjected to electrophoresis using a Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA) under non-reducing and non-denaturing conditions without SDS in the sample buffer and running buffer. After transfer, the migration of mutant and WT kindlin-3 in the native PAGE was measured by Western blotting.

**Statistical analysis**

Statistical comparisons with the Student t test (*P < 0.05; **P < 0.01) were performed using microsoft Excel software.

Reference List