Essential role of zyxin in platelet biogenesis and glycoprotein Ib-IX surface expression

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Platelets, the central regulator of thrombosis and hemostasis, are generated from the cytoplasm of megakaryocytes (MKs) via actin cytoskeleton reorganization. Zyxin is a focal adhesion protein and wildly expressed in eukaryotes to regulate actin remodeling. Zyxin is upregulated during megakaryocytic differentiation; however, the role of zyxin in thrombopoiesis is unknown. Here we show that zyxin ablation results in profound macrothrombocytopenia. Platelet lifespan and thrombopoietin level were comparable between wild-type and zyxin-deficient mice, but MK maturation, demarcation membrane system formation, and proplatelet generation were obviously impaired in the absence of zyxin. Differential proteomic analysis of proteins associated with macrothrombocytopenia revealed that glycoprotein (GP) Ib-IX was significantly reduced in zyxin-deficient platelets. Moreover, GPIb-IX surface level was decreased in zyxin-deficient MKs. Knockdown of zyxin in a human megakaryocytic cell line resulted in GPIbα degradation by lysosomes leading to the reduction of GPIb-IX surface level. We further found that zyxin was colocalized with vasodilator-stimulated phosphoprotein (VASP), and loss of zyxin caused diffuse distribution of VASP and actin cytoskeleton disorganization in both platelets and MKs. Reconstitution of zyxin with VASP binding site in zyxin-deficient hematopoietic progenitor cell-derived MKs restored GPIb-IX surface expression and proplatelet generation. Taken together, our findings identify zyxin as a regulator of platelet biogenesis and GPIb-IX surface expression through VASP-mediated cytoskeleton reorganization, suggesting possible pathogenesis of macrothrombocytopenia.

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INTRODUCTION
Platelets, the central regulator of thrombosis and hemostasis, are generated from the cytoplasm of megakaryocytes (MKs). In bone marrow (BM), hematopoietic stem cells differentiate into MKs in response to thrombopoietin (TPO) [1]. Immature MKs undergo endomitosis to increase their size and ploidy and develop a demarcation membrane system (DMS) that forms the plasma membrane of future platelets [2]. In the meantime, MK progenitors migrate from BM osteoblastic niche to the vascular niche [3, 4], in which proplatelets are formed and released from the matured MKs into the bloodstream. Finally, platelets are shed from proplatelets in the bloodstream.

The actin cytoskeleton plays an important role in platelet biogenesis [5]. Actin filaments localizing around the DMS network generate mechanical forces to initiate and mediate DMS formation [6]. Actin cytoskeleton reorganization guides the bending and bifurcating of the proplatelet shafts to increase the number of proplatelet ends [7]. Therefore, human mutations and gene-targeted mice deficient in the components of the actin cytoskeleton, such as non-muscle myosin heavy chain IIA (NMMHC-IIA), α-actinin, filamin A, tropomyosin 4, diaphanous-related formin 1 (DIAPH1), and tropomodulin 3, have shown thrombocytopenia due to abnormal platelet generation [8–13]. However, the molecular mechanisms underlying the complex process still remain elusive.

Zyxin is a focal adhesion protein wildly expressed in tissues [14]. Zyxin regulates actin cytoskeleton remodeling by providing docking sites for actin regulatory proteins [15–18]. The C-terminal of zyxin contains three LIM domains, which are essential for its localization to focal adhesions [18]. The N-terminus of zyxin contains the binding sites for actin filament crosslinker α-actinin [19], and displays the four proline-rich ActA repeats that can interact with the actin assembly modulator enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) proteins [20]. Mutations in α-actinin cause macrothrombocytopenia by inducing multiple defects of proplatelet formation [9]. We recently demonstrated that zyxin interacts with NMMHC-IIA [21], whose coding gene MYH9 has the most macrothrombocytopenia-causing mutations [8]. Zyxin has been shown to express in platelets [14] and was found to be upregulated during megakaryocytic differentiation [22], whereas it is unclear whether zyxin plays a role in platelet biogenesis.
In the present study, we show that zyxin-deficient mice (Zyx−/−) display macrothrombocytopenia. We further demonstrate that zyxin, through mediating VASP subcellular localization, regulates actin cytoskeleton organization, GPIb-IX expression, and proplatelet generation.

**RESULTS**

**Zyx−/− mice display macrothrombocytopenia**

Zyxin has been demonstrated to express in both human and murine platelets [14, 23]. In order to investigate the role of zyxin in platelet biogenesis, we first performed a hematologic analysis in Zyx−/− mice. Interestingly, we found that Zyx−/− mice displayed severe macrothrombocytopenia, with a 65% reduction of platelet count and a 68% increase of platelet volume compared with wild-type (WT) mice (Fig. 1A, B). The Zyx−/− platelets presented normal shape, but obviously enlarged size (Fig. 1C, D). In contrast, the number and morphology of leukocytes and red blood cells were not altered in Zyx−/− mice (Table S1).

To investigate whether the thrombocytopenia is due to the enhanced platelet turnover, platelet lifespan was measured in Zyx−/− mice. We found that platelet lifespan was not obviously altered in Zyx−/− mice (t1/2 = 49 h for WT vs t1/2 = 51 h for Zyx−/− mice) (Fig. 1E). In addition, the percentage of reticulated platelets was not altered, while the reticulated platelet number was markedly reduced in Zyx−/− mice (Fig. S1). Since TPO is essential for MK proliferation and maturation, serum TPO level was examined. There were comparable levels of serum TPO between Zyx−/− and WT mice. Taken together, these data suggest that the macrothrombocytopenia in Zyx−/− mice is not caused by accelerated platelet clearance or impaired TPO generation.

**Zyxin deficiency impairs MK maturation, DMS development, and proplatelet generation**

To investigate the mechanism for zyxin deficiency-mediated macrothrombocytopenia, we first characterized the megakaryopoiesis in Zyx−/− mice. We found that, compared with WT mice, Zyx−/− mice showed a 50% increase in MK number of the BM sections (16 MKs per visual field for WT vs 24 for Zyx−/− mice, Fig. 2A). Spleen is also an important hematopoietic organ in mice. We found that there was an 83% increase in splenic MK number (6 MKs per visual field for WT vs 11 for Zyx−/− mice, Fig. 2A). Therefore, the thrombocytopenia in Zyx−/− mice does not result from a lack of MKs. We next examined the effect of zyxin deletion on MK differentiation in vitro by culturing mouse fetal liver hematopoietic progenitor cells (FL HPCs). The percentages of CD41 positive (CD41+) (Fig. 2B) and CD61 positive (CD61+) (Fig. S2) cells were comparable between WT and Zyx−/− FL HPC cultures. We further cultured bone marrow hematopoietic progenitor cells (BM HPCs) from Zyx−/− and WT mice. There was also no difference in the percentages of CD41+ and CD61+ cells between the two cultures (Fig. S3). These data suggest that zyxin is not required for MK differentiation.

We next investigated the effect of zyxin deficiency on MK maturation and proplatelet production. Giemsa-stained BM smears showed more Stage I MKs (immature MKs) and fewer Stage II and III MKs (mature MKs) in Zyx−/− mice (Fig. 2C). The ultrastructure of Stage III MKs from BM aspirates was further analyzed by transmission electron microscopy (TEM). WT MKs showed well-organized DMS, but Zyx−/− MKs showed disorganized DMS and a lot of vacuoles (Fig. 2D), suggesting the defective development of DMS in Zyx−/− MKs.

Finally, platelet generation was examined in Zyx−/− FL HPC-derived MKs. Culture-derived MKs started to release CD41+ platelet-sized particles from day 5 (Fig. 2E). On day 5 and day 6, there were 13.6% and 11.8% CD41+ platelet-sized particles from WT MKs, while only 2.6% and 2.9% from Zyx−/− MKs (Fig. 2E). Furthermore, Fig. 2F showed that proplatelet protrusions from Zyx−/− MKs were obviously reduced compared with that from WT MKs. Taken together, these data show that zyxin deletion impairs MK maturation, DMS development, and proplatelet generation.

**Fig. 1**  
**Zyx−/− mice display macrothrombocytopenia.**  
**A, B** Platelet count (A) and volume (B) were analyzed in whole blood from WT (n = 21) and Zyx−/− (n = 17) mice. Means are indicated by horizontal lines. ***p < 0.001 compared with WT mice by Mann–Whitney test in (A) and unpaired Student’s t-test with Welch correction in (B).  
**C** Representative images of Wright–Giemsa stained blood smears from WT and Zyx−/− mice (original magnification ×1,000). Scale bar: 10 μm. Platelets were indicated with arrows.  
**D** Representative transmission electron microscopy (TEM) images of WT and Zyx−/− platelets (original magnification ×13,500). Scale bar: 1 μm. Images were obtained from five mice in each genotype in (C) and (D).  
**E** WT and Zyx−/− mice were intravenously injected with NHS-biotin, and peripheral blood was taken at the indicated time points after injection. The percentage of biotinylated platelets was determined by flow cytometry; n = 10 mice per genotype. Data are expressed as means ± SD.  
**F** Serum TPO levels in WT and Zyx−/− mice; n = 7 mice per genotype. Means are indicated by horizontal lines.
Zyxin deficiency reduces glycoprotein (GP) Ib-IX levels in both platelets and MKs

As known, proteins related to the actin cytoskeleton or microtubular system have been reported to be associated with macrothrombocytopenia [8–13, 24–26]. In addition, deficiency or reduction of the major platelet membrane GPIb-IX and GPIIb/IIIa also cause macrothrombocytopenia named Bernard–Soulier syndrome (BSS) and ITGA2B/ITGB3-related thrombocytopenia, respectively [27, 28]. To further explore the molecular mechanism for zyxin deletion-induced macrothrombocytopenia, differential proteomic analysis was performed to compare the amount of the proteins associated with macrothrombocytopenia between WT
and Zyx−/− platelets. We found that GPIbα, GPIbβ, and GPIX, but not NMMHC-IIα, α-actinin, filamin A, DiAPH1, Tropomyosin 4, tropomodulin 3, Wdr1, Cdc42, Rac1, β1-tubulin, or GPIb/IIa, were significantly reduced in Zyx−/− platelets (Fig. 3A).

We next checked the level of GPIb-IX in Zyx−/− platelets. The surface levels of the three subunits were significantly decreased in Zyx−/− platelets (a 63%, 80%, and 81% decrease in Gp1ba, GPibβ, and GPIX, respectively) (Fig. 3B); in contrast, GPIbα was increased by 40% (due to the enlarged size of the Zyx−/− platelets) (Fig. S4). The total amounts of GPibα, GPibβ, and GPIX were also markedly reduced in Zyx−/− platelets (Fig. 3C).

We further confirmed these findings by detecting platelet function. Consistently, botrocetin-induced platelet aggregation, which is mediated by the interaction of GPIbα and von Willebrand factor, was obviously reduced in Zyx−/− platelets (Fig. 5A). Furthermore, Zyx−/− platelets tend to disaggregate in response to 0.05 U/mL thrombin, consistent with the requirement of GPibα in low dose thrombin-induced platelet activation [29]. However, zyxin ablation did not affect platelet aggregation induced by relatively high doses of thrombin (0.1 U/mL and 0.2 U/mL) (Fig. S5B). In addition, there was no significant difference in ADP- or U46619-induced platelet aggregation between WT and Zyx−/− platelets (Fig. SSC, D). These data suggest that zyxin deficiency impairs only GPIb-IX-dependent platelet function.

We also examined the expression levels of GPIb-IX/GPibα in MKs. The mean fluorescence intensities (MFI) of GPibα, GPibβ, and GPIX were significantly decreased in Zyx−/− MKs in the immunostained BM sections (Fig. 3D). The GPIbα−positive (GPibα+) cells were markedly reduced in Zyx−/− FL HPC-derived MKs (Fig. 3E). Moreover, the GPibα+ platelet-sized particles were also reduced (Fig. 3F). These data indicate that zyxin deficiency results in the reduction of GPIb-IX in both platelets and MKs.

Zyxin knockdown results in lysosome-mediated GPibα degradation in human megakaryocytic Dami cells

GPIb-IX complex is assembled in the endoplasmic reticulum, transported into Golgi, and expressed on the plasma membrane [30]. The presence of all three subunits and proper transportation are required for the efficient surface expression of the whole complex [31]. To explore the reason for the defective GPIb-IX expression in Zyx−/− platelets, we first examined whether zyxin deletion reduced transcription of these three genes. The quantitative real-time PCR (qRT-PCR) results showed that the mRNA levels of the three subunits did not decrease in Zyx−/− platelets (Fig. 4A), excluding the possibility of defective gene transcription. Further analysis of GPibα by Western blot showed additional anti-GPibα antibody-positive bands (60 kDa and 25 kDa) in Zyx−/− platelets (Fig. 4B), suggesting that GPIb-IX complex may be degraded in Zyx−/− platelets.

We next used a human megakaryocytic cell line, Dami cells, which express the GPib-IX complex on the cell surface [32], to explore the role of zyxin in GPIb-IX expression. The small interfering RNAs (siRNAs) targeting zyxin were transduced into Dami cells to knockdown zyxin. We first verified that the zyxin siRNAs significantly reduced the mRNA level of zyxin but not that of GPibα, GPibβ, or GPIX (Fig. 5F). Zyxin siRNAs reduced zyxin expression, and in the meantime, the levels of GPibα and GPIX were obviously reduced (Fig. 4C). Moreover, GPibα and GPIX surface levels were significantly decreased (Fig. 4D). These data suggest that the data with the zyxin−/− platelets and MKs, reveal that knockdown of zyxin reduces GPib-IX level in human megakaryocytic Dami cells.

GPibα was reported to be degraded through a lysosome-dependent manner [30]. We treated Dami cells with lysosomal inhibitors (leupeptin + NH4Cl) and a proteasome inhibitor (MG-132) after zyxin siRNA transfection. As shown in Fig. 4E and F, inhibition of lysosomes but not proteasomes restored GPibα surface expression as well as the total level of full-length GPibα. We further used another lysosomal inhibitor (bafilomycin A1) to verify this. Bafilomycin A1 indeed rescued zyxin knockdown-induced GPibα reduction (Fig. S7). These data demonstrate that zyxin deficiency results in lysosome-mediated GPibα degradation in the human megakaryocytic cell line.

Zyxin deficiency enhances microtubular ring in platelets

Microtubules play critical roles in the extension of proplatelets [5, 7]. The proplatelets from a mouse model of BSS contained twice as many tubulin fibers in the marginal microtubular ring [33]. Thus, we also examined the microtubular system in Zyx−/− platelets. First, we constructed a mouse model of BSS by deletion of GPibα (Fig. S8). The GPibα-deficient (Gpiba−/−) mice presented the typical macrothrombocytopenia phenotype (Table S2). The same as the finding in Gpiba−/− platelets, a brighter and thicker marginal band was found in Zyx−/− platelets compared with that in WT platelets (Fig. 5A). These data suggest that the phenomenon of Zyx−/− platelets is similar to that of the platelets from a mouse model of BSS [32].

Zyxin deficiency alters actin cytoskeleton organization

It has been implied that actin cytoskeleton organization is essential for appropriate insertion or removal of membrane proteins [34]. And alteration of actin cytoskeleton reorganization impairs proplatelet formation leading to macrothrombocytopenia [5]. Zyxin regulates actin assembly [16–18]. Therefore, zyxin ablation may alter actin cytoskeleton organization, contributing to defective GPIb-IX complex expression and proplatelet generation. To test this hypothesis, F-actin organization was visualized by phalloidin staining in platelets spreading on the fibrinogen surface. The stress fibers were bundled along the sides of the WT platelets; in contrast, the stress fibers were disseminated in the whole cytoplasm of Zyx−/− platelets (Fig. 5B). We further detected actin cytoskeleton in MKs from WT and Zyx−/− mice. In MKs spreading on immobilized collagen, only 41% of WT MKs displayed an organized F-actin network along collagen fibers;
whereas 75% of Zyx−/− MKs presented the phenomena (Fig. 5C, D). Furthermore, for MKs spreading on immobilized fibrinogen, there was 61% of WT MKs displaying the lamellipodia, whereas this was only seen in about 26% of Zyx−/− MKs (Fig. 5E, F). The spread areas were not affected by zyxin deficiency on both collagen and fibrinogen surfaces (Fig. S9). These data indicate that actin cytoskeleton organization is altered in Zyx−/− platelets and MKs.

Zyxin deficiency results in VASP mislocalization

We next explored the mechanism for zyxin in regulating actin cytoskeleton organization. α-Actinin and VASP are two important zyxin binding partners [19, 20]. We recently identified NMMHC-IIA as a zyxin binding protein [21]. The three proteins all exist in platelets and MKs and play important roles in actin cytoskeleton reorganization. Zyxin deletion did not affect the expression of the three proteins (Fig. 3A, Fig. S10A). In the absence of zyxin, the distributions of α-actinin and NMMHC-IIA were not obviously altered in platelets spreading on the fibrinogen surface (Fig. S10B). Moreover, zyxin deficiency did not affect the phosphorylation of NMMIIA at Ser1916 and Ser1943, which is related to the activity of NMMIIA [35], in the platelets either under basal condition or stimulated with various agonists (Fig. S11). In contrast, we found that zyxin colocalized with VASP (Fig. 6A) in WT platelets, and the distribution of VASP was obviously disrupted in Zyx−/− platelets compared with that in WT platelets (Fig. 6B),
suggesting the essential role for zyxin in VASP localization. Next, we verified this finding in MKs. Consistent with the finding in platelets, mRNA expression was analyzed by qRT-PCR and determined by a ratio relative to the control GAPDH. The data were expressed as the ratio relative to WT; n = 5 mice per genotype. B Western blot analysis of GPIbα protein in WT and Zyx−/− platelets. Protein concentration was adjusted to the same level between WT and Zyx−/− platelet lysates. Blots are representative of five independent experiments. C, D Dami cells were transfected with siRNAs targeting zyxin (siZYX-1, -2) or negative control siRNA (control). The expression of GPIbα and GPIα in Dami cells was analyzed by Western blot; the blots are representative of five independent experiments (C). The surface level of GPIbα and GPIα was analyzed by flow cytometry (D). E, F Dami cells were treated with or without 10 μg/mL leupeptin plus 10 mM NH4Cl (Leu+NH4Cl) and MG-132 (100 nM) for 12 h after zyxin siRNA (siZYX-1) transfection. The total expression of GPIbα was analyzed with Western blot; the blots are representative of five independent experiments (E). The surface level of GPIbα was analyzed by flow cytometry (F). Data are from five independent experiments in (C–F). Means are indicated by horizontal lines in (A) and (C–F). *P < 0.05, **P < 0.01, ***P < 0.001, by one-way ANOVA followed by Dunnnett’s post hoc test in (C–F). NS, not significant.

Fig. 4 Knockdown of zyxin results in lysosome-mediated GPIbα degradation. A mRNA levels of GPIbα, GPIbβ, and GPIα in WT and Zyx−/− platelets. mRNA expression was analyzed by qRT-PCR and determined by a ratio relative to the control GAPDH. The data were expressed as the ratio relative to WT; n = 5 mice per genotype. B Western blot analysis of GPIbα protein in WT and Zyx−/− platelets. Protein concentration was adjusted to the same level between WT and Zyx−/− platelet lysates. Blots are representative of five independent experiments. C, D Dami cells were transfected with siRNAs targeting zyxin (siZYX-1, -2) or negative control siRNA (control). The expression of GPIbα and GPIα in Dami cells was analyzed by Western blot; the blots are representative of five independent experiments (C). The surface level of GPIbα and GPIα was analyzed by flow cytometry (D). E, F Dami cells were treated with or without 10 μg/mL leupeptin plus 10 mM NH4Cl (Leu+NH4Cl) and MG-132 (100 nM) for 12 h after zyxin siRNA (siZYX-1) transfection. The total expression of GPIbα was analyzed with Western blot; the blots are representative of five independent experiments (E). The surface level of GPIbα was analyzed by flow cytometry (F). Data are from five independent experiments in (C–F). Means are indicated by horizontal lines in (A) and (C–F). *P < 0.05, **P < 0.01, ***P < 0.001, by one-way ANOVA followed by Dunnnett’s post hoc test in (C–F). NS, not significant.

The interaction of zyxin with VASP is required for GPIbα surface expression and platelet production

We further investigated whether zyxin regulates GPIb-IX expression and platelet production through the interaction with VASP. The binding sites for α-actinin and NMMHC-IIA are located in the N-terminal 42 amino acids of zyxin [19, 21], and the binding site for VASP is in the four proline-rich ActA repeats in zyxin [20]. In order to distinguish which site is essential for GPIb-IX expression and platelet production, we constructed zyxin mutants lacking binding sites for α-actinin and NMMHC-IIA (Zyx43–564), and VASP (Zyx4F–A) (Fig. 7A). Zyx−/− FL HPCs were infected with adenoviruses expressing WT zyxin and zyxin mutants, and similar expression levels of these genes were verified with Western blot (Fig. 7B). The expressions of WT zyxin (Zyx1–564) and zyxin mutants (Zyx1–43–564 and Zyx4F–A) in Zyx−/− FL HPCs did not affect the percentage of CD41+ cells compared with that of vector control (Fig. 7C), suggesting that these genes did not affect the MK...
**DISCUSSION**

In this study, we report for the first time that zyxin is essential for platelet biogenesis and GPIb-IX surface expression. Our data show that: (1) Zyx−/− mice display macrothrombocytopenia, (2) zyxin is essential for DMS formation and proplatelet production, (3) zyxin ablation results in GPIba degradation leading to reduction of GPIb-IX surface expression and GPIb-IX-dependent platelet function, (4) deletion of zyxin alters actin cytoskeleton organization, and (5) the interaction of zyxin with VASP is required for proplatelet production and GPIb-IX surface expression.

Defective proplatelet formation has been reported in more than ten forms of inherited thrombocytopenia [36]. Most of these diseases are macrothrombocytopenia caused by mutations in genes encoding for components of the actomyosin cytoskeleton or microtubular system [36]. Reorganization of the actin cytoskeleton is crucial for DMS formation or extension and release of proplatelets [6, 7]. Therefore, defects in genes associated with the actomyosin cytoskeleton, such as MYH9 and ACTN1, results in alteration of proplatelet formation [8–11]. Microtubules composed of α- and β-tubulin are the driving force of proplatelet elongation [5, 7]. Therefore, defects in TUBBI induce abnormalities of microtubule function, affecting proplatelet formation [26]. In line with the reported macrothrombocytopenia, we found that the absence of zyxin resulted in actin cytoskeleton disorganization and enhanced tubulin filaments in the marginal band. Thus, our data support that zyxin deficiency alters the actin cytoskeleton and microtubular system resulting in defective proplatelet formation and macrothrombocytopenia.

Zyxin is essential for actin filament remodeling. Our data showed that zyxin deficiency affects F- actin organization. Without zyxin, VASP is disseminated in the whole platelets. VASP is capable of bundling actin filaments and inducing actin polymerization [37, 38]. The dislocated VASP may induce actin polymerization in
altered places, leading to altered organization of actin skeleton in Zyx−/− platelets and MKs. Therefore, it is reasonable to find that the actin filaments were disorganized in Zyx−/− platelets and MKs, resulting in defective proplatelet formation. Consistently, reconstitution of zyxin lacking VASP binding site in Zyx−/− FL HPC-derived MKs allowed to spread on type I collagen and fibrinogen and stained for VASP (red), F-actin (green), and nuclei (blue). Scale bar: 10 μm. The original magnification of all the images is ×630. Results are representative of five independent experiments.

Interestingly, we found that zyxin deficiency diminished GPIb-IX surface expression both in Zyx−/− platelets and MKs. These findings suggest that the molecular mechanism of zyxin deficiency-induced macrothrombocytopenia may be similar to that of BSS. GPIb-IX complex is assembled in the endoplasmic reticulum and traffics to the plasma membrane for surface expression [30]. Filamin A that binds to actin associates with the cytoplasmic domain of GPIbα anchoring the GPIb-IX complex for surface expression. One of the roles of actin is for membrane trafficking [39]. Therefore, actin organization might be critical for GPIb-IX trafficking and surface expression. In the current study, the data showed that VSAP localized with zyxin, and the interaction of zyxin with VASP is required for GPIb-IX surface expression. Thus, it is most likely that zyxin through VASP-mediated actin reorganization regulates GPIb-IX trafficking and surface expression. According to this theory, loss of zyxin results in actin cytoskeleton disorganization, which impairs GPIb-IX trafficking to the membrane and incurs GPIbα degradation. In support of this, GPIbα degradation bands were observed in Zyx−/− platelets, and knockdown of zyxin in Dami cells results in lysosome-mediated degradation of GPIbα. GPIb-IX complex, the receptor for the von Willebrand factor, plays an important role in thrombosis and hemostasis. This is the first time we have disclosed that zyxin regulates platelet function through regulating the GPIb-IX surface expression.

We selected a human megakaryocytic cell line, Dami cells, to demonstrate our findings. Consistent with the findings with Zyx−/− mouse platelets and MKs, knockdown of zyxin also reduced GPIb-IX surface level in Dami cells. These data provide important evidence that deletion of zyxin in human cells causes the same effects as it does in mice.
We found that Zyx−/− platelets showed brighter and thicker marginal microtubular rings. This is consistent with the findings that microtubules were obviously increased in GPIba and filamin A null platelets, which also showed defective GPIlb-IX surface expression [33, 40]. Furthermore, increased microtubules were observed in giant platelets from patients with May–Hegglin anomaly [41]. These findings suggest the possible relevance of GPIba to the microtubular system. Future study is needed to disclose the relationship between them.

Therefore, these findings delineate the mechanism of zyxin in platelet biogenesis and GPIlb-IX expression. In the absence of zyxin, (1) VASP was mislocalized, leading to disorganization of actin cytoskeleton, (2) GPIIb-IX could not be properly transported to the plasma membrane for surface expression, (3) microtubule filaments were elevated in the marginal ring. These factors independently or mutually promoting impair proplatelet formation which results in macrothrombocytopenia.

The relevance of zyxin with diseases has been reported [42, 43]. Here we show that the absence of zyxin incurs macrothrombocytopenia. Macrothrombocytopenia could cause severe or life-threatening hemorrhage. However, due to the pathogenic mechanism is not totally understood, many patients with macrothrombocytopenia still have not been diagnosed or were misdiagnosed [36]. In this sense, the current study not only identifies the role of zyxin in the pathogenesis of macrothrombocytopenia, more importantly, but also suggests that defect of zyxin or other cytoskeleton proteins may have the potential to incur macrothrombocytopenia in humans as well. Thus, the finding expands our horizons in finding novel macrothrombocytopenia-causing genes.

In conclusion, our study demonstrates that zyxin ablation results in macrothrombocytopenia in mice. Zyxin, through VASP-mediated cytoskeleton reorganization, regulates proplatelet formation and GPIlb-IX surface expression. Our findings help to understand the mechanism of platelet biogenesis and suggest possible pathogenesis of macrothrombocytopenia.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 WT mice were purchased from JOINN Laboratories (Beijing, China). Zyx−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME) [44] and interbred with WT C57BL/6 mice to generate Zyx−/− and WT control littermate mice.

The Gp1ba−/− mouse model was established by CRISPR/Cas9 genome editing technology on a C57BL/6 J background to induce two double-stranded breaks flanking exon 2 of Gp1ba. The optimized sgRNAs were constructed in the pT7-sgRNA plasmid backbone, and then were in vitro transcribed. The sequences of two independent guide RNAs targeting exon 2 of the Gp1ba gene were gRNA1 5′-TCTCACAGTTTACTTCCAGC-3′ and gRNA2 5′-TATTGGGCACAGTGGGCATT-3′. The in vitro-transcribed Cas9 mRNA together with two sgRNAs were microinjected into the cytoplasm of C57BL/6 J zygote. In total, 145 healthy zygotes were transferred to pseudopregnant recipients and allowed to develop to term. Genotyping was performed by PCR amplification across the targeted
region (F: 5′-AGAAGCTCTGTCCTCCAAGGAC-3′, R: 5′-GTTAGTATGGAC-CATGTACGCTGAC-3′) to screen the born 15 pups. In total, ten founder mice were initially established and confirmed with the right PCR product (597 bp). The founder mice were further bred to WT C57BL/6J mice to generate homozygous Gpiba−/− mice.

Mice aged old, and experiments included balanced groups of male and female mice. All animal experiments complied with the regulatory standards of and were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

**Antibodies and reagents**

FITC-conjugated rat anti-mouse CD41 antibody (MWReg30, 553848), mouse BD Fc Block (anti-mouse CD16/32, clone 2.G4, 553142), and propidium iodide (PI, 556465) were purchased from BD Biosciences (San Jose, CA, USA). FITC-conjugated streptavidin (405202), APC-conjugated (139914), and PE-conjugated (139396) rat anti-mouse CD41 antibodies (MWReg30) were purchased from Biologend (San Diego, CA, USA). Rat anti-mouse GPIbα (Xia.G5, M040-0; Xia.G7, M042-0) and GPIbβ (Xia.C3, M050-0) antibodies, FITC-conjugated rat anti-mouse GPIbα antibody (Xia.G5, M040-1), GPIbβ (Xia.C3, M050-1), and GPIA (Xia.B4, M051-1) antibodies, and DyLight 649-conjugated rat anti-mouse antibody (Xia.C5, M053-1) were purchased from Emfret Analytik (Eibelstadt, Germany). Antibodies against β-actin (4970), GAPDH (5174), α-tubulin (2146), α-actinin (3134), VASP (3132), non-muscle myosin heavy chain IIA (NMHC-IIA, 3403), phospho-NMHC-IIA (Ser1943) (5026), and lamin A (4762) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against zyxin (10330-1-AP), PU-1 (45-1064-1-AP), GAP-1 (50430-2-AP), and flag tag (20543-1-AP) were from ProteinTech (Wuhan, China). Rabbit anti-phospho-NMHC-IIA (Ser1943) (5026) was purchased from ECM Biosciences (Versailles, KY, USA). The antibody for Western blot against mouse GPIA (GTX32502) was from GeneTex (Irvine, CA, USA). HRP-conjugated anti-rabbit IgG (A0208), mouse anti-IgG (A0216), and anti-rat IgG (A0192) secondary antibodies, and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-mouse GPIX antibody (PAB1088M01) for Western blot was from Cloud-Clone Corp (Houston, TX, USA). Anti-human GPIX antibody (clone F12062) was from Millipore (Darmstadt, Germany). EasySep buffer (20144), mouse hematopoietic progenitor cell isolation kit (19856), and mouse hematopoietic progenitor cell serum-free expansion medium (SFEM) (09650) were purchased from Stemcell Technologies (Vancouver, Canada). Mouse anti-human CD41 antibody (4F1A2) was from Beckman Coulter, Miami, FL, USA.

**Platelet aggregation**

Platelets in PRP or washed platelets (3 × 10^11/mL) were stimualted with different concentrations of different agonists. Platelet aggregation was recorded in a Chrono-Log lumi-aggregometer at a stirring speed of 1200 rpm at 37°C. Platelet aggregation was monitored continuously over 10 min.

**Platelet lifespan analysis**

WT and Zyx−/− mice were intravenously injected with 600 µg NHS-biotin in buffer containing 140 mM NaCl and 10% DMSO. Whole blood was collected by capillary tube from the retro-orbital venous plexus at various time points and mixed with ACD. Whole blood was stained with PE-conjugated anti-mouse CD41 antibody and FITC-conjugated streptavidin at room temperature (RT) for 1 h. The percentage of biotinylated platelets was determined by FC 500 flow cytometer (Beckman Coulter, Miami, FL, USA).

**Serum TPO measurement**

Mouse blood was collected from postorbital veins and then incubated at 37°C for 1 h and centrifuged to obtain the serum. Mouse serum TPO levels were measured according to the manufacturer’s protocol. Briefly, 50-µL serum was diluted with 50-µL assay buffer and added to the 96-well plate which had been coated with anti-mouse TPO antibody. After incubation, TPO was detected with biotinylated anti-TPO antibody and HRP-conjugated streptavidin. The plate was read at 450 nm in a Variskan Flash spectical scanning multimode reader (Thermo Fisher Scientific).

**Immunofluorescence staining on femora and spleen cryosections**

Mouse femora were isolated, dehydrated in 20% sucrose. Subsequently, the femora were embedded in OCT compound and cut into 10-µm sections. After deparaffinization with xylene and dehydration with 100% ethanol, sections were washed with phosphate-buffered saline (PBS) and then blocked with 10% normal goat serum. Sections were incubated with rabbit anti-GPIX antibody (1:100) for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200) for 1 h at room temperature. Sections were washed three times with PBS and incubated with DAPI (1:10,000) for 10 min. Sections were counterstained with 0.1% crystal violet solution at room temperature for 10 min. After washing, sections were mounted with mounting medium and observed under a microscope. Images were captured using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

**Platelet and MK imaging**

For blood smear, the murine whole blood from postorbital veins anti-coagulated with 1/7 volume of ACD was made into monolayer cells on a glass slide. For BM smear, the femoral BM was harvested and smeared on a glass slide with fetal bovine serum added in advance. Blood and BM smears were then stained with Wright–Giemsa Stain solution and examined by optical microscopy.

For TEM analysis of platelets, washed mouse platelets were centrifuged at 600 × g for 2 min and then fixed in 2.5% glutaraldehyde at 4°C overnight. For TEM analysis of MKs, femora and tibias of mice were isolated and the BM was flushed out by 0.01 M phosphate-buffered saline (PBS) and then centrifuged at 300 × g for 2 min. The platelets or BM pellets were washed with PBS, postfixed in 1.0% osmium tetroxide for 1 h, gradually dehydrated using acetone, and then stained with saturated uranyl acetate. The samples were infiltrated, embedded with resin, and polymerized. Finally, ultrathin sections were observed with an transmission electron microscope (Philips CM 120, Eindhoven, The Netherlands).

**Hematologic analysis**

Murine whole blood was collected from the postorbital veins and anti-coagulated with 1/7 volume of acid-citrate-dextrose (ACD, 2.5% trisodium citrate, 2.0% D-glucose, 1.5% citric acid). Platelet and blood cell counts were performed with Mindray BC-5000vet Hematologic Analyzer (Mindray Corporation, Shenzhen, China).

**Platelet preparation**

For the preparation of washed mouse platelets, whole blood from mice was collected from postorbital veins or inferior vena cava using 1/7 volume of ACD as the anticoagulant. Platelets were washed with CFS buffer (0.123 M NaCl, 0.033 M D-glucose, 0.013 M trisodium citrate, pH 6.5) and resuspended in Modified Tyrode’s buffer (2.5 mM Hepes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO3, 5.5 mM D-glucose, 1 mM CaCl2, 1 mM MgCl2, pH 7.4) to a concentration of 3 × 10^10/mL and allowed to incubate at 22°C for 1–2 h. For preparation of mouse platelet-rich plasma (PRP), whole blood was anti-coagulated with 1/9 volume of 3.8% trisodium citrate, and PRP were obtained by 100×g centrifugation.
For MK counting in mouse spleens, splenic cytocentrifugation was performed in ice-cold acetone and then blocked in 5% BSA/PBS at RT for 1 h. APC-conjugated anti-mouse CD41 (10 μg/mL) was incubated at RT for 2 h. Cell nuclei were stained with 5 μg/mL DAPI. Samples were visualized with a Leica TCS SP8 confocal microscope.

**In vitro differentiation of MKs and platelet production analysis**

Female WT and 2Yx⁻/⁻ mice were sacrificed on day 14.5 of pregnancy. Fetal livers were harvested and single-cell suspension was prepared as previously described [45]. For isolation of HPCs from mouse BM cells, the femora and tibia of 6–8-week-old mice were isolated and the BM was flushed by EasySep buffer and homogenized as previously described [45]. FL and BM HPCs were isolated by a mouse HPC isolation kit based on the manufacturer’s protocol. The isolated HPCs (3 × 10⁷/mL) were cultured in Stemspan™ SFEM in the presence of 1% penicillin, 1% streptomycin, and 20 ng/mL recombinant murine TPO at 37 °C for 6 days.

Total RNA was extracted from washed platelet from 4–8 mice or 1 × 10⁶ Dami cells (ATCC, Manassas, VA, USA) using TRIzol reagent (15596026, Thermo Fisher Scientific). Fetal livers were harvested and single-cell suspension was prepared as previously described [45]. For isolation of HPCs from mouse BM cells, the femora and tibia of 6–8-week-old mice were isolated and the BM was flushed by EasySep buffer and homogenized as previously described [45]. FL and BM HPCs were isolated by a mouse HPC isolation kit based on the manufacturer’s protocol. The isolated HPCs (3 × 10⁷/mL) were cultured in Stemspan™ SFEM in the presence of 1% penicillin, 1% streptomycin, and 20 ng/mL recombinant murine TPO at 37 °C for 6 days.

The cell suspension was collected from day 3 to 6 and centrifuged at 300 × g for 5 min to separate culture-derived MKs (pellet) and platelet-sized particles (supernatant). The cell pellet was washed with PBS and resuspended in cell staining buffer. Non-specific binding was blocked by 20 μg/mL 2.4G2 at RT for 10 min. Then cells were labeled with APC-conjugated anti-mouse CD41 antibody (MRWreg30, 2 μg/mL) and FITC-conjugated rat anti-mouse GPIbα antibody (Xia.G5, 1:10) at RT for 20 min. After being washed with PBS, cells were labeled with 5 μg/mL PI at RT for 10 min and then measured by flow cytometry. The percentage of CD41⁺ and GPIbα⁻ on cells was analyzed in the PI⁻ population. The supernatant was further centrifuged at 3,500 rpm for 2 min, and the pelletted platelet-sized particles were washed with PBS resuspended in cell staining buffer, and labeled with APC-conjugated rat anti-mouse CD41 antibody (MRWreg30, 2 μg/mL) and FITC-conjugated rat anti-mouse GPIbα antibody (Xia.G5, 1:10) at RT for 20 min. After wash with PBS buffer, platelet-sized particles were stained with 5 μg/mL PI at RT for 10 min and then washed and measured by flow cytometry. The percentage of CD41⁺ and GPIbα⁻ on platelet-sized particles was analyzed in the PI⁻ population.

**Proteomic sample preparation**

Washed platelets (1 × 10⁸) from WT and 2Yx⁻/⁻ mice were lysed with 100 μL lysis buffer (0.05 M NH₄HCO₃, 2% sodium deoxycholate, 0.025 M NaCl). After centrifuged at 14,000 × g for 10 min to remove cell debris, protein concentration was measured by BCA kit and adjusted to the same level by lysis buffer. DTT (2 mM) was added to the platelet lysates and incubated at 37 °C for 30 min. Proteins were hydrolyzed by 2.5 μg trypsin (V528A, Promega, Madison, WI, USA) at 37 °C for 4 h and further hydrolyzed by the addition of 100 μL lysis buffer and 2.5 μg trypsin at 37 °C overnight. The reaction was stopped by 1% formic acid. After centrifuged at 14,000 × g at 4 °C for 15 min, supernatant was collected and lyophilized. The differential proteomic analysis was conducted by Human Phenome Institute (Fudan University, Shanghai).

**RNA extraction and qRT-PCR**

Total RNA was extracted from washed platelet from 4–8 mice or 1 × 10⁶ Dami cells (ATCC, Manassas, VA, USA) using TRizol reagent (15596026, Thermo Fisher Scientific). Then, the RNA was reversely transcribed to cDNA using the RevertAid First Strand cDNA synthesis kit (K1622, Thermo Fisher Scientific) according to the manufacturer’s instruction. qRT-PCR was performed using primers specific for GPIX, GPIbβ, GPIβ, and GAPDH on a LightCycler 96 instrument (Roche, Indianapolis, IN, USA). Primer sequences were as follows: GPIX-mus forward, 5'-CTACGTGGCGGAGGATGAG-3'; GPIbβ forward, 5'-AGCAGGCATCTGAGGGCCCA-3'; GPIbβ reverse, 5'-GGAUCUGGGUCACCAGCTT-3'; siZyx-2, sense, 5'-GGACCTGAGGCAACACCCATT-3'; anti-sense, 5'-AUGUGGUUAGACCAGAGCTT-3' (siZyx-1, sense, 5'-GCAACAGGUCACACCCATT-3'; anti-sense, 5'-AUGUGGUUAGACCAGAGCTT-3'), respectively. Then, the complex was mixed and added to the cells and another 1 mL RPMI-1640 medium supplemented with 10% FBS was added to the cells. The cells were cultured for 48 h after transfection and then harvested. To verify the effect of siRNA transfection, qRT-PCR for zyxin gene expression and Western blot for zyxin protein expression were performed.

**Flow cytometry**

For detecting platelet GPIb-IX surface level, platelets in PRP from WT and 2Yx⁻/⁻ mice were labeled by FITC-conjugated anti-mouse GPIbα, GPIβ, and GPIX antibodies (1:5) at RT for 30 min. For detecting reticulated platelets, platelets in whole blood were labeled by thiazole orange (0.5 μg/mL) and PE-conjugated anti-mouse CD41 antibody (5 μg/mL). For detecting platelet GPIb-IX surface level, cells (1 × 10⁶) were collected and washed with PBS. Dami cells were then resuspended in 0.5% BSA/PBS and incubated with 10 μg/mL anti-GPIbα (6F3') and anti-GPIX (FMC25) antibodies at RT for 1 h. After being washed with 0.5% BSA/PBS twice, cells were stained with FITC-conjugated goat anti-mouse IgG (1 μg/mL) at RT for 1 h and then washed twice with 0.5% BSA/PBS. Platelets and Dami cells were examined by flow cytometer.

**Western blot**

For detection of mouse platelet proteins, washed WT mouse platelets (3 × 10⁹/mL) and 2Yx⁻/⁻ platelets (1.5 × 10⁹/mL) were lysed with an equal volume of 2 x lysis buffer (100 mM Tris, pH 7.4, 2% Triton X-100, 20 mM MgCl₂, 300 mM NaCl) containing phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor cocktail on ice for 30 min. For detection of Dami cell proteins, approximately 2 × 10⁶ cells were lysed in 100 μL 1 x lysis buffer on ice for 30 min and the supernatant was separated from insoluble cell fraction by centrifugation at 10,000 g for 10 min at 4 °C. Protein concentration was measured by the BCA protein assay kit and adjusted to the same level by lysis buffer. Proteins were separated by SDS-PAGE and immunoblotted with specific antibodies.

**Confocal microscopy analysis of platelets and MKs**

For confocal analysis of resting platelets, washed mouse platelets (1 × 10⁶ in 100 μL Modified Tyrode’s buffer) were fixed with an equal volume of 4% PFA and spun to slides. For spread platelets, the slides were first coated with 30 μg/mL fibrinogen diluted in 0.1 M NaHCO₃ (pH8.3) at 4 °C overnight and blocked with 5% BSA. Washed mouse platelets (1 × 10⁷/mL) were allowed to adhere and spread on the fibrinogen-coated surface in the presence of 0.1 U/mL thrombin for 2 h at 37 °C. Platelets were fixed with 4% PFA. For spread MKs, the confocal dishes were first coated with 100 μg/mL fibrinogen or 50 μg/mL type I collagen at 4 °C overnight and blocked with 5% BSA. MKs cultured from mouse FL HPCs were resuspended in DMEM medium and allowed to adhere and spread on a fibrinogen- or collagen-coated surface for 3 h at 37 °C with 5% CO₂. MKs were fixed with 4% PFA. Platelets and MKs were then permeabilized with 0.1% Triton X-100 and blocked with 5% BSA at RT. Platelets and MKs were stained with specific primary antibodies (10 μg/mL) and Alexa Fluor 488- or 555-conjugated secondary antibodies (4 μg/mL). For F-actin staining, Alexa Fluor 488-conjugated phalloidin (0.165 μM) was incubated for 15 min. Sufficient washing was performed after each step. Platelets and MKs were observed using a LeICA TCS SP8 confocal microscope with a 63x oil immersion lens.
Co-immunoprecipitation

Washed platelet (3 x 10^6/mL) were lysed with an equal volume of 2 x lysis buffer containing phenylmethylsulfonyl fluoride (1 mM), protease inhibitor cocktail, NaF (2 mM), and NaVO4 (2 mM) on ice for 30 min. After centrifugation at 15,000 x g at 4 °C for 4 min, the supernatants were immunoprecipitated with antibodies overnight. After washing with Proteins G Plus-Agarose beads at 4 °C for 4 h, the beads were washed thoroughly and analyzed by immunoblotting.

Construction of zyxin mutants and adenovirus infection

Adenoviruses expressing WT zyxin and zyxin mutants zyx45-564 (lacking α-actinin/NIMH/M-CII binding site) and zyx45, 95, 105, 115A (zyx4F > A, lacking VASP binding site) were compared by the two-tailed unpaired Student single variant) or two-way (for multiple variants) ANOVA. Two groups were variance, respectively. Numeric data were analyzed using one-way (for a test (when Gaussian distribution was not assumed). Different levels of GFP adenoviruses expressing WT zyxin; zyxin mutants zyx43 fl were analyzed by

μ immunoblotted with specific antibodies.

Statistics analysis

Statistical analysis was performed using GraphPad Prism 8 software. Shapiro–Wilkinson test and Brown–Forsythe test was done for normality and variance, respectively. Numeric data were analyzed using one-way (for a single variant) or two-way (for multiple variants) ANOVA. Two groups were compared by the two-tailed unpaired Student’s t-test or Mann–Whitney test (when Gaussian distribution was not assumed). Different levels of significance were indicated as *P < 0.05, **P < 0.01, and ***P < 0.001. All animal experiments were subject to randomization based on litter. No animals or samples were excluded from the study. The sample size was predetermined based on the variability observed in prior experiments and on preliminary data. Investigators were not blinded to outcome assessment.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
R.Y. and K.D. conceived and designed the study; X.G., N.P., H.Y., L.Y., B.C., K.Z., M.Y., Y.S., and S.Z. performed the experiments; R.Y., X.G., N.P., Z.D., J.L., C.R., and K.D. analyzed the data; J.L. provided technical and material support; R.Y. and K.D. wrote the paper; K.D. initiated and supervised the project. All authors read and approved the final paper.

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COMPETING INTERESTS
The authors declare no competing interests.

ETHICAL APPROVAL
This study is approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

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