SUPPLEMENTARY APPENDIX

Migfilin supports hemostasis and thrombosis through regulating platelet αIIbβ3 outside-in signaling

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SUPPLEMENTAL DATA

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Methods

Reagents and antibodies

U46619 was from Calbiochem (CA, USA). Mepacrine, apyrase, ADP and fibrinogen were from Sigma Aldrich (MO, USA). Collagen, ristocetin and luciferase were from Chrono-Log Corporation (PA, USA). vWF was from Sino Biological (Beijing, China). Tirofiban was from Grand Pharmaceutical (Wuhan, China). Protein A/G-agarose and antibodies against beta3, anti-phospho-β3 (Y747), anti-phospho-β3 (Y759), and anti-migfilin were from Santa Cruz Biosciences (CA, USA). Anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK 1/2 (Thr202/Tyr204), anti-phospho-syk (Tyr525/526), anti-phospho-Src (Tyr416), anti-filamin A, anti-Src, anti-Syk and anti-β-actin were from Cell Signaling Technologies (MA, USA). Anti-GPIbα was from Emfret (Eibelstadt, Germany). Alexa Fluor 488- and 546-tagged secondary antibodies for immune staining were from Molecular Probes (OR, USA). Migfilin peptides were generated as described by ChinaPeptides (Suzhou, China).

Hematologic analysis

Complete blood counts and hematocrits were determined with an automatic cell counter (Sysmex F-820, Kobe, Japan), using the standard parameters for mice.

Electron microscopy

Washed WT or migfilin$^{-/-}$ platelets were fixed with 2.5% glutaraldehyde in modified Tyrode's buffer. After fixation, staining, and dehydration, the platelets were infiltrated

with embedding medium. Thin sections were stained with uranyl acetate and lead citrate. Samples were examined at 80 kV using a Tecnai 10 transmission electron microscope (FEI, Hillsboro, OR), and images were captured with an ES500W (782) camera (Gatan, Pleasanton, CA) using Digital micrograph software (Gatan, Pleasanton, CA).

Flow cytometric analysis

WT or migfilin$^{-/-}$ platelets (10^6) were labeled for 10 minutes at room temperature with antibodies: FITC-conjugated anti-mouse CD41 monoclonal antibody mAb (MWReg30; BD Biosciences, CA, USA), FITC-conjugated anti-mouse CD42b mAb (Xia.B2; Emfret Analytics, Emfret Analytics GmbH & Co.KG, Eibelstadt, Germany), FITC-conjugated anti-mouse GPVI mAb (JAQ1; Emfret Analytics, Germany), PE-conjugated CD62P (1302-D; Emfret Analytics, Germany), PE-conjugated JON/A (M023-2, Emfret Analytics, Germany). Designated agonists or migfilin peptides were added and incubated for 10 min, then stopped with 1 ml 1% formaldehyde. The samples were analyzed with a flow cytometer (EPICS XL; Beckman Coulter, Hialeah, FL).

Platelet aggregation and secretion

Platelet aggregation and secretion were measured using a lumi-aggregometer (Chrono-Log, Havertown, PA, USA) at 37 ℃ under stirring at 1200 rpm. Washed platelets (2×10^8/ml) in modified Tyrode's buffer were stimulated with thrombin, collagen, U46619, ADP (in the presence of fibrinogen), or Mn^{2+} (in the presence of fibrinogen).[1, 2] Platelet secretion was monitored in parallel with aggregation as ATP release with the addition of luciferin/luciferase reagent (Chrono-Log) to the platelet suspension. Inhibitors or migfilin peptides were incubated with the platelets for 5 minutes prior to stimulation.

Serotonin content and release assay

Washed WT or migfilin$^{-/-}$ platelets (2×10^8/ml) were stimulated with agonists in an aggregometer at 37 ℃ with stirring (1200 rpm). The reaction was stopped by adding 2 × stop buffer (6 mM aspirin and 20 mM EDTA) after 5 minutes of stimulation and moved immediately to ice for 8 min, then centrifuged at 15000 ×g for 10 min at 4 ℃, and the

supernatant was stored at -70 ℃ until analysis. Washed WT or migfilin$^{-/-}$ platelets ($1.5\sim3\times10^8$) were resuspended by 0.2 ml distilled water, and the suspension was stored at -70 ℃ until analysis. A serotonin ELISA kit (Enzo, NY, USA) was used according to the manufacturer's protocol to measure serotonin. Experiments were repeated at least 5 times. Results are expressed as mean ± SEM. Statistical significance was evaluated with paired Student t test.

Platelet spreading on fibrinogen or vWF

Glass coverslips were coated with 20 μg/ml fibrinogen in 0.1 M $NaHCO_3$ (pH 8.3) or 30 μg/ml purified vWF at 4 ℃ overnight. Washed platelets (2×10^7/ml) with or without migfilin peptides (5μM) pre-incubation for 5 min at 37 ℃ were allowed to spread on the fibrinogen-coated surfaces at 37 ℃ for 60 min. Ristocetin (1 mg/ml) was also added in vWF-coated glass coverslips.[3] After three washes with PBS, the platelets were fixed, permeabilized, and stained with fluorescein-labeled phalloidin (Molecular Probes, Eugene, OR), as previously described.[4] Adherent platelets were viewed with an inverted fluorescence microscope (Nikon Ti-S, Tokyo, Japan) using an S Plan Fluor lens (×100/1.30 numerical aperture oil objective). Images were acquired using a Nikon DS-Qi1-U3 camera. The platelet-covered area was measured using NIS-D software (Nikon).

Platelet-mediated clot retraction

As previously described,[5] murine platelets were re-suspended using citrated human platelet-depleted plasma to a concentration of 4×10^8/ml. Coagulation was induced by thrombin (0.4 U/ml). The clots were allowed to retract at room temperature and photographed at various times.

Immunoblotting

Washed WT and migfilin$^{-/-}$ platelets (2×10^8 /ml) were stimulated in a Chrono-Log aggregometer (Chrono-Log), the reaction was stopped by adding 2 × lysis buffer (50 mM Tris and 150 mM NaCl, 1% (vol/vol) TritonX-100, pH 7.4) containing protease inhibitor and phosphatase inhibitor. Alternatively, washed platelets (4×10^7/ml) were

allowed to spread on fibrinogen for 60 min, adherent or control platelets were lysed with lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes, which were incubated with primary antibodies. After incubation with the corresponding secondary antibodies (horseradish peroxidase-conjugated IgGs; Jackson ImmunoResearch Laboratories, West Grove, PA), proteins were visualized by enhanced chemiluminescence and imaged using a Syngene G: BOX Chemi XR system and GeneSnap software (Syngene, Frederick, MD).

Immunoprecipitation assay

After stimulation, washed platelets in a Chrono-Log aggregometer (Chrono-Log) were lysed by the addition of 5 × Ripa lysis buffer (50mM Tris pH 7.4, 750 mM NaCl, 5 mM EDTA, 5% Triton X-100, 5% sodium deoxycholate, 50 mM sodium fluoride containing protease inhibitor) at 4 ℃.[6] Lysates were immunoprecipitated by using 5 μl per sample of anti-beta3 (Santa) or anti-GPIbα (Emfret) overnight at 4 ℃, followed by protein A/G-agarose at 4 ℃ for 2–4 hour. After 3 washes with 1 × Ripa lysis buffer and PBS, proteins bound to the beads were eluted with 2 × loading buffer and analyzed by immunoblotting using corresponding primary and secondary antibodies.

Thrombus formation under flow conditions

Thrombus formation *in vitro* was performed in a Bioflux-200 system (Fluxion, South San Francisco, CA), in which the microfluidic plates were coated with collagen (50 μg/ml) as previous described.[1] Fresh isolated mouse whole blood were anticoagulated with heparin (7.5 U/ml) and labeled with mepacrine (100 μM). Blood was perfused over immobilized collagen surface for 5 minutes at indicated shear rates to visualize platelet adhesion and thrombus formation. Adherent platelets were viewed with an inverted fluorescence microscope using an S Plan Fluor lens (×20/0.4 numerical aperture objective). Images were acquired with a Nikon DS-Qi1-U3 CCD camera. The platelet-covered area was measured using Bioflux software (Fluxion).

$FeCl_3$-induced thrombosis

As previously described,[4] anesthetized mice were catheterized *via* the jugular vein, and fluorescently-labeled platelets (10^8 platelets) were injected through the catheter. The intestines were exposed, and the mesentery was spread on the translucent stage of the fluorescence microscope. Injury of mesenteric arterioles (60-80 μm in diameter) was induced by topical application of 10% $FeCl_3$. Arterioles were monitored for 60 min or until complete occlusion occurred (blood flow stopped for >1 min). For the rescuing experiments with migfilin peptides, platelets were fluorescently labeled by DIOC6 injection into the mice (Sigma-Aldrich, Saint Quentin Fallavier, France).[7] Injury to mesenteric arterioles were induced by topical application of 10% $FeCl_3$ 30 min after the injection of the peptides at indicated concentrations.

Confocal microscopy

Platelets were allowed to spread on coverslips coated with Poly-L-Lysine or fibrinogen (20 μg/ml) for 30min, 60min, 90min and 120min. After platelets were fixed and permeabilized, they were incubated with anti-beta3, anti-filamin A primary antibodies overnight and Alexa Fluor 488- and 546-tagged secondary antibodies for 2 hours. Confocal images were taken in a confocal microscope (Olympus IX83-FV3000-OSR) with a plan apochrom at 100×/1.49 NA oil immersion lens (optical slice thickness 0.5 μm).

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was evaluated with Student t tests or Mann-Whitney U tests using statistical software (GraphPad Prism, GraphPad Software, La Jolla, CA).

Figures

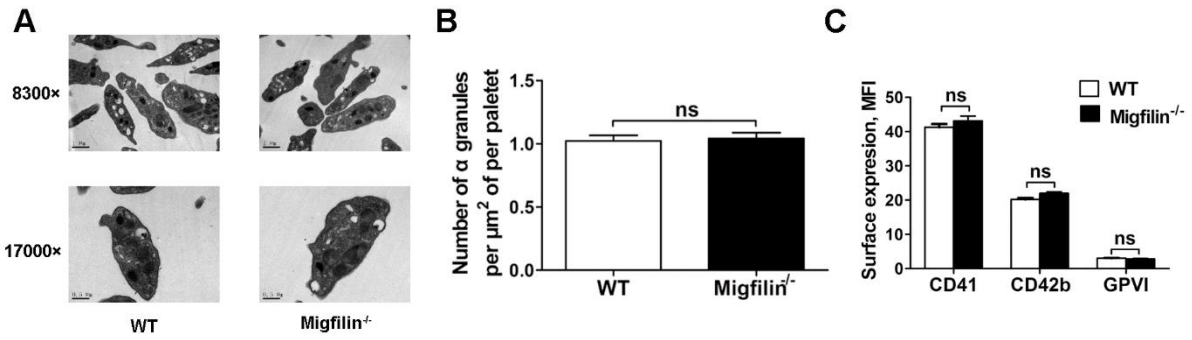

Figure S1. Migfilin$^{-/-}$ platelets had normal discoid morphology and surface expression of the platelet glycoproteins GPVI, CD41 and CD42b.

A. Representative electron microscopic images of WT and migfilin$^{-/-}$ platelets. Scale bars, 1 μm (upper) and 0.5 μm (lower).

B. Quantification of α-granules of WT and migfilin$^{-/-}$ platelets. Under 17000× magnification, α-granules was counted in WT (n = 52) and migfilin$^{-/-}$ platelets (n = 52). The data were expressed as mean ± SEM. Statistical significance was evaluated with Student t test, ns, no significant difference.

C. Surface expression of CD41, CD42b, and GPVI. Mean fluorescence intensity (MFI) was determined by flow cytometry with FITC-conjugated anti-mouse CD41 mAb (MWReg30), FITC-conjugated anti-mouse CD42b mAb (Xia.B2), and FITC-conjugated anti-mouse GPVI mAb (JAQ1). Results are expressed as the mean ± SEM. Statistical significance was evaluated with the Student t test, ns, no significant difference.

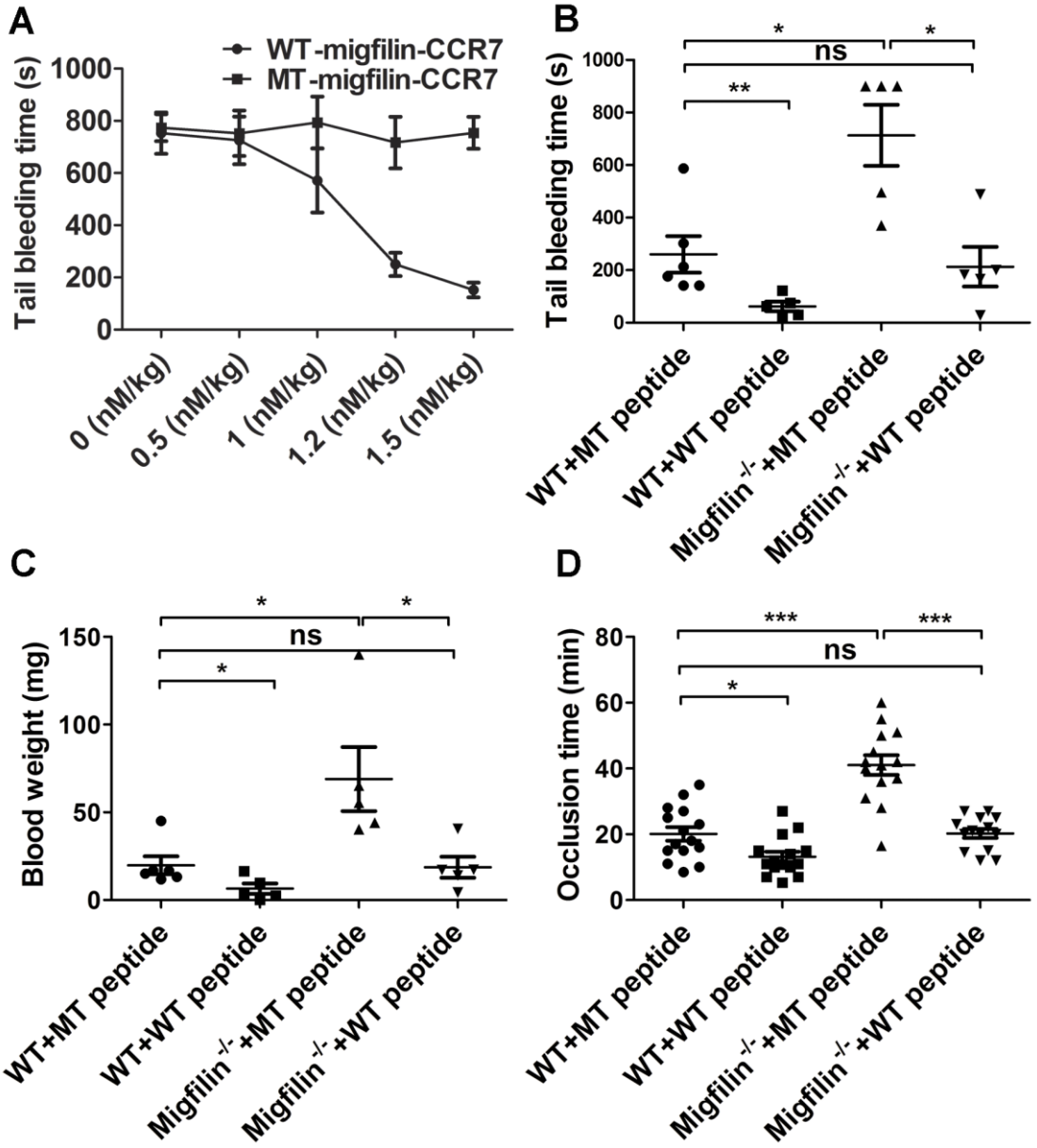

Figure S2. Migfilin peptides rescued hemostatic and thrombotic phenotypes of migfilin$^{-/-}$ mice.

A. WT-migfilin-CCR7 peptide and MT-migfilin-CCR7 peptide (0.5-1.5 nM/kg as indicated) were injected into the migfilin$^{-/-}$ mice via the tail vein. Bleeding times for were then recorded. Means are indicated by horizontal lines.

B. Bleeding times, **C.** Weight of blood loss, and **D.** Occlusion times of $FeCl_3$-induced arteriolar thrombosis in WT and migfilin$^{-/-}$ mice after intravenous injection of WT-migfilin-CCR7 peptide (1.2 nM/kg) and MT-migfilin-CCR7 peptide (1.2 nM/kg) were recorded. Results are expressed as the mean ±SEM, **P* <0.05, ***P* <0.01, ****P* <0.001, ns, no significant difference, evaluated with 2-tailed Mann-Whitney U tests.

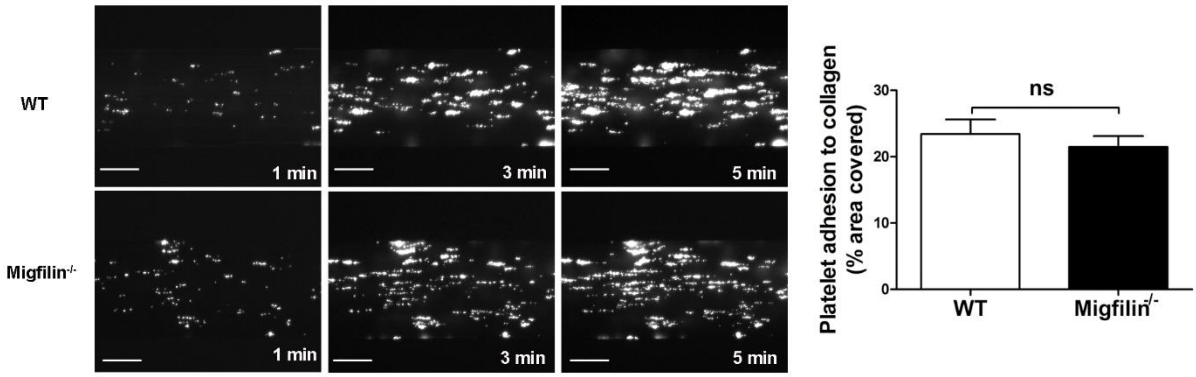

Figure S3. Adhesion of platelets to collagen was not affected by migfilin deficiency. Calcein-labeled platelets (2×10^7/ml) were added to red blood cells (hematocrit 45%) with 2 mM $CaCl_2$. The reconstituted blood was then perfused through fibrillar collagen-coated bioflux plates at a shear rate of 1000 s^{-1} for 5 min. The results are shown as mean ± SEM, ns, no significance, Student t test. Original magnification ×10. Scale bars, 100 μm.

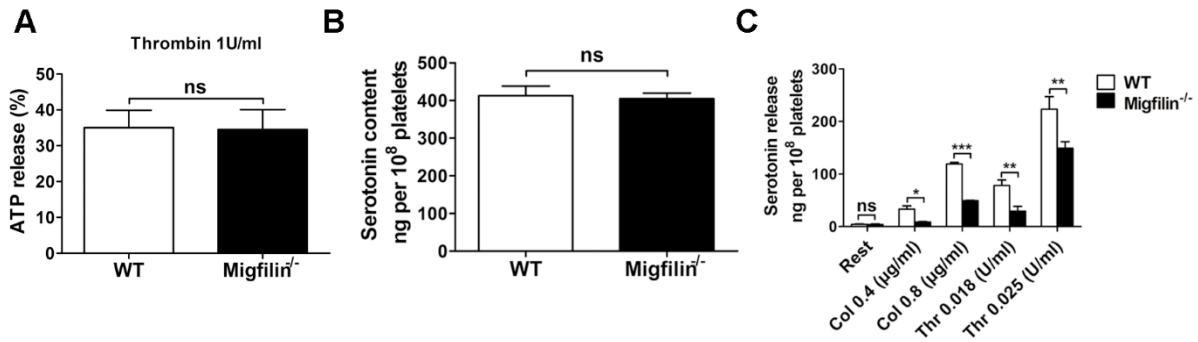

Figure S4. Thrombin- and collagen-induced secretion of serotonin from migfilin$^{-/-}$ platelets was impaired.

A. ATP release was comparable in WT and migfilin$^{-/-}$ platelets stimulated by 1 U/ml thrombin (n = 4), Results are expressed as mean ± SEM. Statistical significance was evaluated with paired Student t test, ns, no significant difference.

B. Serotonin content was comparable in WT and migfilin$^{-/-}$ platelets (n = 10). Results are expressed as mean ± SEM. Statistical significance was evaluated with Student t test, ns, no significant difference.

C. Impaired release of serotonin from dense granule in migfilin$^{-/-}$ platelets stimulated with collagen (0.4, 0.8 µg/ml) or thrombin (0.018, 0.025 U/ml). The results are shown as mean ± SEM (*P <0.05, **P <0.01, ***P <0.001, ns, no significant difference, paired Student t test).

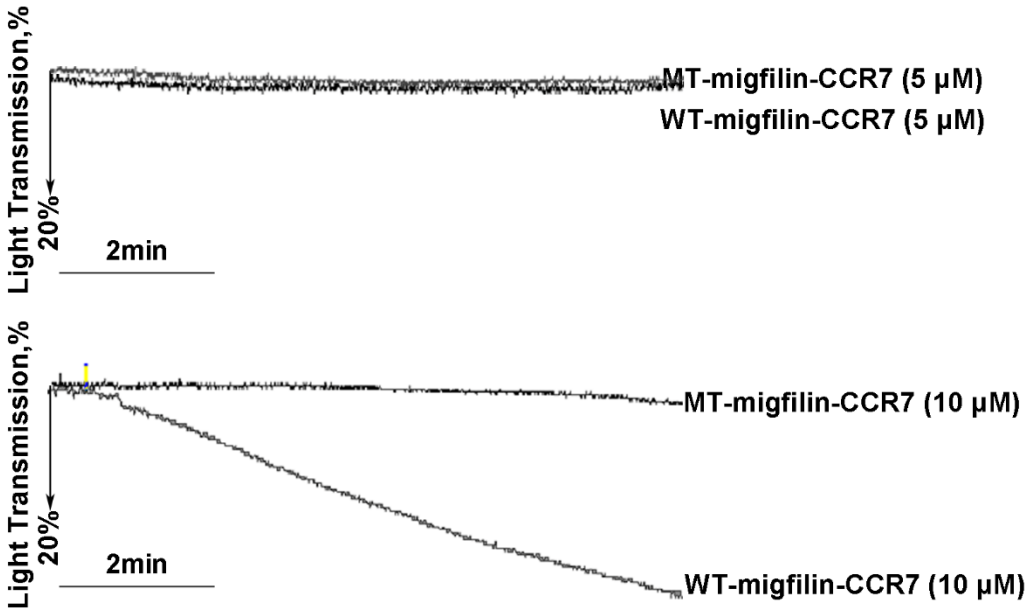

Figure S5. WT-migfilin-CCR7 (5 μM) peptide cannot induce platelet aggregation. Addition of different concentration migfilin peptides to a washed platelet suspension. WT-migfilin-CCR7 (10 μM) induced significant aggregation of the platelets. This effect was not observed at the concentration of 5 μM WT-migfilin-CCR7 or MT-migfilin-CCR7 (5 μM, 10 μM). Traces were representative of at least 3 independent experiments.

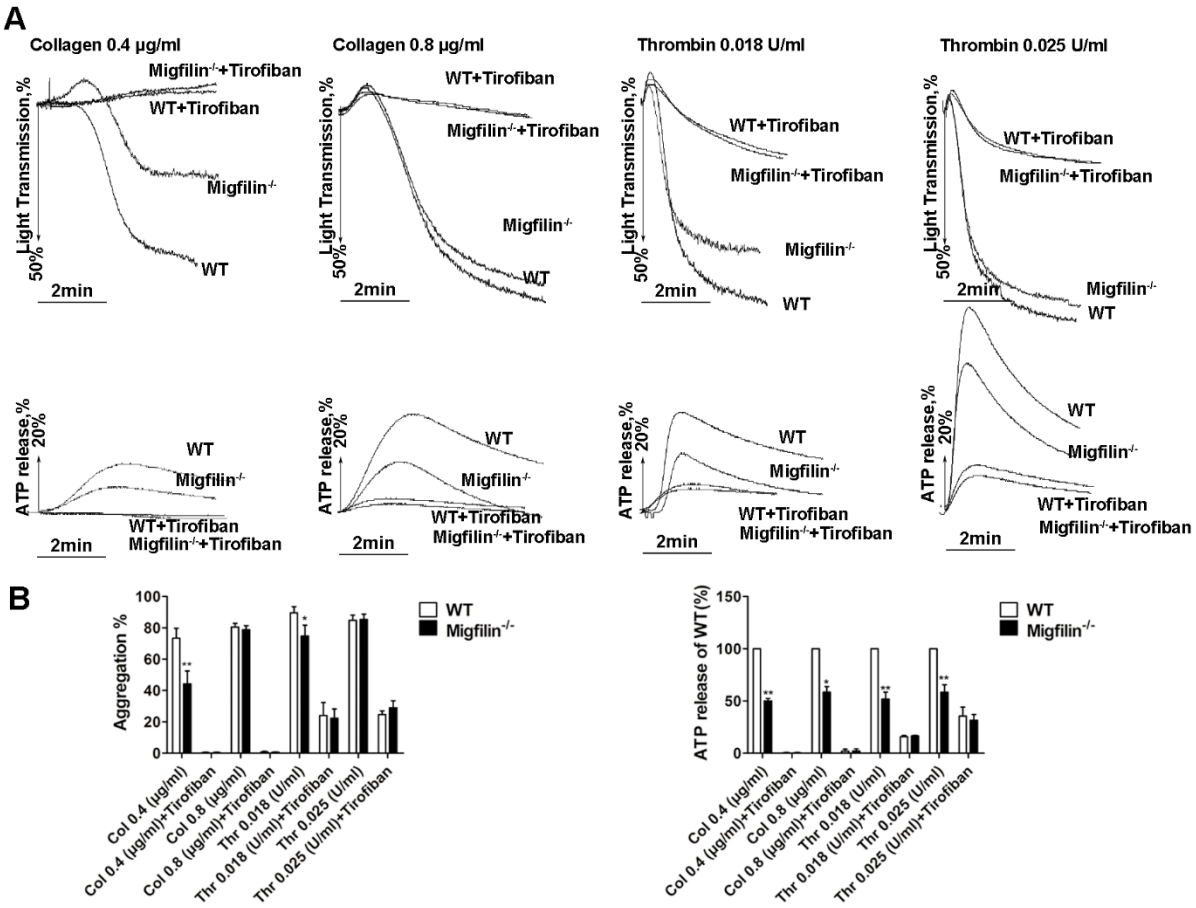

Figure S6. Migfilin-mediated platelet functions are dependent on ligand-αIIbβ3 engagement.

A. Aggregation and ATP release of washed WT or migfilin$^{-/-}$ platelets stimulated with collagen (0.4 µg/ml) or thrombin (0.018 U/ml) in the presence or absence of tirofiban (4 µg/ml) incubated for 10 min.

B. Percentage of platelet aggregation and ATP release from at least 4 independent experiments of panel A . The results are shown as mean ± SEM (**P* <0.05, ***P* <0.01, paired Student t test).

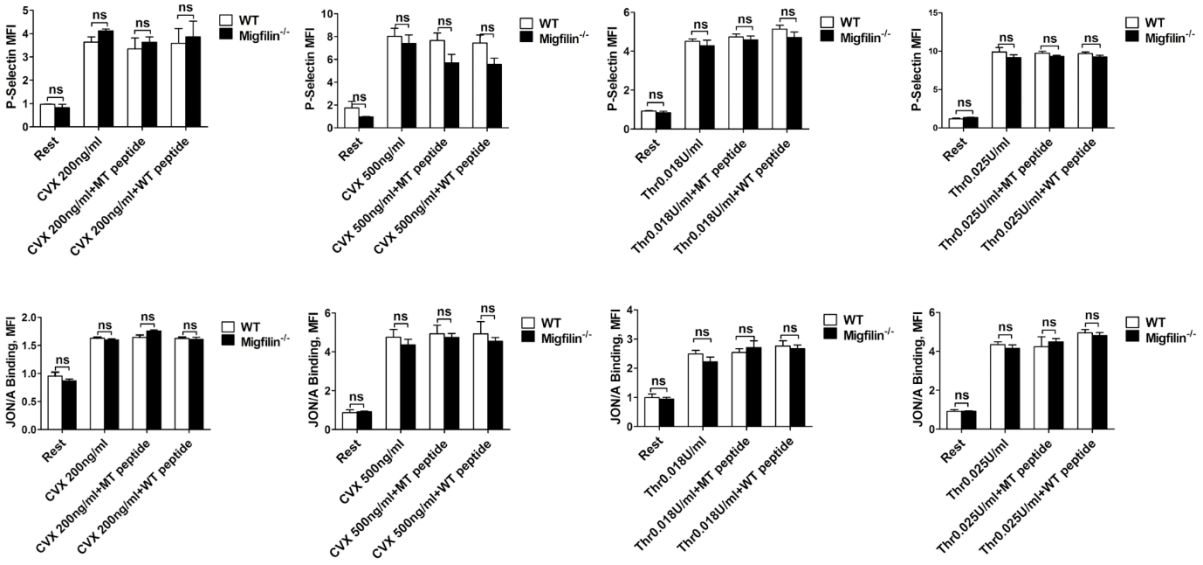

Figure S7. Secretion of α-granules and the conformational change of αIIbβ3 on single platelet was not affected by migfilin.

Flow cytometric analysis of integrin αIIbβ3 activation and P-selectin exposure in response to convulxin (CVX) or thrombin (Thr). Washed WT and migfilin$^{-/-}$ platelets were incubated with WT-migfilin-CCR7 (5 μM) or MT-migfilin-CCR7 (5 μM) peptide before stimulating with CVX (200 ng/ml, 500 ng/ml), Thr (0.018 U/ml, 0.025 U/ml) in the presence of FITC-labeled rat anti–mouse P-selectin mAb (RB40.34) and phycoerythrin-labeled rat anti–mouse integrin αIIbβ3 mAb (JON/A). After fixation, samples were measured in flow cytometer. The results are mean ± SEM, ns, no significance, Student t test. Experiments were repeated at least 3 times.

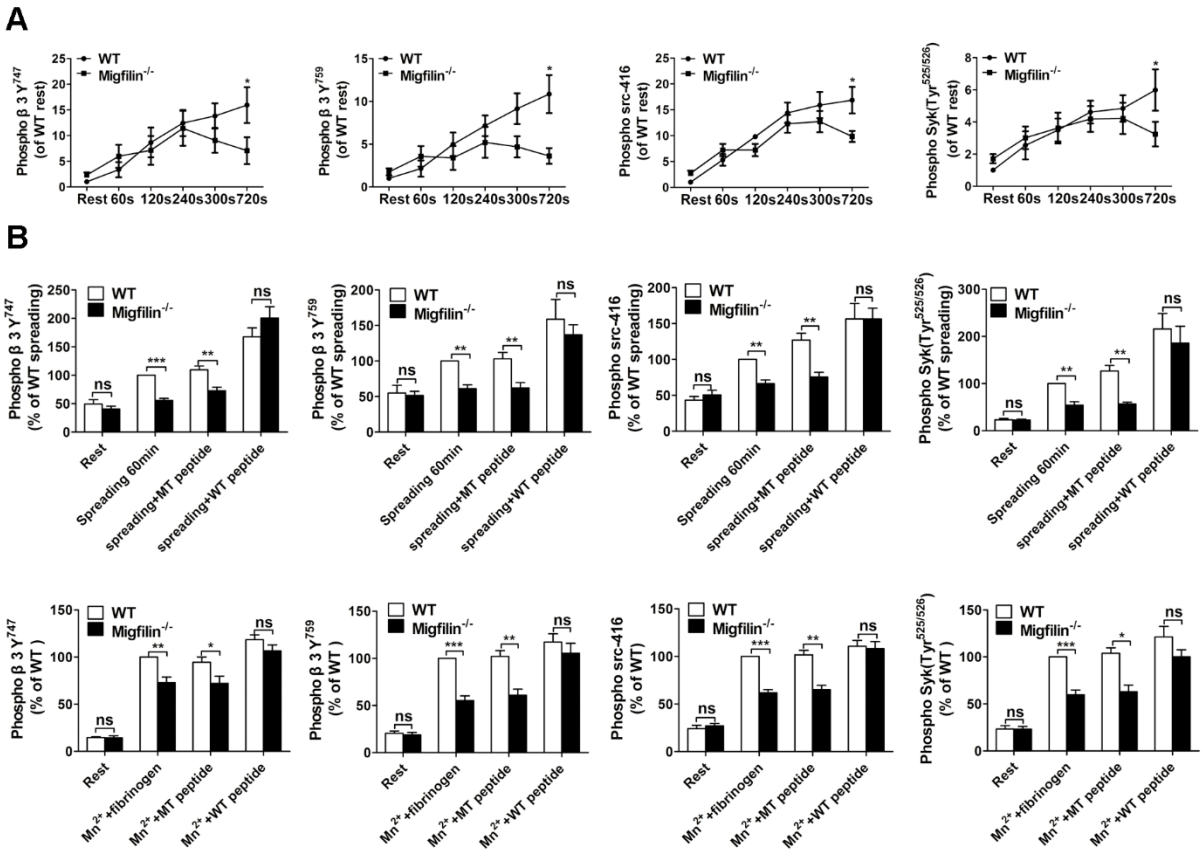

Figure S8. Migfilin deficient platelets displayed impaired early outside-in signaling phosphorylation events and WT-migfilin-CCR7 peptide rescued this defect.

A. Densitometry quantification of β3 Tyr^{747}, β3 Tyr^{759}, SFK Tyr^{416}, Syk $Tyr^{525/526}$ phosphorylation in platelets stimulated with Mn^{2+} (0.5 mM) in the presence of fibrinogen (25 μg/ml) in suspension at different time points. Results are presented as mean ± SEM from at least 3 independent experiments (**P* <0.05, paired Student t test).

B. Densitometry quantification of β3 Tyr^{747}, β3 Tyr^{759}, SFK Tyr^{416}, Syk $Tyr^{525/526}$ phosphorylation of WT and migfilin$^{-/-}$ platelets spread on fibrinogen for 60 min or stimulated with Mn^{2+} (0.5 mM) in the presence of fibrinogen (25 μg/ml) for 10 min, in presence or absence of migfilin peptides (5 μM). Results are presented as mean ± SEM (**P* <0.05, ***P* <0.01, ****P* <0.001, ns, no significance, paired Student t test).

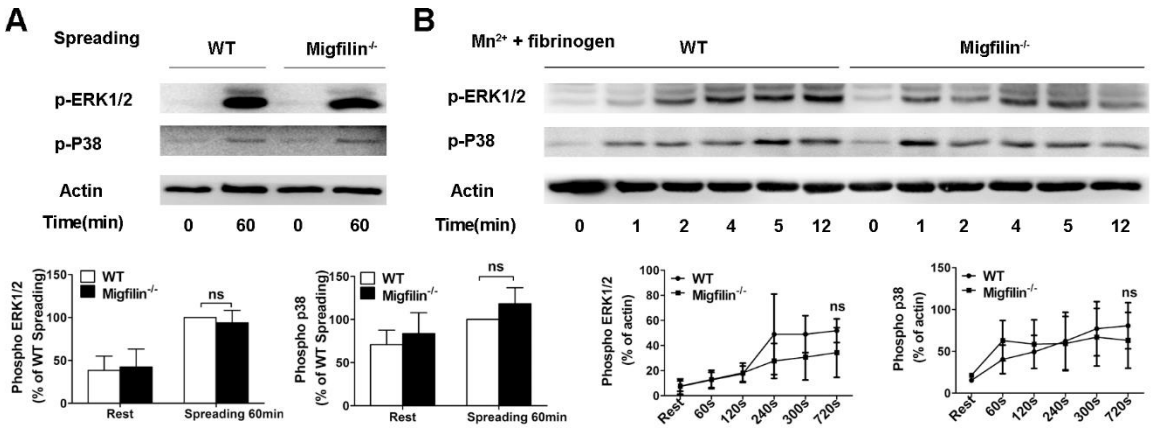

Figure S9. Phosphorylation of ERK and p38, was not drastically changed when provoked by either spread on immobilized fibrinogen or Mn^{2+} stimulation.

A. Measurement of phosphorylated ERK1/2 and p-38 in WT and migfilin$^{-/-}$ platelets spread on fibrinogen or stimulated with Mn^{2+} (0.5 mM) in the presence of fibrinogen (25 μg/ml) in suspension. At indicated time points, platelets were lysed and analyzed by western blot with antibodies recognizing phosphorylated ERK1/2 Thr^{202}/Tyr^{204} (T^{202}/Y^{204}), phosphorylated p38 Thr^{180}/Tyr^{182} (T^{180}/Y^{182}), total β-actin. Experiments were repeated at least 3 times.

B. Densitometry quantification of figure A. Results are presented as mean ± SEM from at least 3 independent experiments (ns, no significance, paired Student t test).

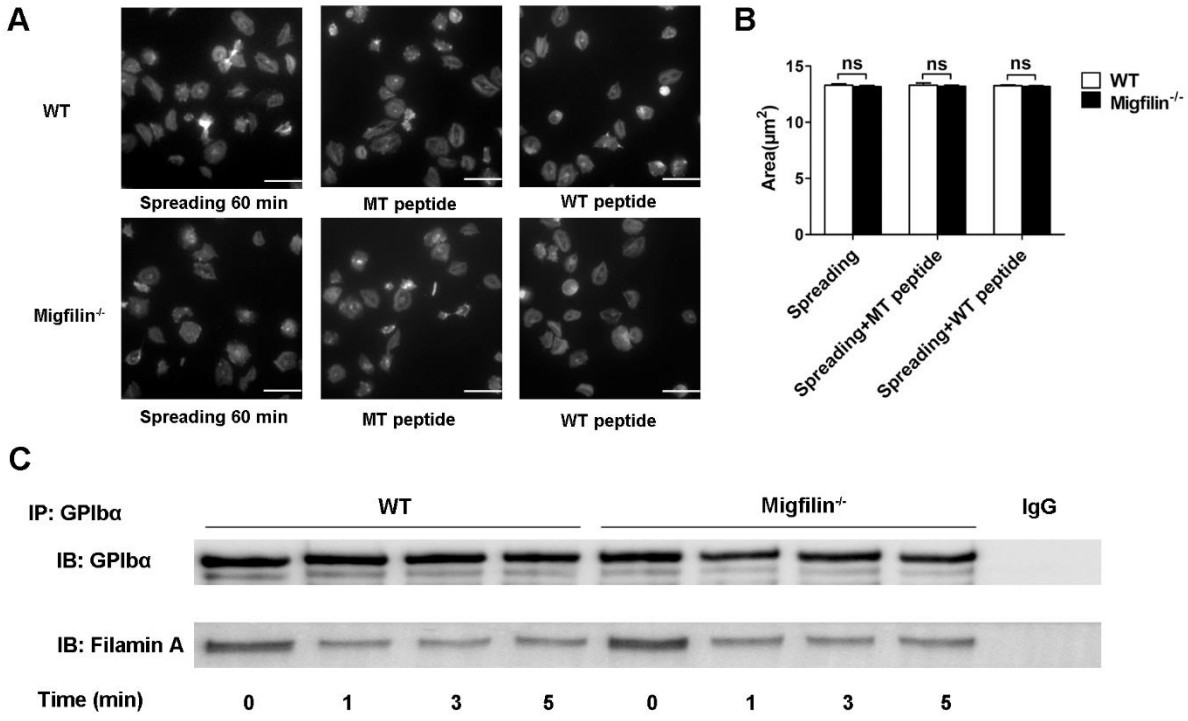

Figure S10. Normal platelet spreading on vWF and an unaltered binding between filamin A and GPIbα upon migfilin-deficiency.

A. Spreading of WT and migfilin$^{-/-}$ platelets on immobilized vWF in the presence or absence of migfilin peptides (5 μM). Ristocetin (1 mg/ml) was present in vWF-coated glass coverslips. Images are representative of 3 independent experiments with similar results. Original magnification ×100. Scale bars, 10 μm.

B. Quantification of the areas of spread (μm^2) of WT and migfilin$^{-/-}$ platelets (mean ± SEM, ns, no significant difference, Student t test).

C. Washed WT platelets in suspension were stimulated with ristocetin (1 mg/ml) in the presence of vWF (10 μg/ml) and lysed at indicated time points. Platelet lysates were immunoprecipitated with antibody against GPIbα and then immunoblotted with antibodies against filamin A and GPIbα, respectively. Representative result from at least 3 independent experiments.

Supplemental Video 1: Imaging of unstable thrombi formation in migfilin$^{-/-}$ whole blood under flow condition *in vitro*.

Whole blood from WT control (upper panel) or migfilin$^{-/-}$ (lower panel) mice were perfused through a collagen-coated surface in a microfluidic system at the shear rate of 1000 s^{-1} during 5 min. Video was recorded on streaming mode during 5 min and displayed within a length of 15 sec (accelerated 20x). Original magnification ×10. Time in mm:ss.

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