Supporting Information

Zhang et al. 10.1073/pnas.1610963114

SI Materials and Methods

Reagents and Antibodies. Thrombin, collagen, ADP, and Chrono-Lume were obtained from Chronolog. BSA, aspirin, pentobarbital sodium, Arg-Gly-Asp-Ser (RGDS), and Nonidet P-40 were from Sigma-Aldrich. Calcein acetylmonethyl ester (calcein-AM) was from Dojindo Molecular Technologies. U46619, LY294002, and human fibrinogen were from Calbiochem. FITC-conjugated anti-mouse P-selectin (Wug.E9), PE-conjugated anti-mouse active integrin αIIbβ3 (JON/A), and anti-mouse GPIbα antibodies were from Emfret Analytics. Mouse anti-RIP1 antibody was from BD Biosciences. RIP3 inhibitor GSK-872 was from Merck Millipore. PE-conjugated anti-mouse CD41 antibody was from BioLegend. FITC-conjugated mouse fibrinogen, anti-mouse phospho-MLKL (Ser345), anti-CD45, and anti-mouse TP antibodies, Annexin V-FITC, GST-tagged active human RIP3 full-length protein, and the PF4 ELISA Kit were from Abcam. Anti-mouse MLKL antibody was from Abgent. The TXB2 Enzyme Immunoassay (EIA) Kit was from ENZO Life Sciences. Anti-mouse RIP3 antibody was from ProSci. Anti- phospho-Akt (Thr308), anti-total Akt, anti-ERK1/2 antibody, anti- phospho-ERK1/2, anti-GAPDH, and anti-human RIP3 antibodies were from Cell Signaling Technology. Alexa Fluor 488-conjugated phallodin was from Life Technologies. Anti-mouse P2Y12 antibody was from Alomone Labs. HRP-conjugated anti-rabbit IgG, anti-mouse PAR-3/4, Gqα, Gi and Gq, anti-6xHis, and anti-GST antibodies, Complete Protease Inhibitor Mixture tablet, Protein A/G Plus Agarose, and rabbit IgG were from Santa Cruz Biotechnology. Botrocetin was kindly provided by Dr Junling Liu, Department of Biochemistry and Molecular Cell Biology, Shanghai Jiaotong University, China. Glutathione-one-conjugated Sepharose beads were from GE Healthcare. Recombinant His-tagged human Gq11 full-length protein was from NewEast Biosciences.

Hematologic Analysis and Tail Bleeding Time. Complete blood cell counts were performed with a Sysmex XP-100 Hematologic Analyzer. In the tail bleeding time experiments, 6- to 8-wk-old mice (equivalent numbers of males and females) were anesthetized with 2% pentobarbital. Tails were amputated 3 mm from the tip and immediately immersed into isotonic PBS at 37 °C. For each tail, time until bleeding had stopped (i.e., no rebleeding within 60 s), or a maximum of 600 s, was recorded.

In Vivo Thrombosis. In the ferric chloride (FeCl3)-induced mesenteric arteriole thrombosis model experiment, platelets were isolated from donor mice and labeled with calcine-AM (5 μg/mL). Male mice were injected i.v. with calcine-labeled platelets (5 × 10^7/g) of matching genotype. The recipient mice were anesthetized. In inhibition experiments, GSK-872 (96 μM) or vehicle (DMSO) was retrobulbarly injected and then circulated for 30 min. The mesentery vascular bed was exteriorized, and one arteriole was chosen and visualized with an inverted fluorescence microscope (Leica Microsystems), and recorded on videotape. Thrombus formation was induced by topical application of a 3-mm² filter paper soaked with 5% FeCl3. The vessel occlusion time was defined the time to complete cessation of blood flow.

Irradiation and Bone Marrow-Derived Cell Repopulation. Male WT and RIP3<sup>−/−</sup> mice (10 wk old) were lethally irradiated with 8 Gy from a cobalt-60 γ radiation source. Bone marrow-derived cells were harvested from 8-wk-old WT and RIP3<sup>−/−</sup> male mice and injected into irradiated recipient mice (1 × 10^7 donor cells per animal). The mice were used in experiments when blood cell counts recovered. RIP3 expression in platelets of each recipient mouse was detected by immunoblotting.

Platelet Aggregation and Secretion. Platelet aggregation and secretion were recorded in a Chrono-Log lumi-aggregometer. Washed platelets (3 × 10<sup>8</sup>/mL) were stimulated with different agonists. Luciferin/luciferase (10 μL) was added to 240 μL of washed platelet suspension within 2 min before stimulation. In some experiments, platelets were pretreated with inhibitors at 37 °C before the addition of agonists. Platelet aggregation was monitored continuously over 5–10 min.

Electron Microscopy. Platelets were fixed in 2.5% glutaraldehyde at 4 °C overnight and then centrifuged at 600 × g for 2 min. Platelet 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 a Hitachi H600 transmission electron microscope. For the measurement of platelet morphology and granule content, micrographs of 20 platelets from WT and RIP3<sup>−/−</sup> mice were analyzed from five different slides for each strain.

Measurement of TXA<sub>2</sub> Generation. TXA<sub>2</sub> generation was assayed as its stable metabolite, TXB<sub>2</sub>, in conditions of platelet aggregation induced by aforesaid agonists. Platelet aggregation was stopped after 8 min of stimulation by adding ice-cold EDTA (1 mM) and aspirin (1 mM). Samples were centrifuged, and the supernatants were stored at −80 °C to avoid repeated freeze-thaw cycles. Levels of TXB<sub>2</sub> were measured using the TXB<sub>2</sub> EIA Kit following the manufacturer’s protocol.

Flow Cytometry Analysis. Washed platelets (3 × 10<sup>8</sup>/mL) were stimulated with agonists. Antibodies were added simultaneously with agonists as indicated. After incubation for 15 min at room temperature, the reaction was stopped by adding PBS, and the samples were analyzed with a Cytomics FC500 MCL flow cytometer (Beckman Coulter).

Assessment of Fibrinogen Binding. Washed platelets were incubated with FITC-conjugated mouse fibrinogen (135 μg/mL), and thrombin or vehicle at 37 °C for 30 min. The reaction was stopped by adding PBS, and flow cytometry analysis was performed.

Immunoblotting Detection. Washed platelets were exposed to U46619 or thrombin for different times. Platelets were then lysed and probed by immunoblotting with the antibodies indicated in the figures.

Coimmunoprecipitation. Washed platelet suspensions (2 × 10<sup>8</sup> platelets) were lysed with equal volumes of 2 × Nonidet P-40 lysis buffer (100 mM Tris pH 7.4, 2% Nonidet P-40, 20 mM MgCl₂, 300 mM NaCl, 2 mM PMSF, 2 mM NaF, and 2 mM Na<sub>2</sub>VO<sub>4</sub>) containing protease inhibitor mixture tablets on ice for 30 min. After centrifugation at 17,000 × g and 4 °C for 10 min, the supernatants were immunoprecipitated with antibodies overnight. After incubation with Protein A/G Plus Agarose beads at 4 °C for 2 h, the beads were analyzed by immunoblotting.
Plasmids and Oligos. The GST sequence in a pGS-21a vector (Genescript) was amplified by PCR and subcloned into pcDNA3.1(+) using NheI and NotI (forward primer, 5′ggagaccaagtctgactcatgccctcctactagta; reverse primer, 5′tctagactcggagccttggccctcctactagta). The RIPC3 gene in pCI-neo plasmid (Promega) and the GST sequence in the pGS-21a vector was amplified by overlap methods using the following primers: GST, forward: 5′ggagaccaagtctgactcatgccctcctactagta; GST-RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta; GST-RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta; RIPC3, forward: 5′tctagactcggagccttggccctcctactagta; RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta. The GST-RIPC3 DNA sequence was subcloned into pcDNA3.1(+) using NheI and NotI. The GST-RIPC3 N-terminal (1–350 aa) sequence was amplified by overlap methods using the following primers: GST, forward: 5′ggagaccaagtctgactcatgccctcctactagta; GST-RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta; RIPC3, forward: 5′tctagactcggagccttggccctcctactagta; RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta. The GST-RIPC3-N DNA was subcloned into pcDNA3.1(+) using NotI and NheI, respectively. The GST-RIPC3 C-terminal (351–518 aa) sequence was amplified by overlap methods using the following primers: GST, forward: 5′ggagaccaagtctgactcatgccctcctactagta; GST-RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta; RIPC3, forward: 5′tctagactcggagccttggccctcctactagta; RIPC3, reverse: 5′tctagactcggagccttggccctcctactagta. The GST-RIPC3-C DNA was subcloned into pcDNA3.1(+) using NheI and NotI. The GSTα13 gene in pCMV3 (Sino Biological) was amplified and subcloned into pcDNA3.1(+) using NheI and NotI at the C terminus of the GSTα13 protein (forward primer: 5′ggagaccaagtctgactcatgccctcctactagta; reverse primer: 5′tctagactcggagccttggccctcctactagta). The constructs were verified by sequencing.

Expression and Purification of Proteins. HEK 293T cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics at 37 °C in a 5% CO2 atmosphere. The HEK 293T cells were transfected by pcDNA3.1(+) containing target DNAs using Lipofectamine 2000 (Thermo Fisher Scientific). The cells were cultured for 48 h after transfection and then harvested. The cells were washed and resuspended in the lysis buffer (1× PBS pH 7.4 and 0.5% Nonidet P-40), containing 1× protease inhibitor mixture at a ratio of 1 mL of lysis buffer per 2 × 107 cells. The cells were lysed by sonication in five cycles (5 s on 30% power, 30 s off) on ice. The crude lysate was centrifuged at 20,000 × g for 30 min at 4 °C. The cleared lysate was then mixed with GST resin, followed by incubation for 2 h at room temperature. The resin was centrifuged, washed, and then resuspended in 500 μL of elution buffer (10 mM reduced glutathione and 50 mM Tris, pH 8.0), followed by another centrifugation at 2,000 × g for 5 min. The supernatant containing the purified protein was assayed by SDS/PAGE and Western blot analysis.

ELISA. Proteins or BSA control (4 μg/mL) was immobilized onto microtiter plates at 37 °C for 1 h, and the wells were blocked with 3% BSA. Increasing concentrations of His-tagged GSTα13 were incubated with immobilized RIPC3 or BSA at 37 °C for 1 h, and the wells were washed three times with PBST (0.2% Tween-20). After incubation with mouse anti-His antibody (5 μg/mL) and HRP-conjugated goat anti-mouse antibody (1:8,000), standard wash substrate (tetramethylbenzidine; Thermo Fisher Scientific) was added to each well, and the plates were incubated at room temperature for 10 min. The reaction was stopped by addition of an equal volume of 2 M H2SO4. The plates were read at 450 nm in a Variskan Flash spectral scanning multimode reader (Thermo Fisher Scientific).

Transfection and Protein-Binding Assays. The pcDNA3.1(+) expressing His-GSTα13 gene was cotransfected with the pcDNA3.1(+) expressing GST or GST-RIPC3 gene into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were cultured for 48 h after transfection in DMEM supplemented with 10% FBS, then harvested and lysed by 1× lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF, and Protease Inhibitor Mixture). The lysate was centrifuged at 12,500 × g for 15 min at 4 °C. The clarified lysates were then mixed with glutathione-conjugated Sepharose beads and incubated at 4 °C overnight. The beads were washed with 1× lysis buffer three times, and the bead-bound proteins were analyzed by immunoblotting.

Platelet Spreading on Immobilized Fibrinogen. Chamber slides with microtiter wells were coated with 10 μg/mL fibrinogen in 0.1 M NaHCO3 (pH 8.3) at 4 °C overnight. Washed platelets (2 × 107/mL) were allowed to adhere to and spread on fibrinogen-coated wells at 37 °C for 2 h with stimulation by thrombin. After washing, the cells were fixed, permeabilized, and stained with Alexa Fluor 488-conjugated phalloidin. Adherent platelets were viewed with an Olympus FluoView FV1000 confocal microscope. Images were acquired and the spreading area of single platelets was measured using ImageJ2x software, with pixel number as the unit of size. Ten randomly selected fields from at least three different tests were used for statistical analysis.

Clot Retraction. Washed mouse platelets (4 × 108/mL) were resuspended in modified Tyrode’s buffer in unused aggregometer tubes and mixed with 150 μg/mL purified human fibrinogen. The clots were initiated by the addition of human α-thrombin to a final concentration of 1 U/mL, followed by incubation at 37 °C for 60 min. Clot retraction was monitored every 5 min and photographed. Clot size was quantified from photographs using Image J2x software.

Statistics. All of the reported figures are derived from at least three independent experiments. Data are expressed as mean ± SEM. Statistical analysis was performed using Prism version 6.0 (GraphPad Software), and the data were compared using the unpaired Student t test (except for the data in Fig. 1, which were compared using the two-tailed Mann–Whitney U test). For all analyses, a P value <0.05 was considered to indicate statistical significance.
Fig. S1. Expression levels of TP and PAR3/4 in WT and RIP3-deficient platelets. Whole blood was collected and anticoagulated with 1/7 volume of ACD. The blood was centrifuged at 200 g for 11 min to yield platelet-rich plasma (PRP). PRP was then washed and resuspended in modified Tyrode’s buffer (3 × 10⁸/mL). The washed platelets were lysed with SDS/PAGE loading buffer, and probed by Western blot analysis with anti-mouse PAR-3/4 (Santa Cruz Biotechnology) and TP (Abcam) antibodies. Blotting to GAPDH (anti-GAPDH antibody; Santa Cruz Biotechnology, CA) was used as a loading control. The figures are representative of three independent experiments.

Fig. S2. RIP3 deficiency does not affect ATP secretion in platelets stimulated with collagen. Washed platelets from WT and RIP3−/− mice were stimulated with 0.5, 1.0, 1.5, and 2.0 μg/mL collagen. ATP secretion was recorded concomitantly with platelet aggregation in the presence of luciferin/luciferase reagent at 37 °C at a stirring speed of 1,000 rpm.

Fig. S3. Effects of apyrase on platelet aggregation in response to low doses of U46619 or thrombin. Washed platelets (3 × 10⁸/mL) from WT and RIP3−/− mice were stimulated with 100 nM U46619 or 0.006 U/mL thrombin in the presence or absence of apyrase (1 U/mL) at 37 °C under constant stirring. Platelet aggregation was monitored using a turbidimetric aggregometer. The traces are representative of three independent experiments.
Fig. S4. Analysis of α-granule secretion in RIP3−/− platelets. Flow cytometry analysis of P-selectin exposure in mouse platelets stimulated with the indicated doses of U46619 (nM) (A) or thrombin (U/mL) (B). Data are expressed as mean fluoresce intensity ± SEM of three independent experiments. There was no statistically significant difference between the two groups.

Fig. S5. PF4 release from WT and RIP3−/− mouse platelets stimulated with U46619 and thrombin. Washed WT and RIP3−/− mouse platelets were stimulated with different concentrations of U46619 (A) or thrombin (B) for 8 min at 37 °C with stirring. The platelet suspensions were centrifuged, and the supernatants were analyzed for PF4 release using the PF4 ELISA Kit (Abcam). Quantitative results are expressed as mean ± SEM of three independent experiments.

Fig. S6. TXB2 generation in the RIP3−/− platelets. TXB2 production in WT and RIP3−/− platelets stimulated with thrombin (0.008 U/mL), ADP (2.5 μM), collagen (0.5 μg/mL), or vehicle (control). All data are expressed as mean ± SEM of three independent experiments. *P < 0.05, Student’s t test.
Fig. S7. PS exposure in mouse platelets stimulated with thrombin and U46619. Washed WT and RIP3−/− platelets were stimulated with different concentrations of thrombin (A) or U46619 (B), or with thrombin (1 U/mL) + collagen (5 μg/mL) (C) for 30 min at 37 °C. The platelets were further incubated with Annexin V-FITC for 10 min and then analyzed by flow cytometry. Results from a representative experiment stimulated with selected concentrations of thrombin and U46619 are shown.
Fig. S8. U46619- and thrombin-induced integrin αIIbβ3 activation in RIP3−/− platelets. (A and B) Flow cytometry analysis of PE-JON/A binding to WT and RIP3−/− platelets stimulated with vehicle (control) or the indicated doses of U46619 (A) or thrombin (B). Results from a representative experiment are shown. Quantitative results are expressed as mean ± SEM of three independent experiments. *P < 0.05. (C) Flow cytometry analysis of FITC-labeled fibrinogen binding to WT and RIP3−/− platelets stimulated with thrombin (0.002 and 0.004 U/mL) or vehicle (control). Results from a representative experiment are shown. Quantitative results from three independent experiments are expressed as mean fibrinogen binding index [total bound fluorescence (thrombin)/nonspecifically bound fluorescence (vehicle)] ± SEM. ***P < 0.001.

Fig. S9. Coimmunoprecipitation of RIP3 with Gi or Gq in mouse platelets. Washed platelets (3 × 10⁸/mL) from WT mice were lysed with equal volumes of 2× Nonidet P-40 lysis buffer containing protease inhibitor mixture tablets on ice for 30 min. After centrifugation at 17,000 × g at 4 °C for 10 min, the supernatants were immunoprecipitated with anti-RIP3 antibody or IgG (control) overnight. After incubation with protein A/G plus-agarose beads at 4 °C for 2 h, the proteins were analyzed by Western blot analysis with anti-Gq and anti-Gi antibodies. The figures are representative of at least three independent experiments.
Fig. S10. Interaction of Gα13 with RIP3 in platelets stimulated with thrombin. Washed platelets (3 × 10⁸/mL) from WT mice were stimulated with 0.006 U/mL thrombin for the indicated times at 37 °C under stirring. The platelets were lysed with equal volumes of 2× Nonidet P-40 lysis buffer containing protease inhibitor mixture tablets on ice for 30 min. After centrifugation at 17,000 × g and 4 °C for 10 min, the supernatants were immunoprecipitated with anti-Gα13 antibody or IgG (control) overnight. After incubation with Protein A/G Plus Agarose beads at 4 °C for 2 h, the proteins were analyzed by Western blot analysis with anti-RIP3 and anti-Gα13 antibodies. The figures are representative of three independent experiments.

Fig. S11. The interaction of Gα13 with RIP3. Increasing concentrations of Gα13 (His-tag; NewEast Biosciences) were incubated with immobilized RIP3 (Abcam) or BSA (control). The binding of Gα13 was assessed with mouse anti-His antibody and HRP-conjugated goat anti-mouse antibody. The absorbance at 450 nm was measured in four independent experiments, and values are presented as the mean ± SD after subtracting the binding of Gα13 to BSA (background). After the background signal had been subtracted, the binding curve was fitted to the equation Y = Bmax × x/(Kd + x), where Y is the specific binding, x is the ligand concentration, Bmax is the binding maximum, and Kd is the equilibrium dissociation constant. For some data points, the error bars are smaller than the symbols.

Fig. S12. Gα13 binds to Gβ in both WT and RIP3−/− platelets. Washed WT and RIP3−/− mouse platelets (3 × 10⁸/mL) were lysed with equal volumes of 2× Triton X-100 lysis buffer containing protease inhibitor mixture tablets on ice for 30 min. After centrifugation at 12,500 × g for 10 min at 4 °C, the supernatants were incubated with 2 μg/mL rabbit IgG and anti-Gα13 (Santa Cruz Biotechnology) antibody for 2 h at 4 °C, and then immunoprecipitated with Protein G Plus Agarose beads at 4 °C overnight. After three washes with lysis buffer, the beads were analyzed by immunoblotting. The figures are representative of three independent experiments.
**Fig. S13.** Role of RIP3 in ERK phosphorylation. Washed platelets were stimulated with 350 nM U46619 or 0.006 U/mL thrombin for the indicated times at 37 °C with stirring. The platelets were lysed and analyzed by Western blot analysis with anti-phospho-ERK1/2 and anti-total-ERK1/2 antibodies. The figures are representative of three independent experiments.

**Fig. S14.** RGDS peptides abolish U46619-induced Akt phosphorylation. Washed WT and RIP3−/− mouse platelets were preincubated with RGDS (2 mM) or vehicle (−RGDS) at 37 °C for 5 min. Then 350 nM U46619 was added to platelets in an aggregometer with stirring for 5 min. The platelets were lysed and analyzed by Western blot analysis with anti-phospho-Akt (Thr308) and anti-total Akt antibodies. The Western blot shown is representative of at least three independent experiments.

**Fig. S15.** The effects of GSK’872 on mouse platelet aggregation. Washed mouse platelets were incubated with GSK’872 (6 μM) or vehicle (DMSO) at 37 °C for 30 min, and then stimulated with U46619 and thrombin. Platelet aggregation was monitored using a turbidimetric aggregometer. The traces are representative of three independent experiments.

**Table S1.** Hematologic analysis of WT and RIP3−/− mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT (n = 27)</th>
<th>RIP3−/− (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, ×10¹²/L, mean ± SEM</td>
<td>7.935 ± 0.113</td>
<td>8.186 ± 0.185</td>
</tr>
<tr>
<td>WBC, ×10⁹/L, mean ± SEM</td>
<td>3.779 ± 0.233</td>
<td>4.421 ± 0.308</td>
</tr>
<tr>
<td>Hemoglobin, g/dL, mean ± SEM</td>
<td>12.37 ± 0.196</td>
<td>12.57 ± 0.265</td>
</tr>
<tr>
<td>MCV, fl, mean ± SEM</td>
<td>51.47 ± 0.274</td>
<td>49.92 ± 0.261*</td>
</tr>
<tr>
<td>MPV, fl, mean ± SEM</td>
<td>5.648 ± 0.029</td>
<td>5.712 ± 0.046</td>
</tr>
</tbody>
</table>

MCV, erythrocyte mean corpuscular volume; MPV, mean platelet volume.

No significant difference between WT and RIP3−/− mice was observed for RBC, WBC, hemoglobin, and MPV.

*P < 0.001.