Animals

Megakaryocyte- and platelet-specific Gα_{13}-deficient mice were generated using the PF 4 Cre/lox P system. Gna13^{fl/fl} mice were crossed with PF 4 Cre mice resulting in Gna13^{fl/fl,PF4-Cre} (Gα_{13}^-/-) mice. Littermate Gna13^{fl/fl} mice served as controls (Gα_{13}^+/-).

Luminometric measurement of ATP release

Washed platelets (240 µl with 0.3x10^6 platelets/µL) were incubated with Luciferase-Luciferin reagent, followed by agonist addition. ATP release and aggregation were measured simultaneously on a Lumi-Aggregometer (Chrono-Log, Havertown, PA).

Intracellular calcium measurements

Washed platelets were suspended in calcium-free Tyrode-HEPES buffer and loaded with fura-2/AM (5 µM; Molecular Probes) in the presence of Pluronic F-127 (0.2 µg/mL; Molecular Probes) for 30 min at 37°C. After labeling, platelets were washed once and resuspended in calcium-free Tyrode-HEPES buffer. Stirring platelets were stimulated with the indicated agonists and fluorescence was measured with a PerkinElmer LS55 fluorimeter (Waltham, MA). Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Each measurement was calibrated using Triton X-100 and EGTA and changes in intracellular Ca^{2+} concentration ([Ca^{2+}]_i) were calculated.

In vivo platelet depletion

To deplete platelets in vivo 2.5 µg p0p/B^3 were i.v. injected into wild-type mice; a second group of wt mice were injected with vehicle and served as control. 24 h after injection the platelet count of the antibody-treated animals was determined. Mice displaying 30-50% of the platelet counts of wt mice were used in experiments.
REFERENCES


Figure S1. Analysis of megakaryocyte numbers and platelet myosin light chain phosphorylation in RhoA−/− mice

(A),(B) Increased number of megakaryocytes (MK) with high ploidy in bone marrow of RhoA−/− mice. (A) Femurae from 8 to 12 weeks old mice were fixed and decalcified (14 days in PBS 10% EDTA; 4°C) and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin. Numbers of MK per visual field (400×) of at least 15 visual fields were counted (n=3). (B) Bone marrow was flushed out of femurae from adult wt and RhoA−/− mice. MK were stained with a FITC-coupled anti-CD41 antibody (BD Biosciences) and propidium iodide. Ploidy levels of the CD41-positive cell populations were determined by flow cytometry (wt: n=3; RhoA−/−; n=5 mice). **P<0.01, ***P<0.001. (C),(D) Western blot analysis of platelet lysates from wt and RhoA−/− platelets after stimulation with the indicated agonists (C) and under unstimulated conditions (D). Expression levels of myosin light chain (MLC) and phosphorylated MLC (MLC-P) were assessed using appropriate antibodies. GPIIIa expression served as loading control. Abbreviations: U46, U46619; thr, thrombin. (E) Numbers of dense granules in transmission electron microscopical pictures of unstimulated RhoA−/− and wild-type platelets were counted manually and the % of platelets containing no (0), 1, 2, 3, or 4 granules was calculated (wt: n=90 platelets; RhoA−/−: n=140 platelets).

Figure S2. Normal glycoprotein expression levels but partially altered α granule release in Go13−/− platelets

(A) Peripheral platelet counts and platelet volume of Go13+/+ and Go13−/− mice were determined with a blood cell counter. Results are mean ± SD of 7 mice per group and are representative of 3 individual measurements. Fl, femtoliter. Glycoprotein expression on the platelet surface was determined by flow cytometry. Diluted whole blood from the indicated mice was incubated with FITC-labeled antibodies, and platelets were analyzed directly. Results are expressed as mean fluorescence intensity ± SD (n=4) and are representative of 3 individual experiments. MPV
indicates mean platelet volume; n.s., not significant. (B) Integrin αIIbβ3 activation and P-selectin exposure in Gα13⁺ and Gα13⁻ platelets after activation. Top, Flow cytometric analysis of integrin αIIbβ3 activation (binding of JON/A-PE) and bottom, degranulation-dependent P-selectin exposure in response to the indicated agonists in Gα13⁺ and Gα13⁻ platelets. Results are mean fluorescence intensities (MFI) ± SD of 4 mice per group and representative of 3 individual experiments. * P <0.05, ** P <0.01.

Figure S3. Decreased ATP release in RhoA⁻ platelets after G₁₃ stimulation but normal Ca²⁺ mobilization upon activation

(A) Washed wt and RhoA⁻ platelets were incubated with Luciferase-Luciferin reagent and ATP release was measured in a Lumi-aggregometer (Chrono Log, Haverton, PA) after stimulation with the indicated agonists. Representative curves of 2 individual experiments with n=2 are shown. (B),(C) Fura-2–loaded washed wt and RhoA⁻ platelets were suspended in calcium-free Tyrode-HEPES buffer and stimulated with 3 µM U46619, 0.005 U/mL thrombin, or 10 µg/mL CRP, changes in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) were calculated. (B) Representative measurements of wt (black line) and RhoA⁻ platelets (gray line) and (C) maximal increase in [Ca²⁺]ᵢ compared with baseline levels before stimulus (Δ[Ca²⁺]ᵢ) ± SD of at least 10 mice per group are shown.

Figure S4 . RhoA⁻ platelets display largely normal spreading morphology

(A–C) Washed platelets of wt and RhoA⁻ mice were allowed to spread on fibrinogen (200 µg/mL) after stimulation with 0.01 U/mL thrombin. (A) Scanning electron microscopy (SEM) pictures of spread wt and RhoA⁻ platelets from the indicated time points. Bar, 2.5 µm. (B) Visualization of filamentous actin (red) and vWF (green) in spread (20 min) RhoA⁻ and wt platelets by stimulated emission depletion (STED) microscopy. Bar, 5 µm. (C) Detailed visualization of actin filaments in spread platelets by STED microscopy. Bar, 2.5 µm. (D) Flow
cytometric determination of F-actin content in wt and RhoA−/− platelets. Washed unstimulated or activated platelets (1 U/mL thrombin) were fixed, permeabilized, stained with phalloidin-FITC (10 µM) and directly analyzed. Values are mean ± SD of at least 4 mice per group. Abbreviation: thr, thrombin. (E) Normal spreading of Gα13-deficient platelets. Washed platelets of Gα13+/+ and Gα13−/− mice were allowed to spread on fibrinogen (200 µg/mL) for 30 min after stimulation with 0.01 U/mL thrombin. Representative differential interference contrast images are shown. Bar, 5 µm.

**Figure S5. Aggregate formation of RhoA−/− platelets on collagen under flow** Whole blood was perfused over a collagen-coated surface at a shear rate of 1700 sec−1 (A),(B), and 7700 sec−1 (C). (A) Results using RhoA-deficient blood with reconstituted platelet counts. Top, Representative phase contrast images at the end of the perfusion time and bottom, mean surface coverage ± SD are depicted (n=5) (B) Results without reconstitution of platelet counts in RhoA-deficient blood. Mean absolute surface coverage (left) and coverage relative to the platelet count (right) ± SD are shown (n=5). Right: Bar, 50 µm. *P<0.05. (D) Stable occlusive thrombus formation in wild-type mice displaying platelet counts and platelet size comparable to RhoA−/− mice. Wild-type mice were either treated with vehicle (control) or 2.5 µg p0p/B (depl.) 24 h prior to injury to deplete platelets to 30–50% of normal levels. Left: comparison of the platelet size of wild-type, RhoA−/−, and platelet-depleted wild-type mice. Platelet size expressed as mean forward scatter (FSC) was determined by flow cytometry. Right: Thrombus formation was induced by a single firm compression of the abdominal aorta with a forceps. The graph shows time to stable vessel occlusion. Each symbol represents one individual. ***P<0.001, *P<0.05.
Figure S1
Figure S2

### Table A

<table>
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<tr>
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<th>$G_{\alpha_{13}}^{++}$</th>
<th>$G_{\alpha_{13}}^{--}$</th>
<th>significance</th>
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<tr>
<td>Count, $x10^3$/µl</td>
<td>982 ± 61</td>
<td>911 ± 113</td>
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<td>MPV, fl</td>
<td>5.5 ± 0.2</td>
<td>5.8 ± 0.4</td>
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<td>GPIb</td>
<td>328 ± 5.7</td>
<td>314 ± 16</td>
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<td>GPV</td>
<td>291 ± 6</td>
<td>278 ± 19</td>
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<td>GPIX</td>
<td>467 ± 7.1</td>
<td>447 ± 27</td>
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<td>CD9</td>
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<tr>
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<tr>
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<tr>
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<td>527 ± 40</td>
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<tr>
<td>CLEC-2</td>
<td>137 ± 7</td>
<td>140 ± 6</td>
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### Diagram B

- **Integrin αIIbβ3 activation**
  - $G_{\alpha_{13}}^{++}$: Dark gray bars
  - $G_{\alpha_{13}}^{--}$: Light gray bars

- **P-selectin exposure**
  - Bars represent different treatments with ADP, U46, thrombin, CRP, CVX, and RC concentrations.

**Legend**
- **MFI (JON/A-PE)**
- **MFI (anti P-selectin-FITC)**
Figure S3
Figure S4
Figure S5