

CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation

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Signaling through the second messengers calcium and diacylglycerol (DAG) is a critical element in many biological systems. Integration of calcium and DAG signals has been suggested to occur primarily through protein kinase C family members, which bind both calcium and DAG. However, an alternative pathway may involve members of the CalDAG-GEF/RasGRP protein family, which have structural features (calcium-binding EF hands and DAG-binding C1 domains) that suggest they can function in calcium and DAG signal integration^{1,2}. To gain insight into the signaling systems that may be regulated by CalDAG-GEF/RasGRP family members, we have focused on CalDAG-GEFI, which is expressed preferentially in the brain and blood¹. Through genetic ablation in the mouse, we have found that CalDAG-GEFI is crucial for signal integration in platelets. Mouse platelets that lack CalDAG-GEFI are severely compromised in integrin-dependent aggregation as a consequence of their inability to signal through CalDAG-GEFI to its target, the small GTPase Rap1. These results suggest that analogous signaling defects are likely to occur in the central nervous system when CalDAG-GEFI is absent or compromised in function.

We previously identified CalDAG-GEFI and found that it activates Rap1 by promoting the release of GDP and loading of GTP in response to calcium and DAG¹. Rap1 has been implicated in the activation of inside-out signaling for a variety of integrin adhesion molecules³, including the platelet integrin $\alpha_{IIb}\beta_3$ (refs. 4–6). Integrin $\alpha_{IIb}\beta_3$ and Rap1 are activated by calcium ionophores⁷, as well as by DAG analogs⁸ — molecules that mimic the second messengers produced by phospholipase C (PLC). We reasoned that CalDAG-GEFI could be a prime candidate to integrate signaling downstream of the rise in calcium and DAG levels that occurs after activation of multiple receptors. We therefore tested this possibility in platelets, in which receptors for collagen and adenosine diphosphate (ADP) undergo synergistic signaling to integrin $\alpha_{IIb}\beta_3$ for platelet aggregation⁹.

To generate CalDAG-GEFI-knockout mice, we engineered mouse embryonic stem cells with a deletion in the gene *CalDAG-GEFI* (also known as *Rasgrp2*) that results in a frameshift mutation and a premature stop codon at amino acid 37 of CalDAG-GEFI. The engineered embryonic stem cells were used to derive chimeric mice that yielded germline transmission of the CalDAG-GEFI mutation (Crittenden, J. *et al.*, unpublished data). To confirm that CalDAG-GEFI knockout mice lacked CalDAG-GEFI expression, we carried out immunohistochemistry and western blot analyses with polyclonal and monoclonal antibodies against CalDAG-GEFI. Western blot analysis of platelet-rich plasma demonstrated that knockout mice lacked wild-type CalDAG-GEFI (Fig. 1a). Similarly, sections of bone marrow demonstrated strong immunoreactivity for CalDAG-GEFI in wild-type megakaryocytes (platelet progenitors) from wild-type mice but none in those from knockout mice (Fig. 1b,c).

Tail-bleed assays indicated a remarkable bleeding diathesis in CalDAG-GEFI-knockout mice, relative to sibling controls: after tail transection, 95% of the wild-type and *CalDAG-GEFI*^{+/-} heterozygous mice ceased bleeding within 8 min, whereas bleeding continued in the knockout mice for the full 30-min period of testing (Fig. 2a). The bleeding phenotype in CalDAG-GEFI knockout mice did not result from defects in clotting factors, as demonstrated by prothrombin and partial thromboplastin time (PT and PTT) assays (see **Supplementary Table 1** online). Assays for blood in stool samples from knockout mice were negative (data not shown), and hematocrit and hemoglobin levels were not significantly lower ($P = 0.14$ and 0.09 , respectively) than in controls (**Supplementary Table 2** online). These findings indicate that the loss of CalDAG-GEFI does not result in spontaneous hemorrhaging or anemia.

The CalDAG-GEFI-knockout mice had major defects in platelet function. Aggregation in response to calcium ionophore (A23187), collagen, ADP and the thromboxane A₂ (TxA₂) analog U46619, was strongly impaired in platelets from knockout mice, suggesting a critical function for CalDAG-GEFI in the inside-out activation of integrin $\alpha_{IIb}\beta_3$, the major integrin that mediates platelet aggregation (Fig. 2b). By contrast, CalDAG-GEFI knockout mice were deficient in platelet

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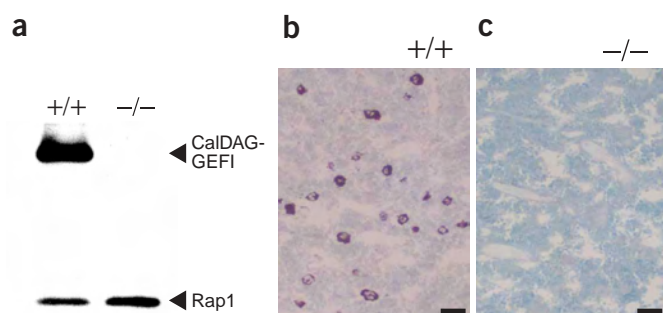


Figure 1 Loss of CalDAG-GEFI expression in platelets and megakaryocytes of CalDAG-GEFI knockout mice. **(a)** Western blot of platelet lysates from wild-type (+/+) and CalDAG-GEFI knockout (-/-) mice incubated successively with a monoclonal antibody to CalDAG-GEFI and a polyclonal antibody to Rap1. The monoclonal antibody against CalDAG-GEFI identifies a band with a relative molecular mass of ~68,000 (top); this band is absent in knockout samples. Similar results were obtained with three additional antibodies (one monoclonal and two polyclonal) raised against CalDAG-GEFI. The subsequent incubation with the Rap1 antibody shows a control for sample loading (bottom). **(b,c)** Sections through bone from wild-type and CalDAG-GEFI-knockout mice showing enriched immunostaining for CalDAG-GEFI in megakaryocytes in wild-type **(b)**, but not knockout **(c)**, bone specimens. Scale bar, 50 μm **(b,c)**.

aggregation at low, but not high, concentrations of thrombin or a DAG analog, phorbol 12-myristate 13-acetate (PMA), demonstrating that platelets can mobilize CalDAG-GEFI-independent signaling pathways to activate $\alpha_{\text{IIb}}\beta_3$ (Fig. 2b).

To determine whether defective inside-out signaling to integrin $\alpha_{\text{IIb}}\beta_3$ was responsible for the impaired aggregation response of platelets in these mice, we measured $\alpha_{\text{IIb}}\beta_3$ activation in the platelets of the knockout mice by flow cytometry using the binding of JON/A, a monoclonal antibody that detects the activated form of mouse integrin $\alpha_{\text{IIb}}\beta_3$ (ref. 10). In agreement with the results obtained by stan-

dard aggregometry, we found that $\alpha_{\text{IIb}}\beta_3$ activation was strongly reduced in CalDAG-GEFI-deficient platelets stimulated with 1 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$ collagen-related peptides (CRP) (91% and 66%, respectively; Fig. 2c). By contrast, $\alpha_{\text{IIb}}\beta_3$ activation was reduced by only 35% and 13% when platelets were activated with 0.1 and 1 U/ml thrombin, respectively (Fig. 2c). Similar results were obtained when the platelets were stained for surface-bound fibrinogen to assess activation (data not shown). The reduction in $\alpha_{\text{IIb}}\beta_3$ activation did not result from decreased expression of $\alpha_{\text{IIb}}\beta_3$ receptors on platelets from CalDAG-GEFI knockout mice, as demonstrated by flow cytometry with an anti-

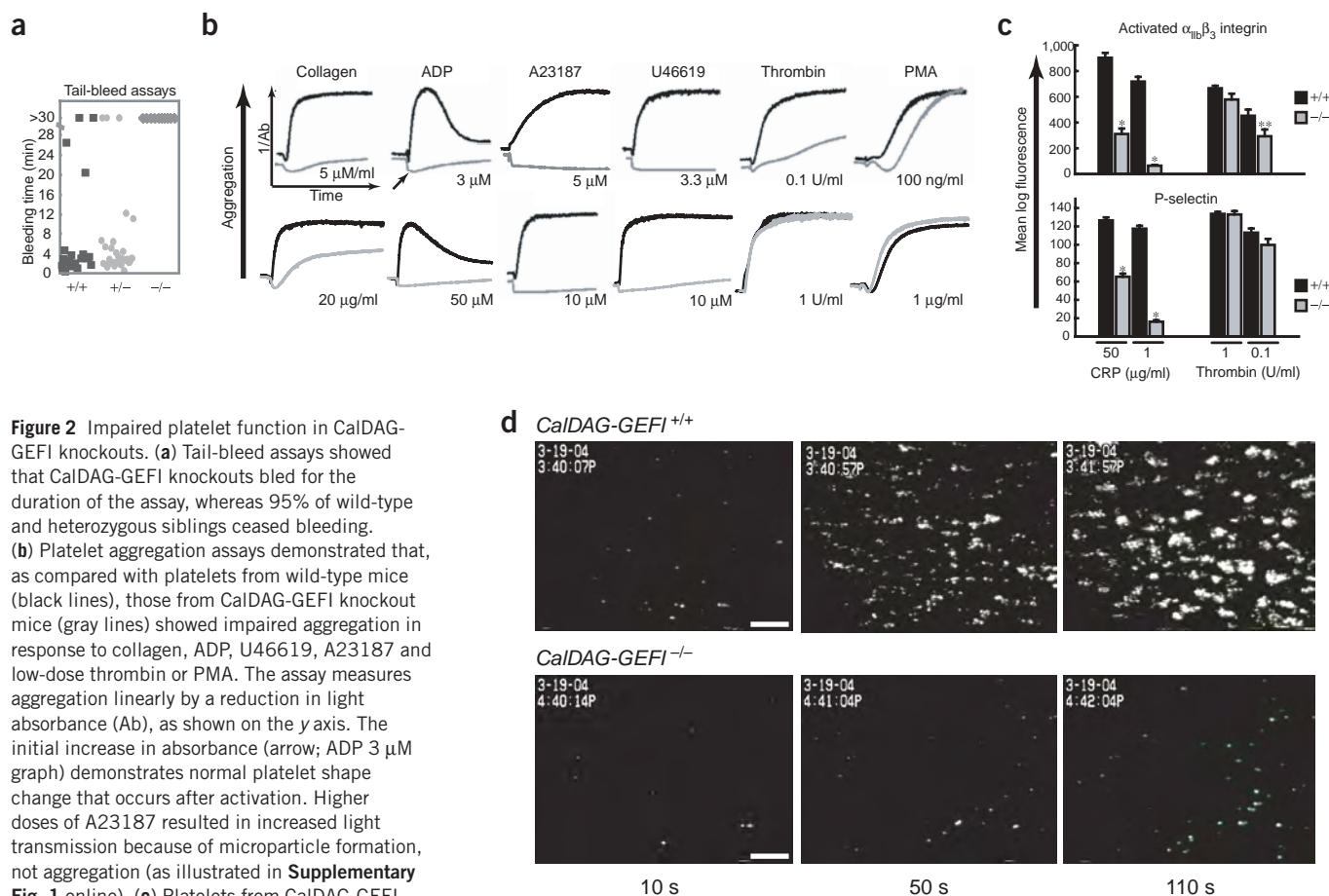
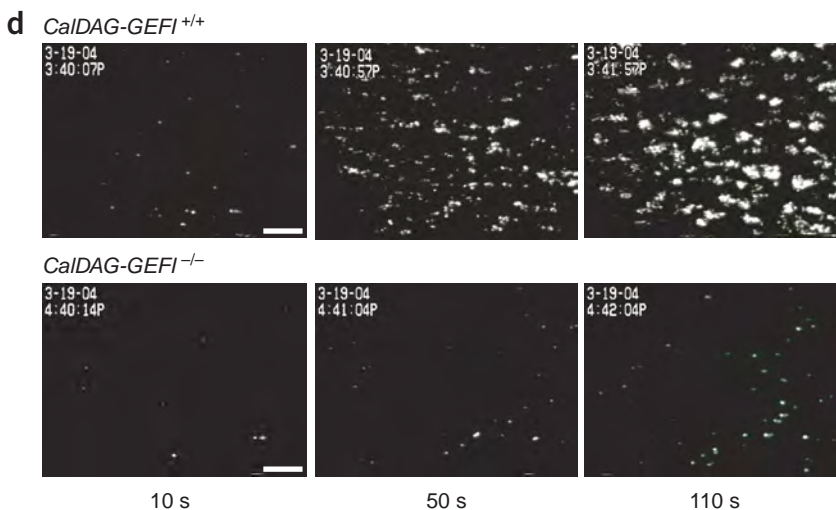


Figure 2 Impaired platelet function in CalDAG-GEFI knockouts. **(a)** Tail-bleed assays showed that CalDAG-GEFI knockouts bled for the duration of the assay, whereas 95% of wild-type and heterozygous siblings ceased bleeding. **(b)** Platelet aggregation assays demonstrated that, as compared with platelets from wild-type mice (black lines), those from CalDAG-GEFI knockout mice (gray lines) showed impaired aggregation in response to collagen, ADP, U46619, A23187 and low-dose thrombin or PMA. The assay measures aggregation linearly by a reduction in light absorbance (Ab), as shown on the y axis. The initial increase in absorbance (arrow; ADP 3 μM graph) demonstrates normal platelet shape change that occurs after activation. Higher doses of A23187 resulted in increased light transmission because of microparticle formation, not aggregation (as illustrated in **Supplementary Fig. 1** online). **(c)** Platelets from CalDAG-GEFI knockout mice showed reduced activation of integrin $\alpha_{\text{IIb}}\beta_3$ (JON/A immunoreactivity) and surface expression of P-selectin (P-selectin immunoreactivity), relative to those from wild-type mice, in response to collagen-related peptides. Responses of CalDAG-GEFI knockout mouse platelets to thrombin were reduced only at low doses. * $P < 0.0001$ and ** $P < 0.05$; $n = 6$ per genotype. **(d)** CalDAG-GEFI knockout mouse platelets in whole blood failed to form thrombi when perfused over a collagen surface. The surface area covered by platelets from CalDAG-GEFI knockout mice after 2 min of perfusion was $1.9 \pm 0.05\%$ of the flow chamber area, as compared with $24.5 \pm 1.0\%$ for platelets from wild-type mice. $P < 10^{-6}$; $n = 5$ of each genotype; scale bar, 50 μm .



body that measures total levels of $\alpha_{IIb}\beta_3$ integrin (data not shown). To assess the function of CalDAG-GEFI in platelet granule release, we tested activated platelets for the presence of P-selectin — an α -granule-associated molecule that is displayed on the platelet surface after exocytosis. CRP-induced expression of P-selectin was markedly reduced in the platelets of CalDAG-GEFI knockout mice (48% and 86% for 50 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$ CRP, respectively), but no significant difference was observed when the cells were activated with thrombin (Fig. 2c).

Together, these findings indicate that CalDAG-GEFI signaling in platelets is critical for the inside-out activation of $\alpha_{IIb}\beta_3$ as well as for granule secretion by platelets in response to specific agonists. By contrast, the activation-dependent change in platelet shape from discoid to spherical seemed to be normal in platelets from knockout mice for all of the agonists tested (Fig. 2b). This result indicates that CalDAG-GEFI is not essential for the early-phase cytoskeletal reorganization that accompanies platelet activation, a process mediated by the small GTPase Rho¹¹.

To test whether CalDAG-GEFI functions in platelet activation under conditions that mimic physiological blood flow in arteries, we compared thrombus formation in blood from knockout and wild-type mice during perfusion over a collagen surface — a process requiring binding by multiple platelet receptors, including integrin

$\alpha_{IIb}\beta_3$ (refs. 12,13). Platelets from CalDAG-GEFI-knockout mice were indistinguishable from wild-type platelets in their ability to tether to the collagen surface (Fig. 2d and Supplementary Video 1 online). However, platelets from CalDAG-GEFI knockout mice were unable to attach firmly to the collagen surface and did not form stable thrombi throughout the perfusion period (Fig. 2d and Supplementary Video 1 online). Thus, CalDAG-GEFI signaling is critical to collagen-induced integrin activation and platelet aggregation under flow conditions.

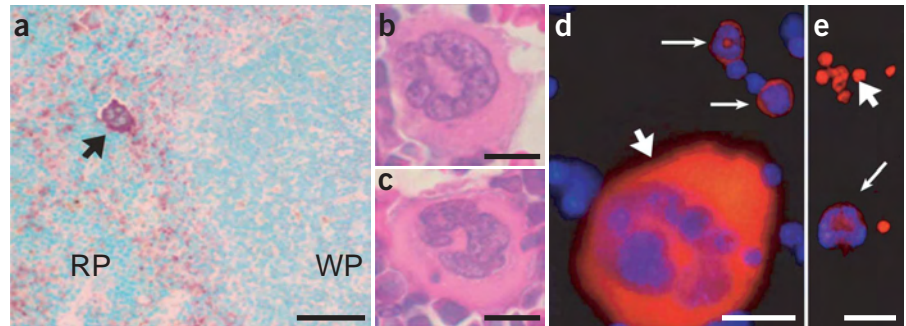


Figure 3 Expression of CalDAG-GEFI in the hematopoietic system. (a) CalDAG-GEFI immunostaining in platelets and megakaryocytes (arrow) within the red pulp of the spleen (RP) but not in white pulp (WP). (b,c) Megakaryocyte morphology in H&E-stained sections from the bone marrow of wild-type (b) and knockout (c) mice, showing similar morphology in the two genotypes. (d) CalDAG-GEFI immunostaining of bone marrow cells prepared by cytopspin, illustrating modest expression in developing neutrophils (thin arrows) and strong expression in megakaryocytes (thick arrow). (e) CalDAG-GEFI immunostaining of peripheral blood, showing CalDAG-GEFI expression in neutrophils (thin arrow) and platelets (thick arrow). Scale bars, 50 μm (a) and 10 μm (b–e).

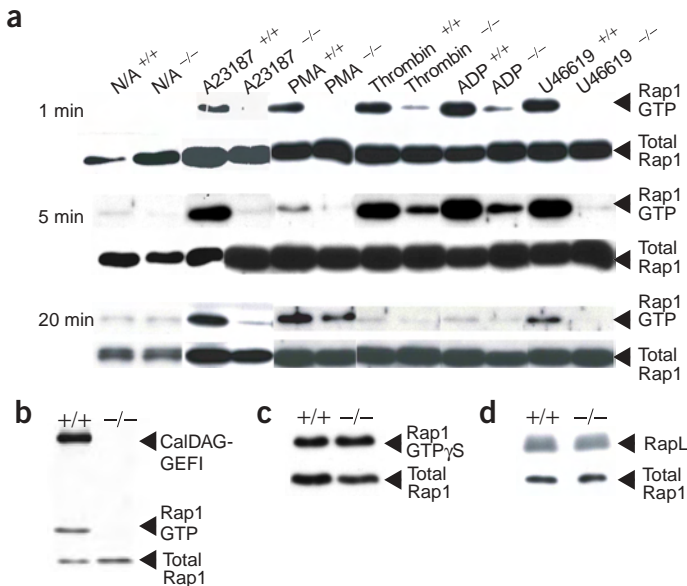


Figure 4 Impaired Rap1 activation in CalDAG-GEFI knockout mice. (a) Western blots of Rap1–GTP (activated Rap1) as compared with total Rap1 showing severely diminished Rap1 activation in CalDAG-GEFI knockout mouse platelets (–/–), relative to that in wild type (+/+), treated with A23187, PMA, thrombin, ADP or U46619 for 1, 5 or 20 min (N/A, untreated samples). (b) Western blot of affinity precipitated Rap1–GTP sequentially incubated with antibodies to Rap1 and CalDAG-GEFI, illustrating coprecipitation of CalDAG-GEFI with Rap1 in platelets treated with thrombin from wild-type, but not knockout mice. (c) Incubation of platelet extracts with GTP γ S showed equivalent loading of GTP onto Rap1 in platelets from wild-type and CalDAG-GEFI knockout mice. (d) Anti-RapL antibody detected an ~25-kDa band in platelet extracts from both wild-type and CalDAG-GEFI knockout mice. In a–d, total Rap1 was detected in separate western blots that were prepared from the same platelet lysates used in the Ral RBD precipitation and RapL experiments. (e) Multiple platelet receptors signal through PLC β and PLC γ , mobilizing calcium and DAG, which converge to activate CalDAG-GEFI. This convergence results in the activation of Rap1 and platelet integrin $\alpha_{IIb}\beta_3$, ultimately resulting in platelet aggregation and thrombus formation. In addition to stimulating CalDAG-GEFI, thrombin functions in a CalDAG-GEFI-independent pathway to mediate platelet aggregation through unidentified intermediates.

As an *in vivo* test of the importance of CalDAG-GEFI in platelet activation, we determined the susceptibility of CalDAG-GEFI knockout mice to thrombosis induced by collagen injection. This treatment resulted in the death of all of the wild-type mice ($n = 4$) within 10 min, but of only 1 of 6 CalDAG-GEFI knockout mice ($P < 0.024$). Thus, deletion of CalDAG-GEFI conferred resistance to collagen-induced thrombosis.

A primary site of mouse platelet production is the red pulp of the spleen¹⁴, where we observed expression of CalDAG-GEFI in megakaryocytes and platelets (Fig. 3a). This finding prompted us to analyze platelet production and megakaryocyte morphology in the CalDAG-GEFI-knockout mice. The morphology of megakaryocytes in the CalDAG-GEFI knockouts was normal (Fig. 3b, c). Platelet counts from the peripheral blood of knockout mice were slightly elevated, indicating that platelet dysfunction, and not a deficit in total platelet number, accounted for the increased bleeding time in CalDAG-GEFI knockout mice (Supplementary Table 2 online).

Circulating neutrophil counts were elevated approximately twofold in knockout mice, relative to wild-type siblings ($P < 10^{-4}$; Supplementary Table 2 online). CalDAG-GEFI was detected in developing and mature neutrophils from bone marrow (Fig. 3d) and peripheral blood (Fig. 3e); the function of CalDAG-GEFI in this cell type is under investigation. No CalDAG-GEFI immunoreactivity was observed in the white pulp of the spleen (Fig. 3a) or in the thymus (data not shown), suggesting that CalDAG-GEFI expression is low or absent in mouse lymphocytes. The lineage-restricted expression of CalDAG-GEFI within the hematopoietic system is consistent with the distinct expression profiles of other CalDAG-GEF family members: CalDAG-GEFII/RasGRP1 expression is preferential to T cells¹⁵, CalDAG-GEFIII/RasGRP3 to B cells^{16, 17} and RasGRP4 to mast cells¹⁸.

Multiple receptors for platelet activation stimulate the small GTPase Rap1B^{19–21}. To test for Rap1 activation in platelets from CalDAG-GEFI knockout mice, we measured the levels of Rap1 in the active, GTP-bound state as compared to the inactive, GDP-bound state. Rap1–GTP was selectively precipitated from platelet homogenates with the Ras-binding domain (RBD) of RalGDS (a protein that binds Rap1–GTP but not Rap1–GDP²²) and western blot analysis of the precipitates was performed with an anti-Rap1 antibody. In platelets from wild-type mice, treatment with A23187, PMA, thrombin, ADP and U46619 for 1, 5 and 20 min all resulted in activation of Rap1 (Fig. 4a). By contrast, treatment of mice from CalDAG-GEFI knockout mice with the same agonists induced only poor activation of Rap1, despite equivalent amounts of total Rap1 being present in platelet lysates from the knockout and wild-type mice (Fig. 4a). Further, Rap1–GTP precipitates from platelets from wild-type mice contained coprecipitated CalDAG-GEFI, as shown by western blotting with an anti-CalDAG-GEFI antibody (Fig. 4b). This evidence suggests that CalDAG-GEFI is complexed with Rap1 in platelets.

To test whether Rap1 from the platelets of CalDAG-GEFI knockout mice is competent to undergo GTP-loading, we used the Ral RBD precipitation assay to measure the amount of Rap1–GTP in knockout mouse platelet lysates that were preincubated with GTP γ S, a form of GTP that cannot be hydrolyzed to GDP by the intrinsic GTPase activity of Rap1. We detected equivalent levels of Rap1–GTP in knockout and wild-type samples, indicating that Rap1 from the platelets of CalDAG-GEFI knockout mice does not have intrinsic activation defects (Fig. 4c). The Rap1-binding protein RapL promotes integrin activation in leukocytes, suggesting that RapL may link activation of Rap1 to integrin-dependent adhesion²³. We therefore tested whether lysates of platelet-rich plasma were positive for RapL; indeed they were, as shown by western blot analysis, suggesting that RapL could link Rap1 to platelet integrin $\alpha_{IIb}\beta_3$ (Fig. 4d).

Our results demonstrate that CalDAG-GEFI is a key signal integrator in the cascade leading through Rap1 and integrin $\alpha_{IIb}\beta_3$, to platelet aggregation and thrombus formation (Fig. 4e). That CalDAG-GEFI knockout mice are resistant to collagen-induced thrombosis, and do not undergo spontaneous hemorrhaging, suggests that CalDAG-GEFI could be a promising new target for antithrombotic therapy.

METHODS

Mouse maintenance. All protocols were approved by the Massachusetts Institute of Technology and CBR Center for Biomedical Research Committees on Animal Care. Mice were maintained under a standard light/dark cycle with free access to food and water. Routine genotyping of CalDAG-GEFI knockout mice was carried out using the following three primer sequences in a single polymerase chain reaction: 5'-AACAGTTC-CCAGGCTAGAGATAGAGAGTTCTCC-3', 5'-ACCAGACTCTAGGCCA-GAACCTACC-3' and 5'-AGTGTGCTGTGGTAAATGCAGCCATTCC-3'. Analysis of the wild-type mice yielded a 208-base-pair product with the first two primers, whereas analysis of the knockouts yielded a 286-base-pair product with the second two primers.

Bleeding time. Measurements were made in 3-week-old mice before genotyping. Mice were anesthetized with 120 mg/kg ketamine and 16 mg/kg xylazine. A 2-mm section of the tail tip was transected, and the tail was immersed in 37 °C PBS to score time to cessation of blood flow.

Platelet aggregation. Whole blood was collected by retro-orbital bleeds from isoflurane-anesthetized mice and used to prepare platelet-rich plasma or washed platelets. Platelets were adjusted to a concentration of 3×10^8 platelets/ml with modified Tyrode's buffer containing 1 mM CaCl₂ and maintained at 37 °C with stirring. Aggregation was initiated by adding agonists at the following concentrations: 5 or 20 μ g/ml collagen (Nycomed), 3.0 or 50 μ M ADP, 3.3 or 10 μ M U46619, 0.1 or 1 U/ml thrombin, 100 ng/ml or 1 μ g/ml PMA, or 5, 10 or 100 μ M A23187 (Sigma). Aggregation was monitored by light transmission with a Chronolog aggregometer (Chrono-Log).

Flow cytometry. Activation of $\alpha_{IIb}\beta_3$ integrin and surface expression of P-selectin were measured by flow cytometry. Briefly, washed platelets from wild-type and knockout mice were activated for 5 min with thrombin (0.1 or 1 U/ml) or collagen-related peptides (1 or 50 μ g/ml; a gift from J. Hartwig), stained for 10 min with fluorophore-labeled antibodies against activated $\alpha_{IIb}\beta_3$ integrin (EMFRET Analytics), P-selectin (Pharming), or α_{IIb} integrin (Pharming) and analyzed on a FACScalibur (Becton Dickinson).

Flow-chamber studies. Platelets obtained from the blood of wild-type or CalDAG-GEFI knockout mice were washed, labeled with 2.5 μ g/ml calcein, and added back to the respective platelet-poor whole-blood samples immediately before perfusion in a parallel-plate flow-chamber system, as described previously²⁴.

Thromboembolism. Mice were anesthetized with ketamine and xylazine followed by retro-orbital injection of 1.6 mg/kg of Horm collagen (Nycomed). Death was scored as cessation of breathing. CalDAG-GEFI knockout mice were given a lethal dose of sodium pentobarbital and all mice were used for preparation of platelets and lung histology, which confirmed that wild-type mice died from thrombosis.

Blood cell counts, PT/PTT and stool blood. Blood was collected by retro-orbital bleeds from isoflurane-anesthetized mice into tubes containing EDTA (Becton Dickinson) for cell counts or tubes containing sodium citrate (Sarstedt) for PT/PTT assays. Blood cell counts were done with a Hemavet Coulter counter (CBC Tech). PT, PTT and stool blood tests were carried out by IDEXX with Thromboscreen (Curtin Matheson Scientific) and Hemocult SENSE (Beckman Coulter) kits.

Antibody production and immunohistochemistry. Polyclonal antibodies to CalDAG-GEFI were isolated from rabbits immunized by Covance with the peptide N-PEIREEEVQTVEDGVFDIHL-C (ref. 25) generated by Synpep.

Generation of monoclonal antibodies against CalDAG-GEFI was described previously¹. Tissues were processed for paraffin sectioning according to standard protocols and anti-CalDAG-GEFI polyclonal antibody was used at a dilution of 1:1,000 followed by a 1:200 dilution of Cy3-conjugated anti-rabbit secondary antibody (Amersham Pharmacia) or ABC amplification (Vector Laboratories), according to the manufacturer's instructions.

Rap1 activation. Amounts of activated Rap1 were measured as previously described⁷, but with minor modifications: platelet-rich plasma was isolated by spinning whole blood at 200g for 10 min, collecting the supernatant and adding agonists at final concentrations of 10 μ M (A23187), 100 ng/ml (PMA), 0.1 U/ml (thrombin), 3 μ M (ADP) or 3.3 μ M (U46619). Platelets were incubated with agonists for 1, 5 or 20 min at room temperature, before lysis in TLB (50 mM Tris-HCl at pH 7.4, 500 mM NaCl, 1% Nonidet P-40, 2.5 mM MgCl₂ and 10% glycerol) with Complete Protease Inhibitor Cocktail lacking EDTA (Roche). GTP γ S-loading experiments were performed with platelet extracts according to the manufacturer's instructions (Upstate Biotechnology). Affinity precipitation of Rap1 was performed according to the manufacturer's instructions with RalGDS-RBD coupled to agarose beads (Upstate Biotechnology). Proteins were separated on a 12.5% SDS-PAGE gel and transferred to PVDF membranes (Millipore). Rap1 was detected with rabbit polyclonal antibodies (121; Santa Cruz Biotechnology) followed by anti-rabbit antibodies conjugated to horseradish peroxidase (Vector Laboratories). Immunoreactivity was detected by Western Lightning enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer Life Sciences).

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests (see the *Nature Medicine* website for details)

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