Materials

Low molecular weight Lovenox (enoxaparin sodium; Sanofi-Aventis, Bridgewater, NJ), heparin-coated capillaries (VWR, West Chester, PA), bovine serum albumin (BSA, fraction V), prostacyclin (PGI_2), and human fibrinogen (type I) (all from Sigma-Aldrich, St Louis, MO), 2-methylthio-AMP triethylammonium salt hydrate (2-MesAMP, P2Y12 inhibitor, BioLog, Bremen, Germany), U46619 (Cayman Chemical), fibrillar collagen type I (Chronlog, Havertown, PA), polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), clopidogrel (Plavix, Sanofi-Aventis, France), and PAR4-activating peptide (Advanced Chemtech, Louisville, KY) were purchased. Convulxin was provided by Kenneth J. Clemetson (Theodor Kocher Institute, University of Berne, Switzerland). Monoclonal antibodies directed against the activated form of murine αIIbβ3, JON/A-PE, or GPIX, and antibodies for platelet depletion in mice were purchased from Emfret Analytics (Wuerzburg, Germany).

Western blotting

Platelet lysates were separated on 15% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. CalDAG-GEFI and CalDAG-GEFIΔC1 were detected with a rabbit polyclonal antibody raised against the C-terminus of CalDAG-GEFI (Thermo Fisher Scientific, Huntsville, AL). After incubation with horseradish peroxidase-coupled anti–rabbit antibodies, (Vector Laboratories), immunoreactivity was detected by Western Lightning enhanced chemiluminescence (G-Biosciences).

Immunofluorescence

Resting platelets were fixed for 10 minutes in 3.7% formalin, followed by permeabilization with 0.5% tritonX-100 for 2 minutes. Samples were blocked for 30 minutes with 3% BSA solution, stained with Alexa594-labeled antibodies to CalDAG-GEFI, and analyzed on an Nikon Ti-U inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a Retiga EXL monochrome camera (QImaging, Surrey, Canada). Images were acquired using Nikon NIS Elements software (NIS-Elements Advanced Research; Melville, NY, USA) and a 100x oil objective. 15 cross-sections were taken at 0.25 µm spacing, and images were deconvolved using the „nearest neighbor“ iteration (NIS Elements software).

Aggregometry. Platelets were washed as described previously [12] and re-suspended at a concentration of 2 x 10^8 platelets/ml in modified Tyrode's Buffer (137 mM NaCl, 0.3 mM Na_2HPO_4, 2mM KCl, 12 mM $NaHCO_3$, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mM glucose, pH 7.3) containing 0.35% BSA and 1 mM $CaCl_2$. The experiment was performed at 37°C in the presence of 50 µg/ml fibrinogen and under stirring conditions (1200 rpm). After addition of agonist, light transmission was recorded over 5 min on a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

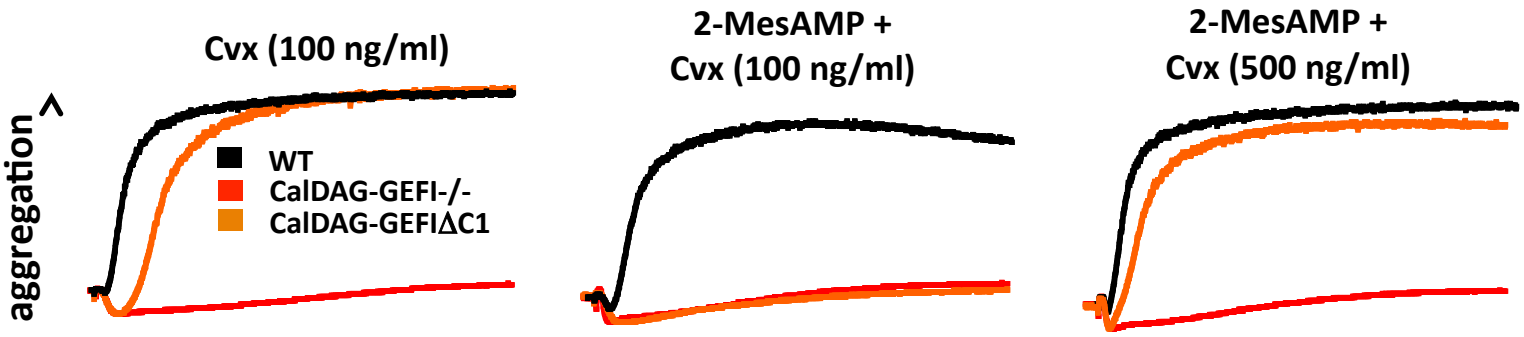

Figure S1. Aggregation of CalDAG-GEFIΔC1 platelets. Aggregation induced by 100 ng/ml convulxin in the absence (left panel) and presence (middle panel) of the P2Y12 inhibitor, MesAMP. The right panel shows aggregation induced by 500 ng/ml convulxin in the presence of MesAMP. Results are representative of three experiments.