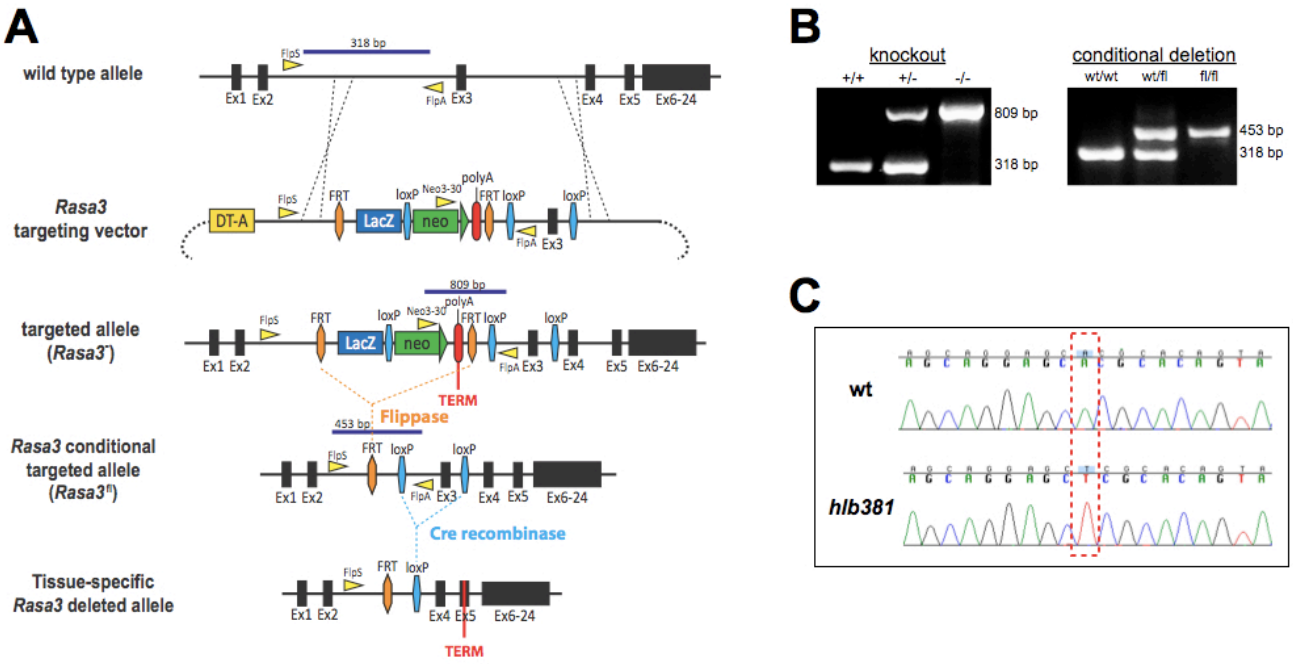
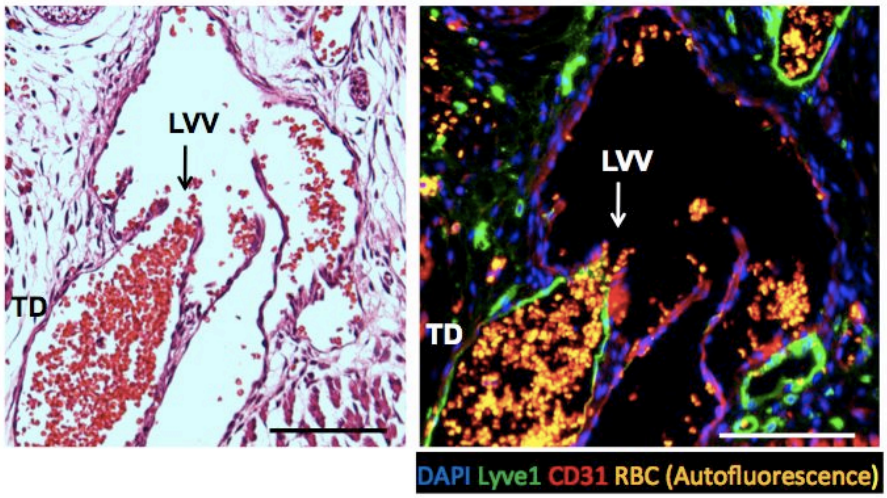
Supplementary Figure 1



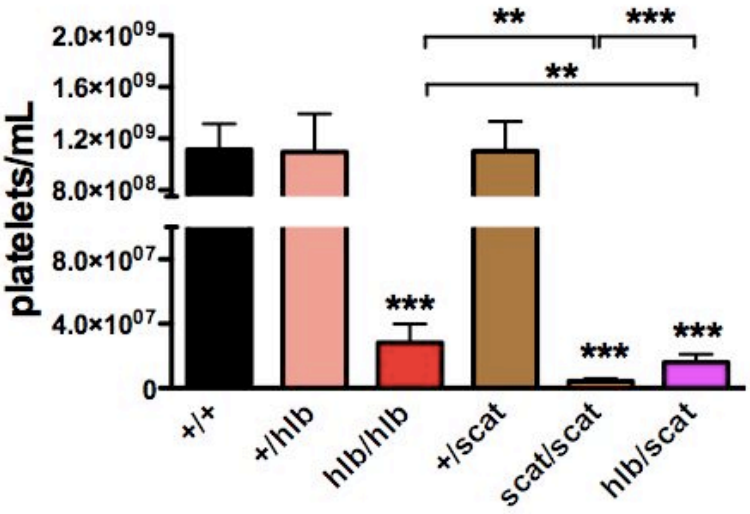
Generation of Rasa3 knockout mice. (A) Scheme of the targeting strategy. The *Rasa3* targeting vector consists of an IRES/LacZ reporter and a promoter-driven neomycin selection marker (Neo) flanked by FRT sites, inserted upstream of a loxP-flanked exon 3 (http://www.mousephenotype.org/martsearch_ikmc_project/martsearch/ikmc_project/48704). The first allele to be generated was a non-expressive form (*Rasa3*$^{-}$) since the LacZ/Neo cassette has a strong polyadenylation signal that truncates the Rasa3 mRNA upstream of exon 3 (TERM). Chimeric mice bearing the *Rasa3*$^{-}$ allele were crossed with germline deleter Flp mice to remove the LacZ/Neo cassette and create the conditional Rasa3 allele (*Rasa3*^{fl}) that restores wild type RASA3 expression. Subsequently conditional *Rasa3*$^{+/fl}$ mice were crossed with mice expressing the Cre recombinase under the megakaryocyte-specific Pf4 promoter. Cre-mediated recombination results in excision of exon 3, a frameshift throughout exon 4, and the generation of a premature stop codon in exon 5 that leads to nonsense-mediated mRNA decay. **(B)** Genotyping of *Rasa3* knockout mice. Genomic DNA isolated from tail snips of the indicated mice was analyzed by PCR using the primer pairs shown in (A). **(C)** Identification of the *hlb381* mutation. The mutation in *hlb381* was fine-mapped in an F2 intercross to an interval on mouse chromosome 8 that overlapped the *scat* critical interval. Following positive allele testing with *scat*, we sequenced *Rasa3* in *hlb381* homozygotes to identify the mutation. For sequencing, oligonucleotides were designed using public sequence data (Ensembl, http://www.ensembl.org; NCBI, http://www.ncbi.nlm.nih.gov). PCR amplification products were purified (AMPure; Agencourt Biosciences) and sequenced using the automated dye termination technique (ABI Prism Model 3700 genetic analyzer; Applied Biosystems). Sequences were analyzed with Sequencher 4.1 licensed software.

Supplementary Figure 2



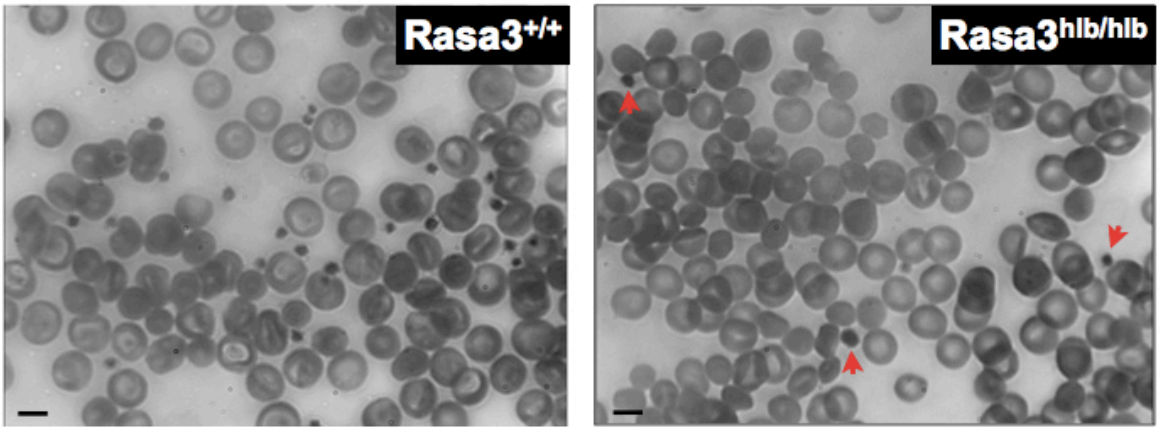
Lympho-venous junction of a *Rasa3*$^{fl/fl}$*PF4-Cre*$^{+}$ embryo in the transverse plane. Left: H&E stained section. Right: Sample stained for LYVE-1 (green) to label lymphatic vessels, and CD31 (red) to label blood vessels, along with DAPI staining (blue) and autofluorescent RBCs (yellow) reveals a physical connection between blood and lymphatic vessels allowing blood backflow into the thoracic duct. LVV, lympho-venous valve; TD, thoracic duct. Scale bars represent 100 μm.

Supplementary Figure 3



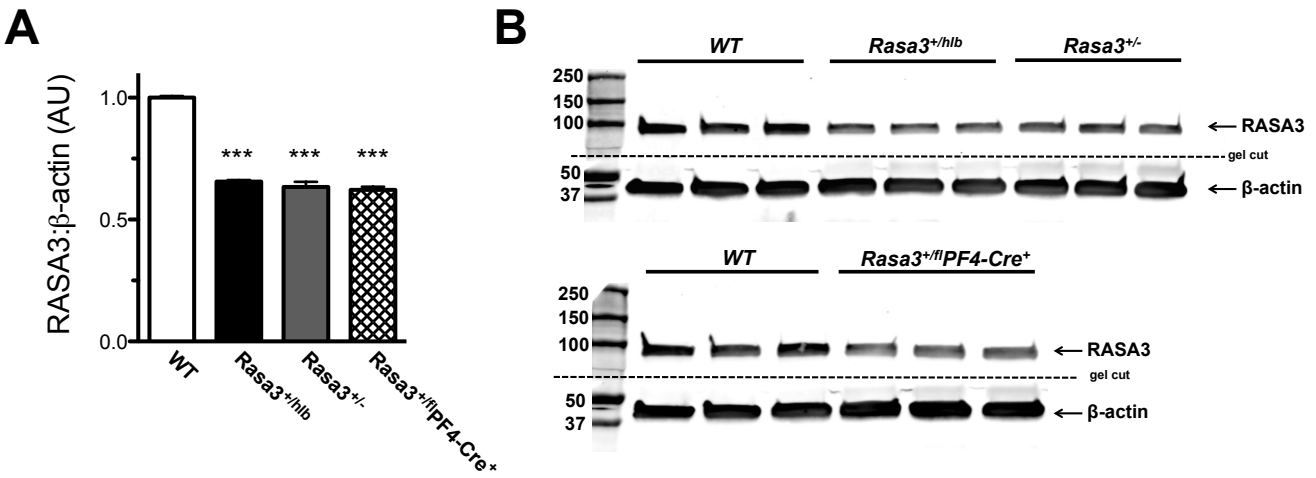
Peripheral platelet counts in mice of the indicated genotypes. Note the severe thrombocytopenia in compound heterozygous *Rasa3*$^{hlb/scat}$ mice. Statistical significance was determined by 2-way ANOVA with Bonferroni posttest. (n=10 – 23).

Supplementary Figure 4



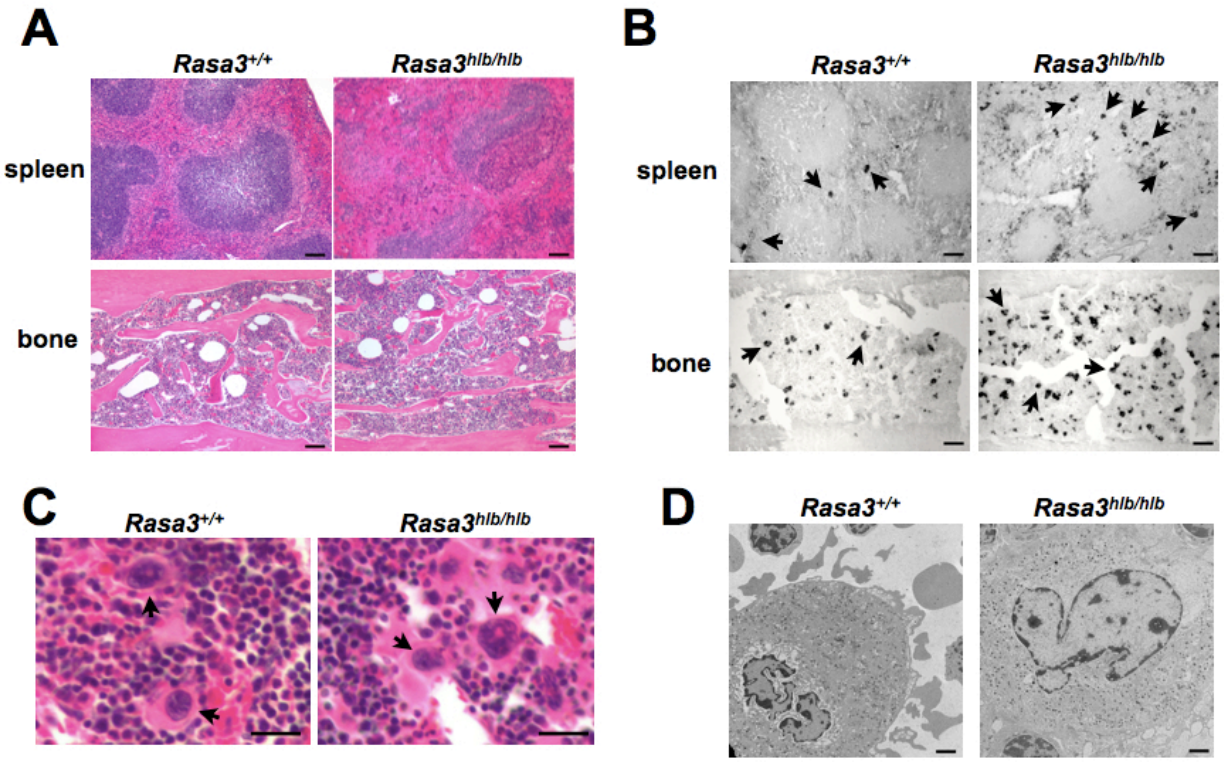
Blood smears from *Rasa3*$^{+/+}$ and *Rasa3*$^{hlb/hlb}$ mice. The red arrows point to the few platelets observed in blood from *Rasa3*$^{hlb/hlb}$ mice. Scale bar = 5 µm. Results are representative of 3 independent experiments.

Supplementary Figure 5



Reduced RASA3 expression in platelets from Rasa3$^{+/hlb}$ mice. (A) Quantification of RASA3 protein expression relative to β-actin levels in platelets from the indicated genotypes. Note the marked reduction in the RASA3 level of platelets from Rasa3$^{+/hlb}$ mice, comparable to that observed in Rasa3$^{+/-}$ or Rasa3$^{+/fl}$PF4-Cre^{+} platelets. RASA3 expression in Rasa3$^{+/+}$PF4-Cre^{+} platelets was comparable to that in WT control platelets (not shown). Statistical significance was determined by 2-way ANOVA with Bonferroni posttest. (n = 5). **(B)** Representative Western blot for RASA3 and β-actin in platelet lysates from three *WT*, *Rasa3$^{+/hlb}$*, *Rasa3$^{+/-}$*, and *Rasa3$^{+/fl}$PF4-Cre^{+}* mice.

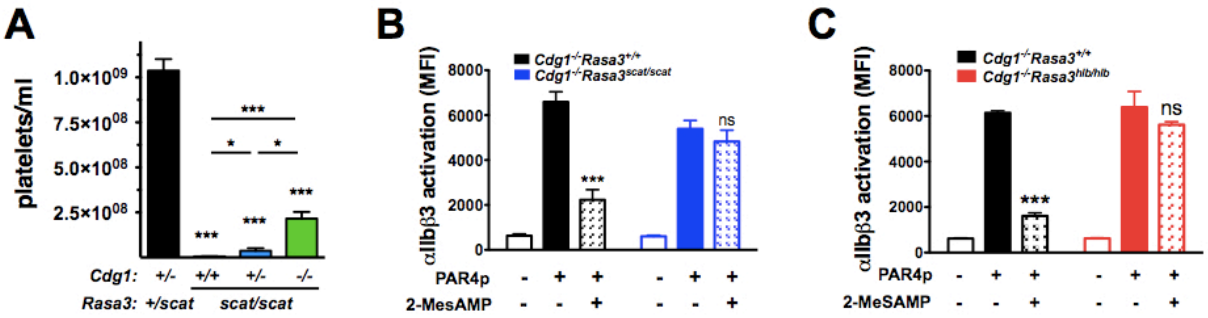
Supplementary Figure 6



Expansion of mature megakaryocytes in spleen and bone of Rasa3$^{hlb/hlb}$ mice. (A) H&E (Hematoxylin and Eosin) stained sections of spleens (upper panels) and femurs (lower panels) isolated from 3 month-old *Rasa3*$^{+/+}$ or *Rasa3*$^{hlb/hlb}$ mice; scale bar = 100 μm. **(B)** Cryosections of spleens (upper panels) and bones (lower panels) stained for acetylcholinesterase. Arrows point to megakaryocytes; scale bar = 100 μm. **(C,D)** No systematic differences in spleen megakaryocyte morphology (C; arrows point to megakaryocytes; scale bar = 50 μm) and bone megakaryocyte ultrastructure (D; scale bar = 2 μm) were observed. Images are representative for three independent experiments.

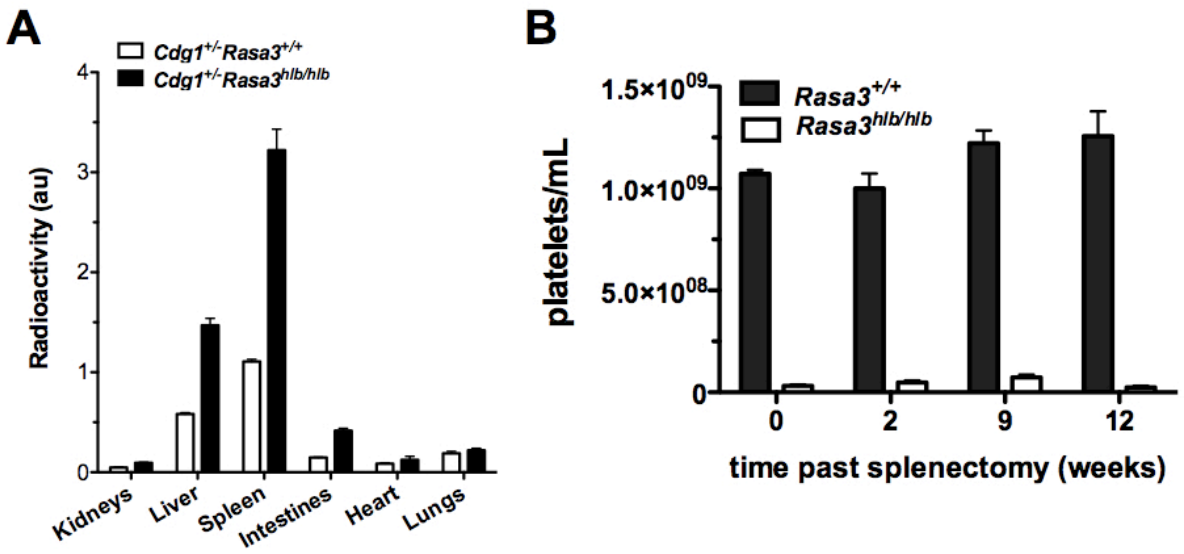
`

Supplementary Figure 7



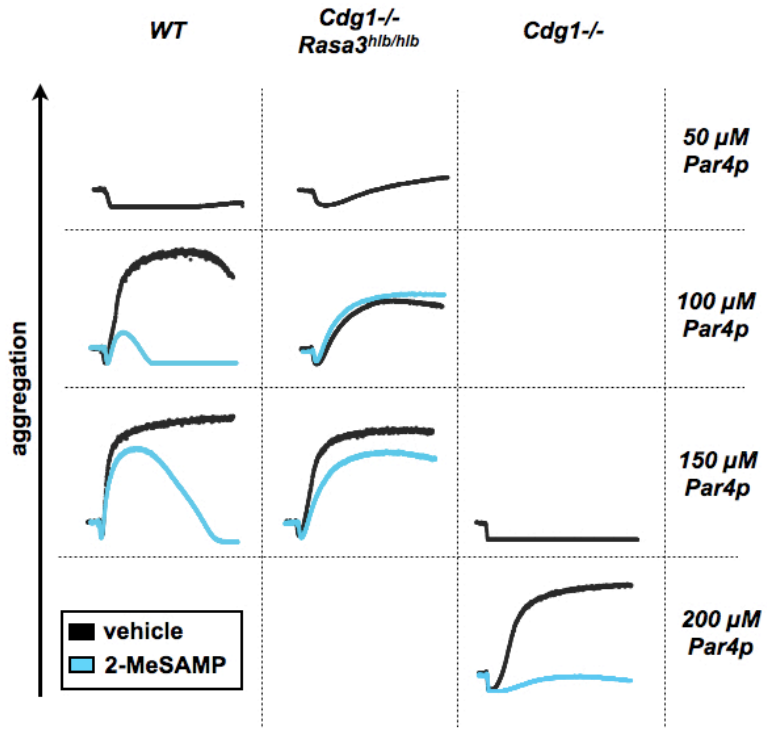
Platelet count and integrin activation response in *Rasa3*^{scat} and *Rasa3*^{hlb} mice. (A) Peripheral platelet count in *Rasa3*$^{scat/scat}$ mice is partially restored by reducing CalDAG-GEFI expression. *Rasa3*$^{scat/scat}$ mice were crossed with *Caldaggef1*$^{-/-}$ mice and the platelet count was determined by flow cytometry in whole blood isolated from the indicated genotypes. Statistical significance was determined by 2-way ANOVA with Bonferroni posttest. *p<0.05, ***p<0.0001. (n = 6). **(B,C)** Agonist-induced αIIbβ3 activation in RASA3 mutant platelets is insensitive to P2Y12 inhibition. Flow cytometry analysis of αIIbβ3 activation (JON/A-PE binding) in *Caldaggef1*$^{-/-}$ (*Cdg1-/-*, black bars), *Caldaggef1*$^{-/-}$*Rasa3*$^{scat/scat}$ (blue bars) or *Caldaggef1*$^{-/-}$*Rasa3*$^{hlb/hlb}$ (red bars) platelets stimulated with 250 μM PAR4p in the presence (checkered bar) or absence (solid bar) of 2-MeSAMP (100 μM). Statistical significance between platelets activated in the presence or absence of 2-MeSAMP was determined by student t-test. ***p<0.0001; ns: p>0.05. (n = 6).

Supplementary Figure 8



Accumulation of *Caldaggef1*$^{+/-}$*Rasa3*$^{hlb/hlb}$ platelets in spleen and liver. (A) *Caldaggef1*$^{+/-}$*Rasa3*$^{hlb/hlb}$ (black bars) or *Caldaggef1*$^{+/-}$*Rasa3*$^{+/+}$ control platelets (open bars) were radiolabeled with Copper-64 and transfused into WT recipient mice (n=3). Twenty-four hours post transfusion, organs were harvested and radioactivity was measured with a gamma-counter. Consistent with the increased clearance of *Rasa3* mutant platelets, platelet-associated radioactivity in blood samples at t=24 hrs was markedly lower in mice transfused with *Caldaggef1*$^{+/-}$*Rasa3*$^{hlb/hlb}$ compared to controls (*Caldaggef1*$^{+/-}$*Rasa3*$^{hlb/hlb}$ over *Caldaggef1*$^{+/-}$*Rasa3*$^{+/+}$ = 0.45). To account for the blood remaining in the excised tissue, results were normalized to Copper-64 signals in peripheral blood of the respective mice. **(B)** Peripheral platelet counts at the indicated time points after splenectomy in *Rasa3*$^{+/+}$ and *Rasa3*$^{hlb/hlb}$ mice. (n=5).

Supplementary Figure 9



Aggregation response of *wild type (WT)*, *Caldaggef1*$^{-/-}$*Rasa3*$^{hlb/hlb}$ (Cdg1$^{-/-}$*Rasa3*$^{hlb/hlb}$) or *Caldaggef1*$^{-/-}$ (Cdg1$^{-/-}$) platelets to increasing doses of Par4 agonist peptide (Par4p) in the presence of vehicle (black traces) or of the P2Y12 inhibitor 2-MeSAMP (light blue traces). Results are representative of three independent experiments. Note the very limited effect of the P2Y12 inhibitor 2-MeSAMP on the aggregation response of *Caldaggef1*$^{-/-}$*Rasa3*$^{hlb/hlb}$ when compared to *WT* or *Caldaggef1*$^{-/-}$ platelets. A similar result was observed when platelets were activated in the presence of the PI3-kinase inhibitor, wortmannin (not shown).

VIDEO CAPTIONS

Supplementary Video 1. Formation of a hemostatic plug in an injured venule of a *wild-type* mouse.

Supplementary Video 2. Formation of a hemostatic plug in an injured venule of a *Caldaggef1*$^{-/-}$ mouse.

Supplementary Video 3. Formation of a hemostatic plug in an injured venule of a *Caldaggef1*$^{-/-}$ *Rasa3*$^{hlb/hlb}$ mouse.

Supplementary Video 4. Formation of a hemostatic plug in an injured venule of a *wild-type* mouse treated with clopidogrel bisulfate.

Supplementary Video 5. Formation of a hemostatic plug in an injured venule of a *Caldaggef1*$^{-/-}$ mouse treated with clopidogrel bisulfate.

Supplementary Video 6. Formation of a hemostatic plug in an injured venule of a *Caldaggef1*$^{-/-}$ *Rasa3*$^{hlb/hlb}$ mouse treated with clopidogrel bisulfate.