Supplementary Materials

Detailed Materials and Methods

Platelet preparation

Blood was drawn from the retro-orbital plexus into heparinized tubes. Platelet-rich plasma (PRP) was obtained by centrifugation at 100*g* for 5 min. PRP was centrifuged at 700*g* in the presence of PGI_2 (5 µM) for 5 min at room temperature. After two washing steps, pelleted platelets were resuspended in modified Tyrode's Buffer (0.137 mM NaCl, 0.3 mM Na_2HPO_4, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mM glucose, pH 7.3) containing 1 mM CaCl2.

Spreading

Platelet spreading experiments were performed as previously described[1]. Briefly, washed platelets were applied to glass coverslips coated with 100 µg/ml human fibrinogen. After 45 minutes at room temperature, platelets were fixed for 10 minutes with 4% paraformaldehyde/PBS and stained with FITC-phalloidin. Images were acquired with an Olympus FV1000 confocal microscope and platelet area calculated using ImageJ software.

Aggregometry

Washed platelets were re-suspended at a concentration of 2 x 10^8 platelets/ml in modified Tyrode's Buffer containing 0.35% BSA and 1mM $CaCl_2$. The experiment was performed at 37°C in the presence of 50 µg/ml fibrinogen and under stirring conditions (1200 rpm). Platelet agonists were added and light transmission was recorded until it reached a plateau on a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

Rap1 activation assay

Washed platelets (1.2 x 10^8 platelets/sample) were stimulated with 150 µM Par4-AP for 20 seconds or 5 minutes at 37°C in a standard aggregometer. Reactions were stopped with ice-cold 2x lysis buffer (100 mM Tris/HCl pH 7.4, 400 mM NaCl, 5 mM $MgCl_2$, 2% Nonidet P-40, 20% glycerol and protease inhibitor cocktail lacking ethylenediaminetetraacetic acid). Cell lysis was completed on ice for 15 minutes. The cell lysates were incubated for 45 minutes with RalGDS-RBD beads (Millipore, Billerica, MA) to pull-down Rap1-GTP[2,3]. After 3 washing steps the pellets were solubilized in sample buffer (75 mM Tris/HCl pH 6.8, 10% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-Mercaptoethanol, 0.004% Bromophenol blue) for the detection of Rap1 by immunoblot. Small aliquots of each sample were saved to control that samples contained similar amounts of protein.

Western blotting

Proteins were separated by SDS–polyacrylamide gel electrophoresis on 4-20% gradient gels and transferred to polyvinylidene fluoride membranes. Standard western blotting procedures were used. Rap1 was detected with the Odyssey Infrared Imaging System (Li-Cor Biosystems).

1. Petrich BG, Fogelstrand P, Partridge AW, et al. The antithrombotic potential of selective blockade of talin-dependent integrin αIIbβ3 (platelet GPIIb–IIIa) activation. *J. Clin. Invest.* 2007;117(8):2250–2259.
2. Franke B, Akkerman JW, Bos JL. Rapid Ca2+-mediated activation of Rap1 in human platelets. *The EMBO Journal*. 1997;16(2):252–259.
3. Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood*. 2009;114(12):2506–2514.

Supplementary Figures and Figure Legends

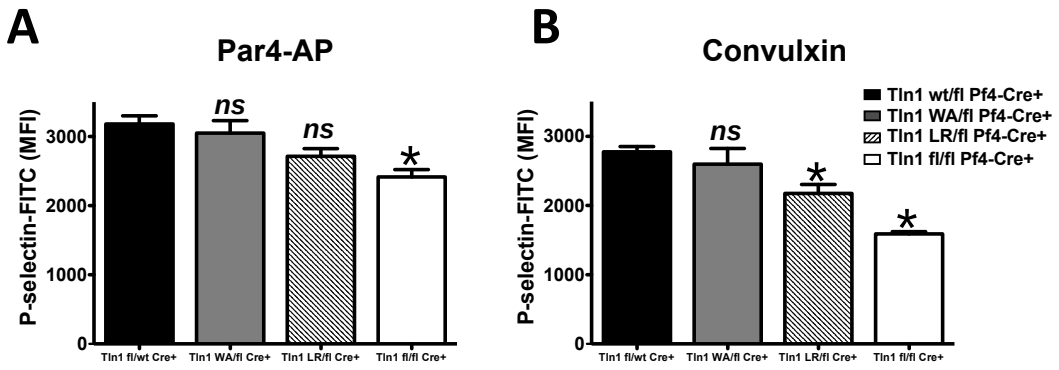

Figure S1. Secretion of α-granule (anti-CD62P antibody binding) in stimulated talin mutant platelets. Washed platelets isolated from mice with the indicated genotypes were stimulated with 600μM PAR4 activating peptide (Par4-AP) (**A**) or 900ng/ml convulxin (**B**). Bar graphs represent mean fluorescence intensity (MFI) ± SEM (n= 6, 3 independent experiments). * p<0.05.

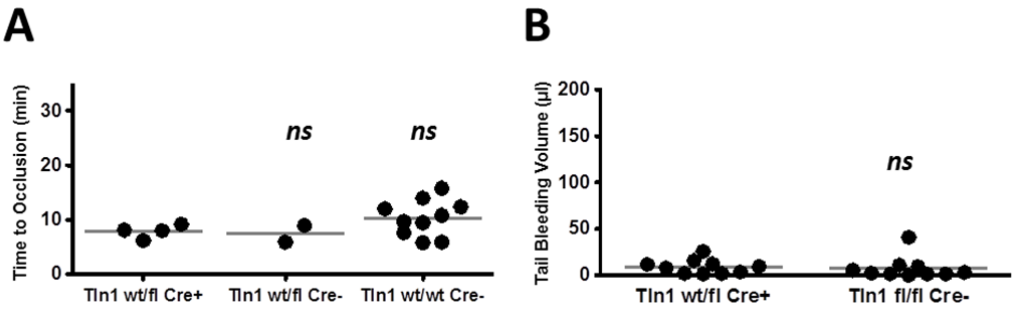

Figure S2. Carotid artery thrombotic occlusion times and tail bleeding volumes in Tln1 wt/fl Cre+ mice. A) Time to carotid artery occlusion following injury with 10% FeCl3 in mice with the indicated genotypes. **B**) Blood loss volumes were measured in Tln1 wt/fl Cre+ and Tln1 fl/fl Cre- mice during the 10 minutes following tail resection as described in Methods.