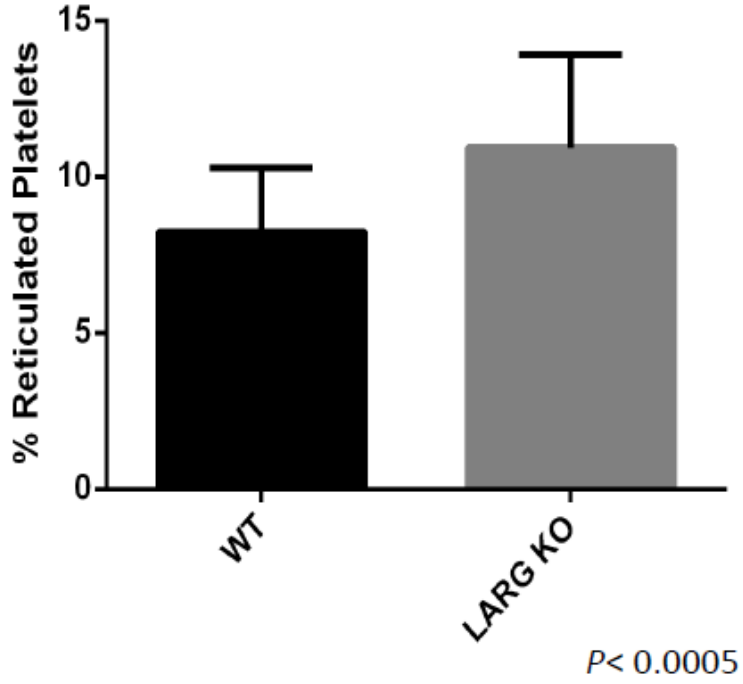
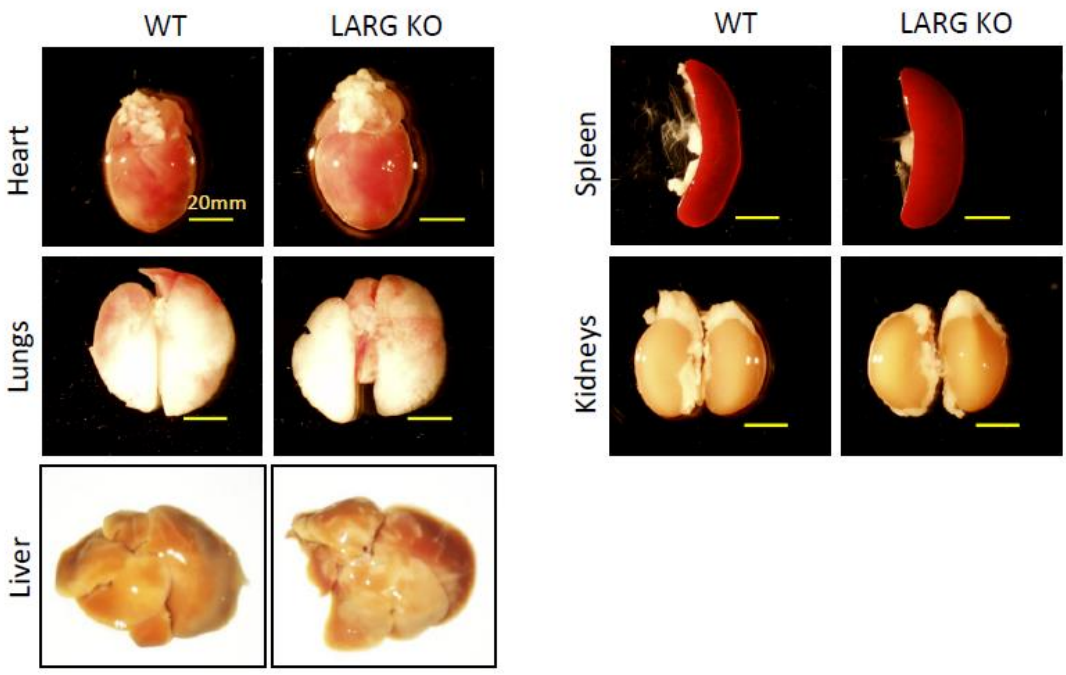
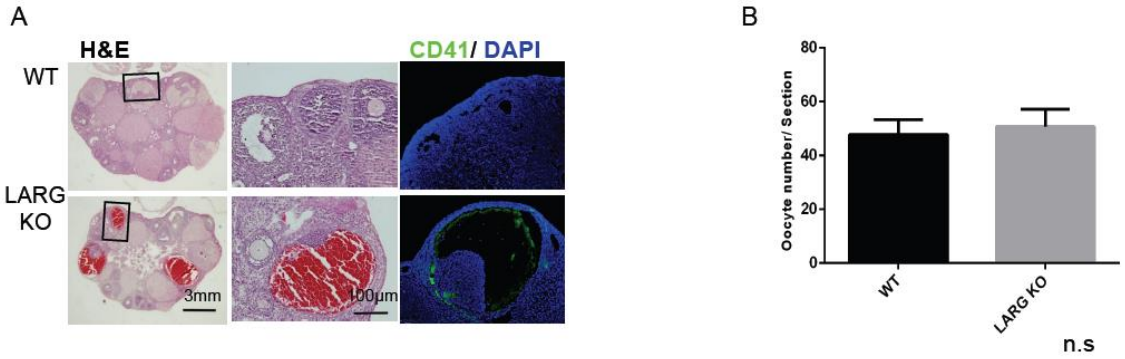
Supplementary Material to Zou et al. "Leukaemia-associated Rho guanine nucleotide exchange factor (LARG) plays an agonist specific role in platelet function through RhoA activation" (Thromb Haemost 2016; 116.3)



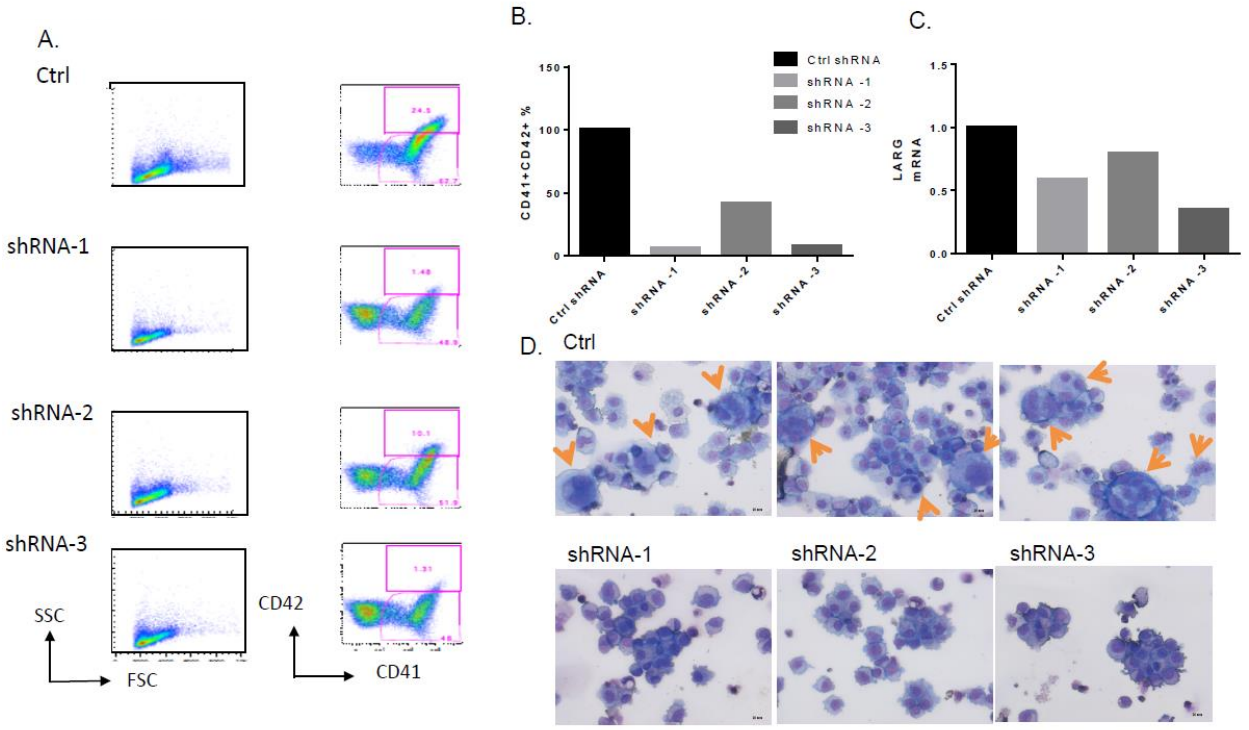
Suppl. Figure 1: *Larg* KO mice have significantly higher reticulated platelet percentage. The mean and SD of percentage of reticulated platelets as thiazole orange positive via flow cytometry is graphed. (WT n = 22; KO n = 32).



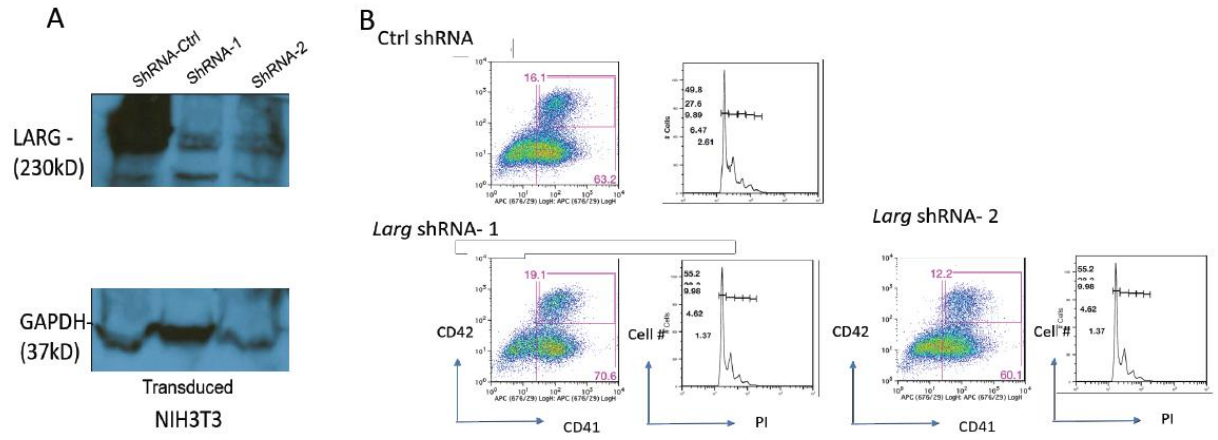
Suppl. Figure 2. Necropsy analysis on major organs of 8-week old WT versus LARG KO There are no obvious gross defects in the KO.



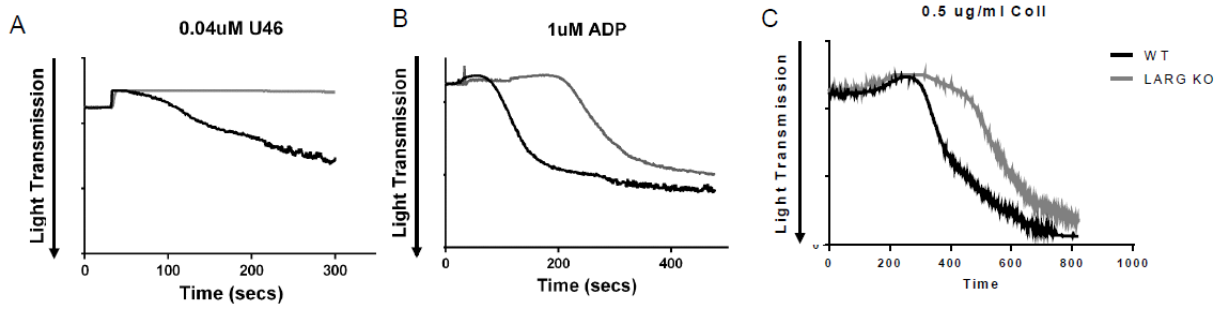
Suppl. Figure 3. Analysis of ovaries reveals hemorrhage, but no change in oocyte numbers. (A) H&E and CD41/DAPI staining of ovarian sections from WT and KO littermates. (B) Oocyte number was determined using ovarian sections from the largest cross-sectional area obtained (n=3 mice/genotype).



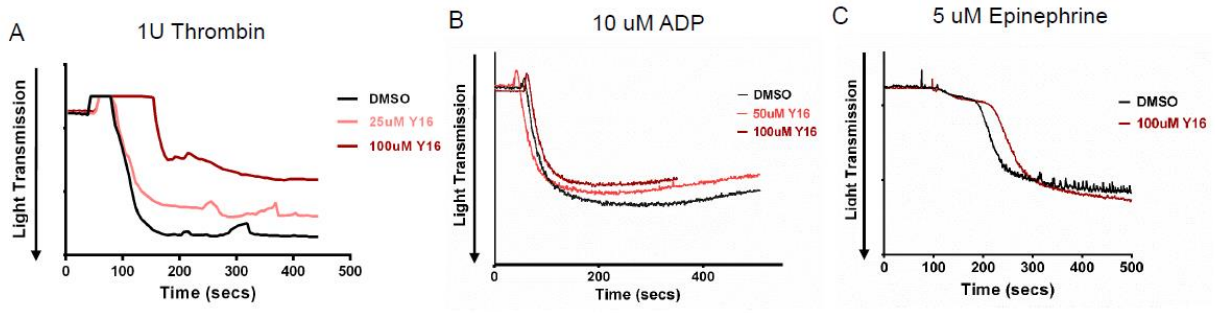
Suppl. Figure 4. Different *LARG* shRNA KD levels correlate with the degrees of inhibition of human MK maturation. Representative data from 1 experiment are shown. (A) Flow cytometric analysis of human MK cultures (starting from lineage depleted mobilized peripheral blood cells) after puromycin selection with control (top) and 3 different shRNAs against *LARG*. (B) Summary of mature (CD41+/CD42+) MK percentages with control and 3 different shRNAs. (C) Degree of *LARG* knockdown assessed by qRT-PCR in puromycin selected cell populations. (D) Morphological analysis of H&E stained cytospins revealed the absence of polyploid megakaryocyte (arrowheads in Ctrl) in the *LARG* knockdown group.



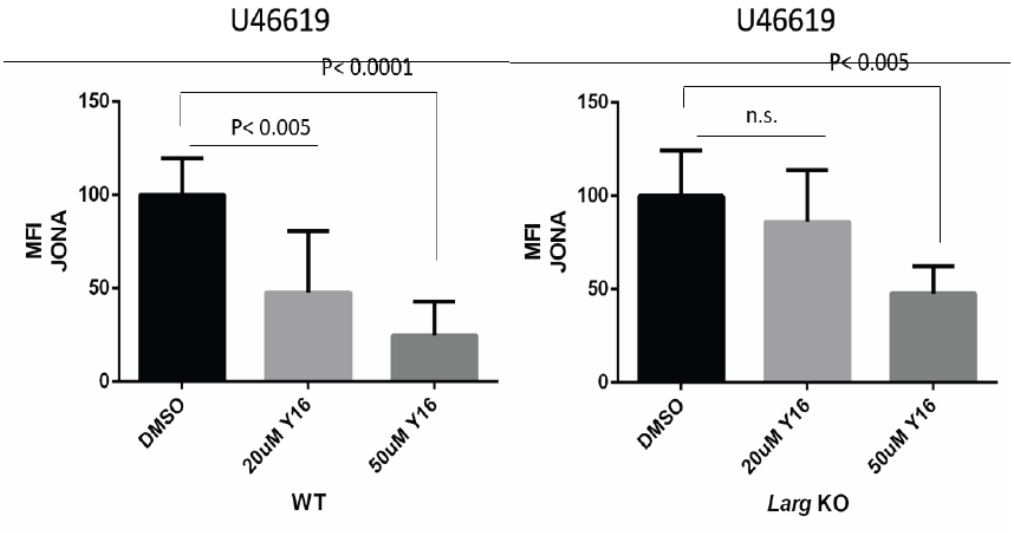
Suppl. Figure 5. Larg shRNA mediated knockdown does not affect murine MK maturation.
(A) Western blot validation of *Larg* knockdown levels in Ctrl or *Larg* shRNA-1/2 (TRCN0000109960; TRCN0000109963) virus transduced NIH3T3 Cells. GAPDH serves as loading control. (B) WT murine bone marrow cells in expansion medium were transduced with Ctrl or *Larg* shRNA-1/2 virus and cultured in the presence of puromycin to select for transduced cells prior to flow cytometric analysis for ploidy, CD41 and CD42. No obvious MK differentiation or polyploidization defects are seen in these three groups. (Representative data shown, 2 independent experiments were performed).



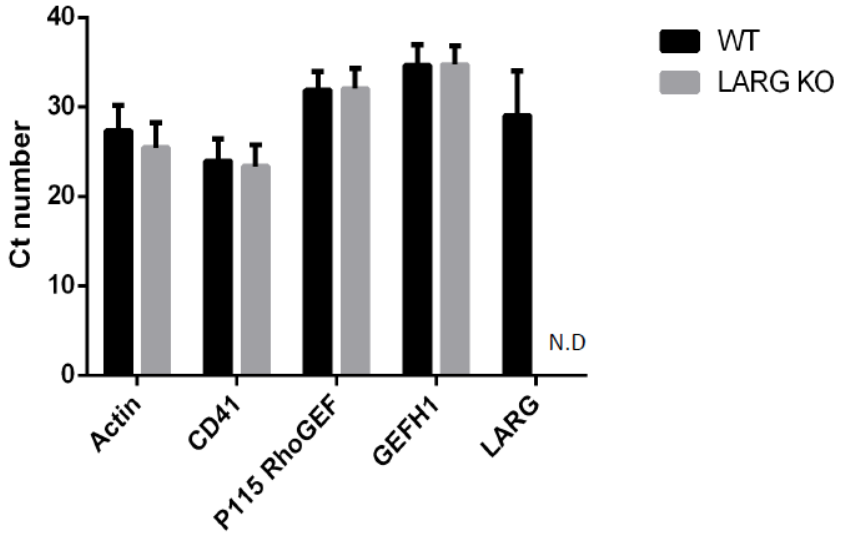
Suppl. Figure 6. Ligand-specific effect on aggregation of WT and *Larg* KO platelets in response to low doses of agonists. (A) Decreased aggregation of LARG KO platelets in response to low dose U46619. (B-C) A slight delay in platelet response, but no significant difference in maximum aggregation, of *Larg* KO platelets in response to low dose ADP and collagen.



Suppl. Figure 7. Y16 shows agonist-specific inhibition of human platelet aggregation. Y16 inhibits thrombin induced human platelet aggregation in a dose-dependent manner (A), but has no effect on ADP (B) or Epinephrine (C) stimulation. Representative data from 3 experiments with different donors are shown.



Suppl. Figure 8. Y16 has greater effect on WT than *Larg* KO platelet activation in response to U46619. Whole blood of WT (left) and *Larg* KO (right) mice was treated with 20 uM or 50 uM Y16 together with agonist (U44619) for 20 min prior to flow cytometric analysis. Relative Mean Fluorescence Intensity (MFI) is normalized to DMSO control for each experiment to allow for comparisons between experiments (3 independent experiments, n=3–6 per genotype). Note that 20 uM Y16 has a statistically significant effect on WT, but not *Larg* KO, platelet activation. At 50 uM, Y16 has a stronger effect on WT than KO, but it affects both suggesting that Y16 is not 100 % specific for murine Larg.



Suppl. Figure 9. No significant changes in the mRNA levels of other GEF genes in *Larg* KO versus WT platelets. Total RNA of washed platelets from WT and KO mice was analyzed by qRT-PCR of shown genes. (n=3/ genotype); ND= Not detected. *Larg* KO does not affect mRNA levels for P115RhoGEF or Gefh1 (Arhgef2) in platelets.

Supplemental Table 1

Comparison of platelet phenotypes of RhoA , Gα13, PF4-LARG KO and LARG KO mice

	RhoA KO (PF4-Cre)[1]	Gα13 KO (Mx-Cre)[2]	LARG KO (PF4 Cre)[3]	LARG KO[4]
Plt Trait	Increased volume Decreased count	No Change	Normal	Increased volume Decreased count
Aggregation	Defective with Thrombin, TXA2, PAR4-AP Normal with ADP	Defective with TXA2, PAR4-AP	Defective with TXA2 and PAR4-AP, Normal with ADP	Defective with TXA2 and Thrombin, Normal with ADP
Integrin α2bβ3 Activation	Defective with Thrombin, TXA2+ ADP Normal with ADP	Normal	Not tested	Defective with TXA2; Normal with ADP
α-granule Secretion	Defective with Thrombin, TXA2+ ADP	Defective with TXA2, PAR4-AP	Not tested	Defective with Thrombin, PAR4-AP (low dose)
Shape Change	Defective	Defective	Not tested	Normal
Spreading	Normal	Not done	Normal	Normal
Bleeding Time	Prolonged	Increase	Not tested	Prolonged
Thrombus Formation	Defective	Defective	Defective	Defective
RhoA Activation	Not tested	Compromised	Decreased baseline RhoA activity	Baseline unaffected, Activation compromised
MLC-Phosphorylation	Compromised	Compromised	N.A	Compromised

[1]Pleines et al., 2012; [2]Moers et al., 2003; [3]Williams et al., 2015; [4]Current study