Supplementary Materials

The secreted tyrosine kinase VLK is essential for normal platelet activation and thrombus formation

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Supplementary Methods

Immunofluorescence

Human platelets were isolated from whole blood obtained by venipuncture from a control individual and a patient with Hermansky-Pudlak syndrome (HPS) subtype 1 as previously described,[1] with approval from the Institutional Review Board at Beth Israel Deaconess Medical Center (BIDMC). Cells were stimulated with 150 µM thrombin-receptor activating peptide (TRAP) SFLLRN (Sigma-Aldrich) for 15 minutes or incubated on collagen coated coverslips for 30 minutes. Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes, blocked overnight with 10% normal goat serum and 1% BSA, incubated with anti- p-Tyr P100 antibody (Cell Signaling Technologies, #9411), and AlexaFluor-488–conjugated secondary antibody (ThermoFisher Scientific). Filamentous actin (F-actin) was stained last using Alexa Fluor 568 Phalloidin (Life Technologies) diluted in methanol to permeabilize and visualize platelets. Coverslips were mounted with Aqua-Poly/Mount (Polysciences) and evaluated by fluorescence microscopy.

Electron microscopy

Electron microscopy of mouse platelets was performed as previously described (Battinelli et al., *Blood* 2019). Samples were examined with a Tecnai G2 Spirit BioTWIN electron microscope (FEI company) at an accelerating voltage of 80 kV. Images were recorded with an AMT 2K CCD camera with AMT digital acquisition and analysis software (Advanced Microscopy Techniques). Representative images are shown from platelets isolated from 3 mice per genotype.

Platelet isolation

Human and mouse washed platelets were isolated from citrated whole blood by differential centrifugation with HEPES Tyrode buffer in the presence of prostaglandin E1 (PGE-1) as previously described.[2,3] After the last wash, platelets were treated with indicated agonists for 15 minutes at 37ºC, separated from supernatants by centrifugation, and lysed with RIPA buffer (150

mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) containing protease and phosphatase inhibitors. For each experimental replicate with mouse platelets, washed platelets were isolated from citrated blood collected by retro-orbital puncture with glass capillaries pooled together from 3 to 5 mice per genotype to ultimately achieve $5x10^8$ cells per mL.

Platelet aggregation and dense granule secretion assay

Platelet aggregation and ATP release from dense granules were measured from washed platelets as described before.[2] Aggregation in response to PAR4-agonist AYPGKF (Sigma-Aldrich), collagen (Chrono-log Corporation), and ADP (Sigma-Aldrich) was determined with ChronoLog 680 aggregometer (Chrono-log Corporation) at indicated concentrations. An agonist dose curve was run to determine the lowest dose at which aggregation was achieved for each independent experiment. Chronolume reagent (Chrono-log Corporation) was used to monitor ATP release from dense granules.

P-Selectin surface expression assay

P-selectin surface expression was measured on platelets from *Vlk*$^{f/f}$ and *Vlk-cKO* mice (n = 3 per genotype) by flow cytometry using FITC–labeled anti-mouse CD62P antibody (BD Pharmingen) in response to indicated concentrations of AYPGKF (Sigma-Aldrich) as previously described.[4] Data is expressed as percentage of P-selectin surface expression compared to stimulated control.

Hematological analysis of whole blood

Hematological parameters for 5 mice per genotype were determined using a Hemavet 850FS (Drew Scientific). Parameters analyzed include platelet volume (PLT Vol.); platelet (PLT), white blood cell (WBC) and red blood cell (RBC) count; hemoglobin (Hb) and hematocrit (HCT) levels.

Statistics

Statistical significance for all methods, except for laser-induced thrombosis, was determined using Student's t test. For laser-induced thrombosis, AUC was calculated for platelet and fibrin after laser injury. Mann-Whitney test was used for statistical comparison between genotypes. Statistical analyses were performed with GraphPad Prism 8.

Supplementary Tables

Table S1. Tyrosine phosphosites mapped to proteins with luminal, secreted, or extracellular domains of transmembrane proteins as annotated in UniprotKB detected in phosphoproteomics of human platelets.

Protein Name (Gene name)	Phosphosite	Reference(s)
Fibrinogen alpha chain (*FIBA*)	Y277	Izquierdo et al., *Thromb Haemost* 2020
Fibrinogen gamma chain (*FIBG*)	Y135	Izquierdo et al., *Thromb Haemost* 2020
Golgi integral membrane protein 4 (*GOLIM4*)	Y673	Beck et al., *Blood* 2017
Glycosaminoglycan xylosylkinase (*XYLK/FAM20B*)	Y138	Izquierdo et al., *Thromb Haemost* 2020
Latent-transforming growth factor beta-binding protein 1 (*LTBP1*)	Y443 Y523	Izquierdo et al., *Thromb Haemost* 2020
Lysosome-associated membrane glycoprotein 1 (*LAMP1*)	Y336	Izquierdo et al., *Thromb Haemost* 2020
Platelet basic protein (*PPBP*)	Y58	Izquierdo et al., *Thromb Haemost* 2020; Beck et al., *Blood* 2017
Platelet glycoprotein Ib alpha chain (*GP1BA*)	Y292 Y294	Zahedi et al., *J Proteome Res* 2008
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 (*PLOD2*)	Y323	Zahedi et al., *J Proteome Res* 2008
P-selectin (*SELP*)	Y346	Izquierdo et al., *Thromb Haemost* 2020
P2X purinoceptor 6 (*P2RX6*)	Y64	Maguire et al., *Proteomics* 2002
Secreted phosphoprotein 24 (*SPP24*)	Y109	Izquierdo et al., *Thromb Haemost* 2020
Thrombospondin 1 (*THBS1*)	Y1126	Izquierdo et al., *Thromb Haemost* 2020

Table S3, related to Figure 3. Hematological parameters in *Vlk-cKO* mice are comparable to *Vlk*$^{f/f}$ control littermates.

	***Vlk*$^{f/f}$**	***Vlk-cKO***	**P-value**
PLT Vol. (fL)	4.2 ± 0.24	4.2 ± 0.13	0.841
PLT X 10/µL	692 ± 85.46	616 ± 107.2	0.250
WBC 10/µL	4.22 ± 1.23	4.58 ± 2.00	0.744
RBC 10^6/µL	8.64 ± 0.77	8.04 ± 0.86	0.281
Hb (g/dL)	12.28 ± 1.05	12.90 ± 0.82	0.330
HCT (%)	42.30 ± 3.28	38.52 ± 3.75	0.128

Platelet volume (PLT Vol.); platelet (PLT); white blood cell (WBC) and red blood cell (RBC) count, hemoglobin (Hb) and hematocrit (HCT) levels; data are expressed as mean ± SD; n=5 per genotype.

Supplementary Figures

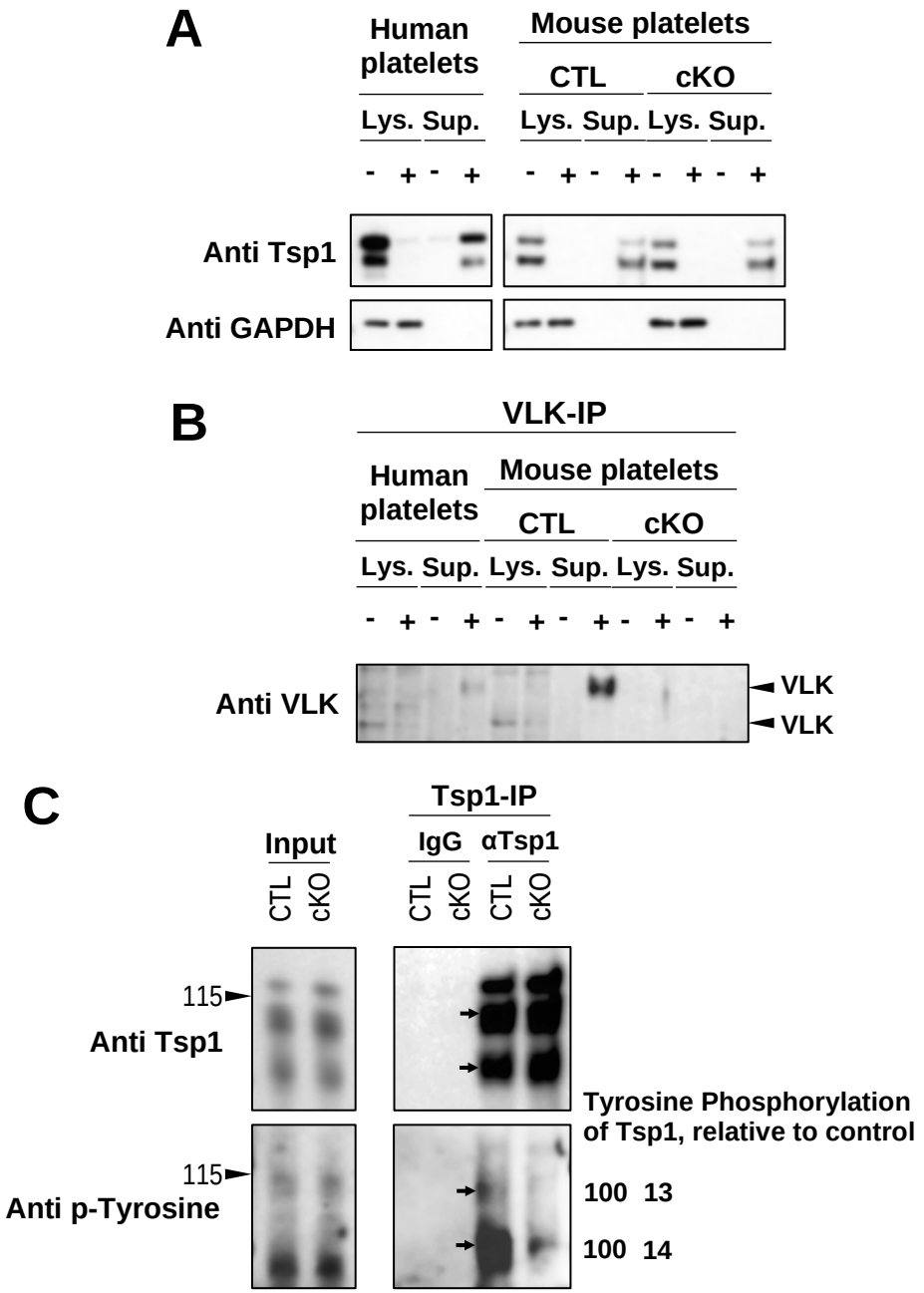

Figure S1. VLK is in the supernatant of thrombin-stimulated mouse platelets, related to Figures 3. (A) Immunoblots of lysates (Lys.) and supernatants (Sup.) from human, and *Vlk*$^{f/f}$ (CTL) and *Vlk-cKO* (cKO) platelets resting or stimulated with 5 U/mL thrombin for 15 minutes. Anti- Tsp1 antibody was used as marker of platelet activation, and anti-GAPDH antibody was used to examine total protein levels in lysates. **(B)** Lysates from A were used to examine the presence of VLK in lysates and supernatants by immunoblot of VLK immunoprecipitates (VLK-IP). **(C)** Input and Tsp1 IP fraction from thrombin-stimulated supernatants analyzed by SDS-PAGE with 8% gels and immunoblotting with anti-Tsp1 and anti-p-Tyr antibodies. IgG was used as negative control for non-specific binding to beads. Black arrows indicate bands decreasing in cKO compared to CTL. Quantifications were normalized to total Tsp1 levels in IP fraction.

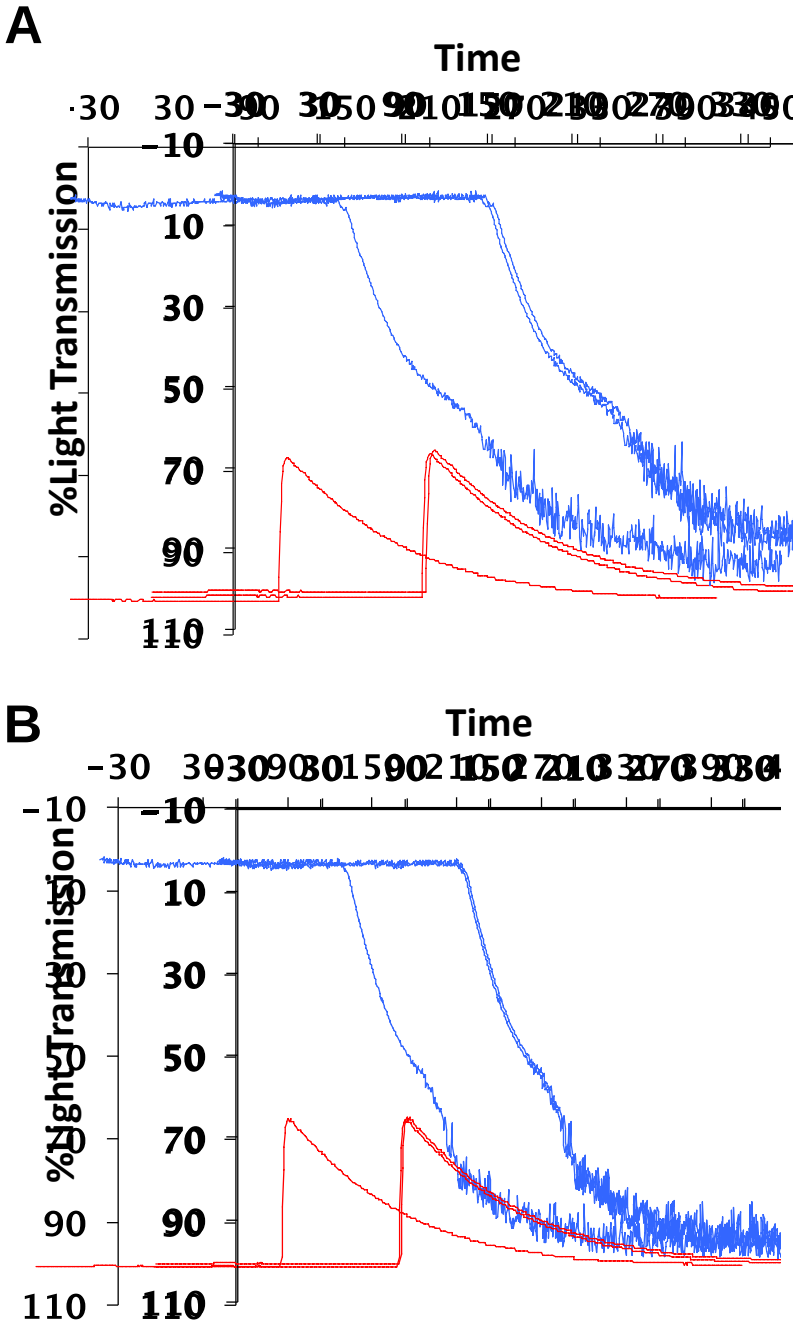

Figure S2. Platelets from *Pf4*-specific *Vlk-cKO* mice demonstrate normal ADP stores in response to high dose thrombin, related to Figure 4. (A, B) Representative aggregation (*blue*) and dense granule release (*red*) tracings in response to 5 U/mL thrombin in *Vlk*$^{f/f}$ **(A)** vs. *Vlk-cKO* **(B)** platelets.

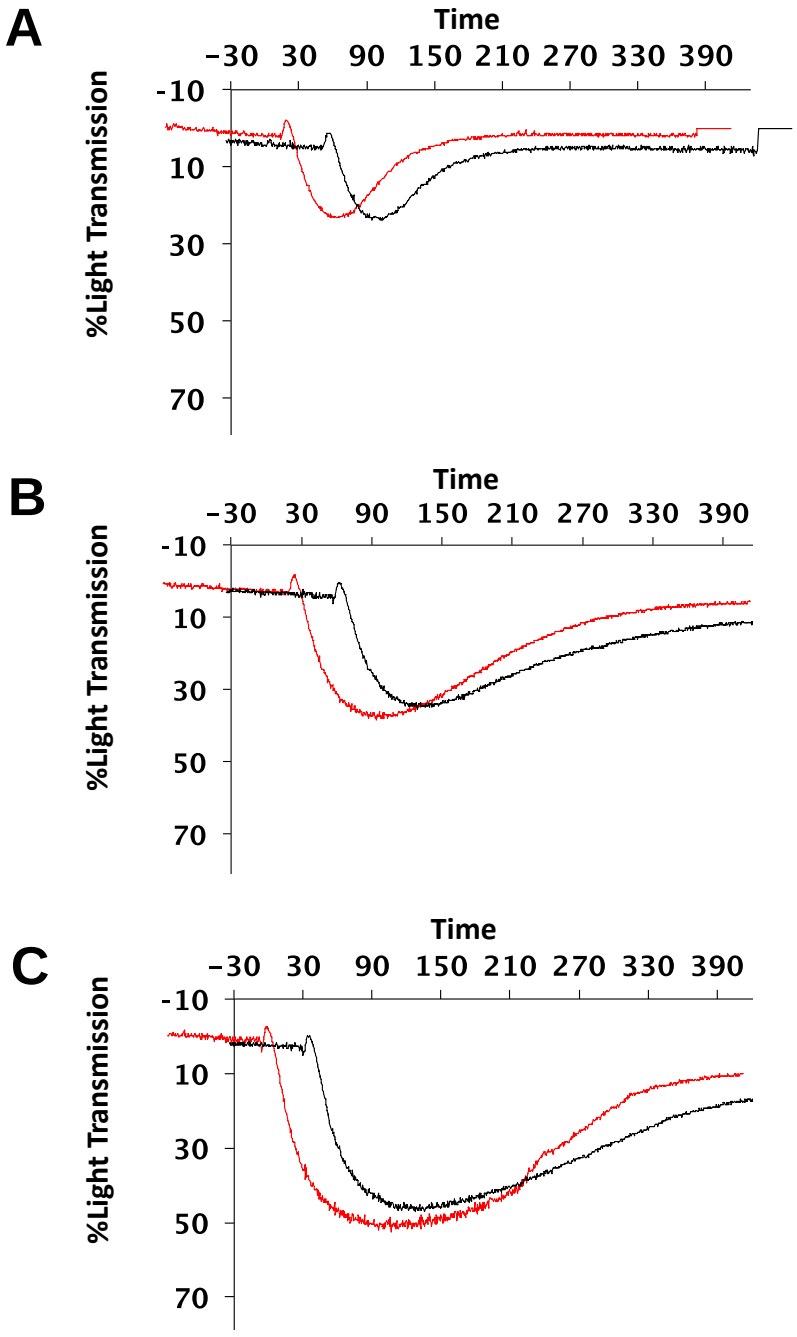

Figure S3. Platelet aggregation in response to various concentrations of ADP remains unaffected in *Vlk-cKO* platelets compared to control, related to Figure 4. (A-C) Representative aggregation tracings in response to low (10 μM) **(A)**, intermediate (40 μM) **(B)**, and high (100 μM) **(C)** doses of ADP in *VLK-cKO* platelets (*red*) compared to control (*black*).

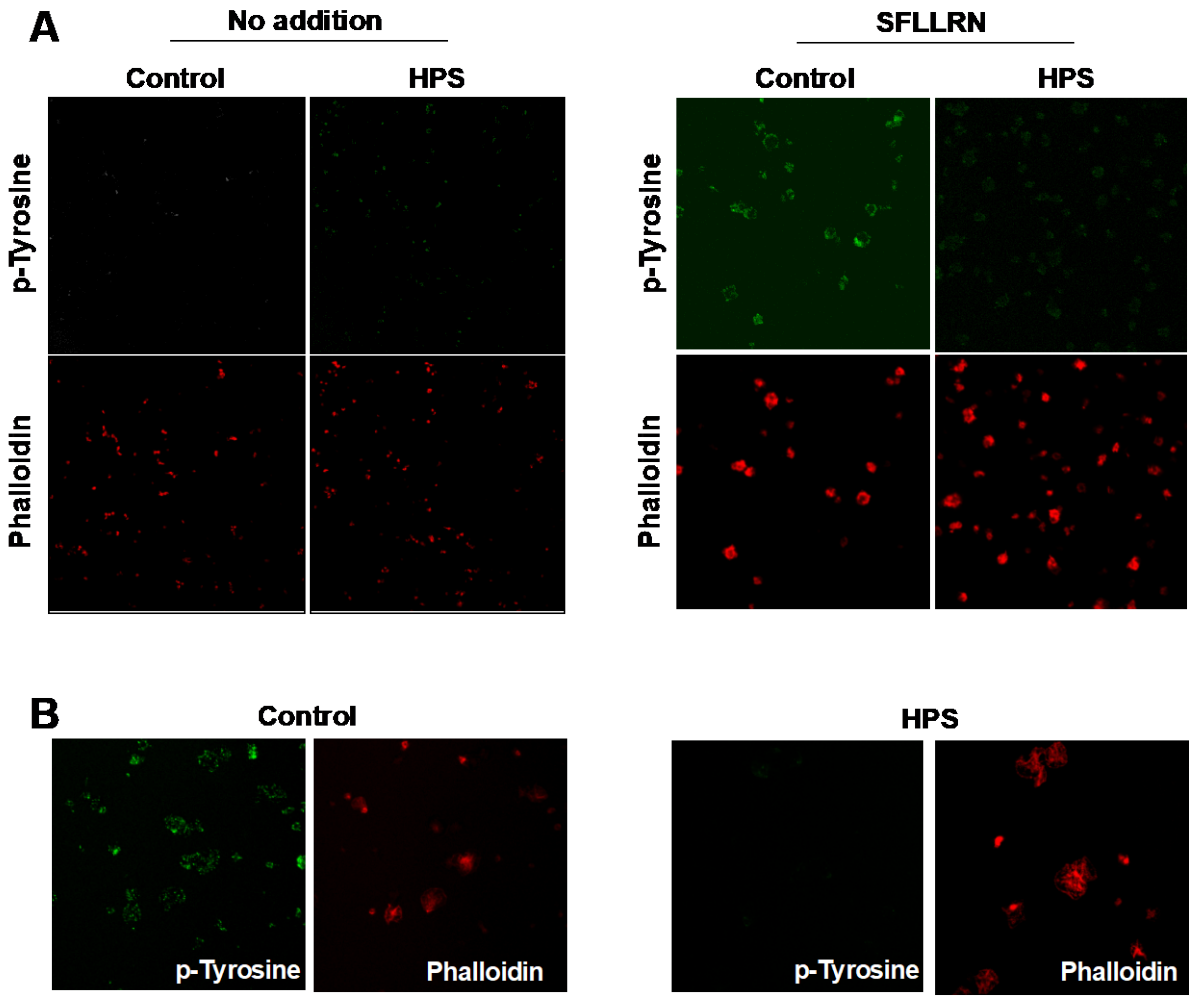

Figure S4. Impaired extracellular tyrosine phosphorylation in Hermansky-Pudlak syndrome, related to Figure 1. (A) Platelets from a control subject and a patient with Hermansky-Pudlak syndrome (HPS) were stimulated with 150 µM SFLLRN for 15 minutes. p-Tyrosine on the platelet surface was visualized by staining nonpermeabilized platelets using an anti p-Tyr antibody. Following p-Tyrosine staining, filamentous actin (F-actin) was stained with Alexa Fluor 568 phalloidin diluted in methanol to permeabilize and visualize platelets on slides. (B) Spreading platelets allowed to adhere for 30 minutes on collagen coated coverslips were stained using an anti p-Tyr antibody and Alexa Fluor 568 phalloidin.

Supplementary References

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