LIM KINASE-COFILIN DYSREGULATION PROMOTES MACROTHROMBOCYTOPENIA IN SEVERE VON WILLEBRAND DISEASE-TYPE 2B

SUPPLEMENTAL METHODS AND DATA

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MATERIALS AND METHODS

Materials

DMSO, α-monothioglycerol, phosphatidyl-choline, cholesterol and oleic acid were from Sigma-Aldrich. Alexa Fluor® 488-labeled phalloidin, Alexa Fluor® 594-labeled DNase I, Hoechst 33342, sheep anti-rat IgG Dyna-beads were from Thermo Fisher scientific. Fibrinogen was obtained from HYPHEN BioMed SAS. Recombinant human TPO and stem cell factor (SCF) were from Miltenyi Biotec. The RhoA kinase inhibitor Y-27632 was purchased from Calbiochem.

List of antibodies

Antibody	Company/#
Western blot	
Cofilin	Cell signaling/D3F9
Cofilin-P	Cell signaling/77G2
MPL	R&D/AF1317
14-3-3ζ	Santa Cruz/C16
Lyn-P	Abcam/Ab40660
PLCY2-P	Cell signaling/3841
PLCY2	Cell signaling/3872
LIMK1/2-P	Cell signaling/3841
RhoA	Cell signaling/67B9
Rac	Cytoskeleton, Inc/ ARC03
IQGAP1	Santa Cruz/H-109
p38-P	Promega/V1211
Flow cytometry	
GPIb (CD42b)	Emfret/clone Xia-B2
Integrin αIIbβ3 (active)	Emfret/clone JON/A
Integrin αIIbβ3/CD41	BD Biosciences /MWReg30
Immunofluorescence	
α-tubulin	Sigma/T6074
VWF	DAKO/A0082
In vivo: platelet depletion	
GPIb	Emfret/R300
Preparation of megakaryocytes	
Ter119	BD Biosciences/clone TER-119
B220	BD Biosciences/clone RA3-6B2
CD4	BD Biosciences/clone GK1.5
CD5	BD Biosciences/clone 53-7.3
CD11b	BD Biosciences/clone M1/70
Gr-1	BD Biosciences/ clone RB6-8C5

: catalog or clone number

Determination of TPO levels in plasma

Blood was collected on sodium citrate 0.138M. TPO levels were determined in plasma using commercially available species-specific kits (R&D Systems).

Generation and quantification of megakaryocytic colonies and CFU-C

Mouse bone marrow cells ($1.5x10^5$ per slide) or spleen cells ($3.25x10^5$ per slide) were cultured in Megacult-C complete kit supplemented with human recombinant TPO (10 nM) and mSCF (50 nM, Miltenyi Biotech) according to the manufacturer's instructions (for mice; StemCell Technologies). The number of

megakaryocytic colonies (colony-forming units–megakaryocyte [CFU-MKs]) was determined in a semi-solid culture according the manual instruction of manufacturer (StemCell Technologies). The total number of colonies was counted 6 days later. For myeloid colony-forming unit (CFU-C), 2.10^4 mouse bone marrow cells were plated per dish in a methylcellulose medium (MethoCult GF M3434; Stem Cell Technologies). After 7 to 8 days of growth at 37 °C, colonies were scored with an inverted microscope based on their morphology.

Bone marrow hematopoietic stem and progenitor cells and megakaryocyte ploidy

Unfractionated bone marrow cells were stained with APC (allophycocyanin) mouse lineage antibodies cocktail against CD3, CD45R, Ly6C, Ly6G, CD11b, and Ter-119 forming the lineage-committed cells (Lin+) (BD Mouse Hematopoietic Stem and Progenitor Cell Isolation Kit, BD Biosciences). Mouse hematopoietic stem and progenitor cells were detected with various antibodies in the Lin^- cells population: PE-Cy™7 anti-mouse Sca1, PE anti-mouse c-kit, PerCP-Cy™5.5 anti-mouse CD16/32 (FcγRIII/II), FITC anti-mouse CD34 or FITC anti-mouse CD41. DAPI (4',6'-diamidino-2-phenylindole) (100 µg/ml; Invitrogen) was used to exclude dead cells. The kit contains matching isotype controls conjugated to the same fluorochrome as one of the specific antibodies, at the same concentration. Acquisitions were made with a BD CANTO 2 flow cytometer. The gating scheme used is delineated in Supplemental Figure 4. MEP: megakaryocyte-erythrocyte progenitors identified as $Lin^-c\text{-}kit^+Sca1^-CD34^-$ cells, Pre-MK: pre-megakaryocytes, identified as $Lin^-c\text{-}kit^+Sca1^-CD41^+$.

After *in vitro* differentiation of Lin^- cells, the percentage of MK precursors ($CD41^+CD42b^-$) and mature MKs ($CD41^+CD42b^+$) and the expression of CD41 (integrin αIIb), CD42b (GPIbα) at the surface of MK were analyzed by flow cytometry (FACSCalibur, BD Biosciences).

To determine megakaryocyte ploidy, bone marrow cells were co-stained with FITC-conjugated CD41 and with Hoechst 33342. The DNA content was determined with a flow cytometer BD LSR II (BD Biosciences) at wavelength emission of 450 nm. All results were analyzed with DIVA software (BD Biosciences), a pool of three mice with each genotype were analyzed three times for both experiments.

Femur/spleen preparation and vWF staining

Femur and spleen tissues were fixed in 2% paraformaldehyde in Phosphate-Buffered Saline (PBS) for 24 hours. Femurs were subsequently decalcified by immersion in a daily fresh 0.5 M EDTA solution for 10 days as previously described (1). After rinsing in PBS, femur and spleen were embedded in paraffin, dehydrated in ethanol (70%-96%-100%) and cleared in xylene. Longitudinal sections were cut every 10 µm using a HM360 microtome (Microm) and were deparaffinized in xylene, followed by decreasing series of ethanol concentrations and distilled water. Megakaryocytes in spleen sections were stained for vWF. Subsequently, endogenous peroxidase activity was blocked by a 20 min incubation in methanol containing 0.3% H_2O_2 (Merck) at RT and then saturated with 10% pre-immune goat serum, 1% BSA, 0.2% triton X-100 during 30 min at RT before application of the primary rabbit anti-human vWF antibody (1/300; A0082; DAKO) applied O/N at 4°C. The secondary goat anti-rabbit antibody coupled to biotin was applied 1h15 at RT. vWF signals were then visualized with 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich) at RT. Sections were then run through a series of increasing percentages of ethanol and finally xylene. The sections were mounted with Eukitt. Negative control included a section incubated with buffer alone. MK size was measured in the bone marrow with Fiji software.

Bone marrow explants analysis

Bone marrow explants were analyzed as previously described (2). Briefly, bone marrows from WT or 2B mice were obtained by flushing femora and were cut in transverse sections of 0.5 mm and placed in a 24-wells plate containing Tyrode's buffer (137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 12 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 7.4) supplemented with 5% WT mouse serum. Explants were maintained at 37°C for 6 hours. Megakaryocytes at the periphery of the tissue were observed under transmission optical microscope. Cells were classified according to their morphology as round shaped, MK deformed, MK with membrane protrusions and MK with proplatelets. Three mice with each genotype were analyzed.

Proplatelets formation over vWF matrix in flow condtions

Following purification by BSA gradient, murine mature MKs ($4x10^5$ cells/ml) were perfused at 18 dyn/cm^2 in a microfluidic platform (BioFlux200®, Fluxion Biosciences) during 45 min 37°C in complete medium

displaying a viscosity of 1 cP (0.98-1.05 cP). Prior to perfusion, microchannels were coated with murine rvWF as previously described (3). The microfluidic plate was positioned on the stage of an inverted microscope (Axiovert 135, Zeiss). A CCD camera (Sony and Q-click Imaging) was used to visualize cells, using a 20x PlasDIC modulation contrast objective. Continuous recording was performed with a digital image recorder (Replay Software, Microvision Instruments) at 1 image/s. The number of platelets and proplatelets released were quantified at various times (10, 20, 30 and 40 min) in 25 fields.

Immune thrombocytopenia

Thrombocytopenia was induced in 6-weeks-old WT and 2B mice by intravenous injection of a mixture of anti–mouse GPIbα antibodies (R300: 1 μg/g of mouse weight). Blood was collected before injection and then at various times after injection. Blood counts were determined with an automatic cell counter at various times (Scil Vet ABC Plus, Horiba Medical).

RhoA and Rac1 activation assay

Pull-down assays were performed following manufacturer instructions (Cytoskeleton). Briefly, washed mature MKs were lysed (50mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal) and 30 µl of the samples of the cell lysate were saved as a RhoA and Rac1 control. Then, samples were incubated for 1 hour with glutathione S-transferase (GST)– tagged Rhotekin-RBD protein on agarose beads (50 µg/ml) or GST-tagged PAK-PBD protein on agarose beads (10 µg/ml) at 4°C while rotating. The beads were then washed and Guanosine 5'-triphosphate (GTP)-bound RhoA and Rac1 were eluted with SDS sample buffer, separated by 4-12% SDS-PAGE, and immunoblotted with anti-RhoA antibody (1/1000) or anti-Rac1 antibody (1/500).

Immunoblotting

MKs were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 µM phenylarsine oxide, 1% SDS, 5 µg/ml leupeptin, 10 µg/ml aprotinin, pH 7.4). The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with various primary antibodies: cofilin (1/1000), p-cofilin (1/1000), IQGAP1 (1/1000), PLCγ2

and p-PLCγ2 (1/1000), p-p38 (1/1000), p-Lyn (1/5000), p-LIMK1/2 (1/1000), 14-3-3ζ (1/1000), MPL (1/1000). Immunoreactive bands were visualized using Enhanced Chemiluminescence Detection Reagents (Pierce). Images of the chemiluminescent signal were captured using G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene).

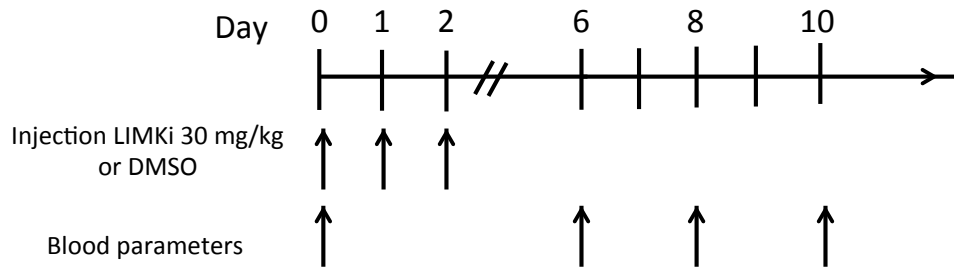
Transmission electron microscopy (TEM) of platelets and bone marrow

Washed platelets (4) were fixed in 1.25% glutaraldehyde and 0.1 M phosphate buffer pH 7.2.

Bone marrows were collected and fixed in 2.5% glutaraldehyde/PFA/Cacodylate and 0.1 M phosphate buffer at 4°C overnight. One hour after fixation in 1% osmium tetroxide and 0.1 M cacodylate buffer at 4°C, samples were dehydrated in graded series of alcohol and embedded in epoxy resin. Ultrathin sections, approximately 70 nm thick, were made using a Reichert ultracut ultramicrotome (Leica Microsystems), mounted on 200 mesh copper grids, contrasted with uranyl acetate and lead citrate. Sections were examined using a JEOL JEM1400 transmission electron microscope equipped with a Gatan Orius 600 camera and Digital Micrograph software (Lyon Bio Image, Centre d'Imagerie Quantitative de Lyon Est). Some thick bone marrow sections were completely dried, stained with 1% toluidine blue/2% borate for 1–2 minutes, and then gently rinsed rinsed with distilled water prior to TEM.

LIMK inhibition *in vitro* and *in vivo*

The LIMK inhibitor (Merck Millipore) was diluted in DMSO to reach a final concentration of DMSO in cells culture of 0.2% (1/500, vol/vol). *In vivo*, WT and 2B mice were injected subcutaneously with LIMKi (30 mg/kg, corresponding to 1.6 µl/g of mouse weight) or DMSO as control (1.6 µl/g of mouse weight), daily for 3 days. Blood counts were determined with an automatic cell counter at various times (Scil Vet ABC Plus, Horiba Medical). The following scheme describes this protocol:



Measure of bleeding time

Bleeding time assays were performed on 8-week old mice by cutting off the tip of the tail (3 mm from the tip) and immediately immersing it in saline. We then recorded the time taken for the bleeding to stop. Tail bleeding was monitored for at least 60 seconds beyond this time point, to ensure that bleeding did not start again. Tail bleeding assays were stopped at 600 seconds if the bleeding did not stop.

Flow cytometric analysis

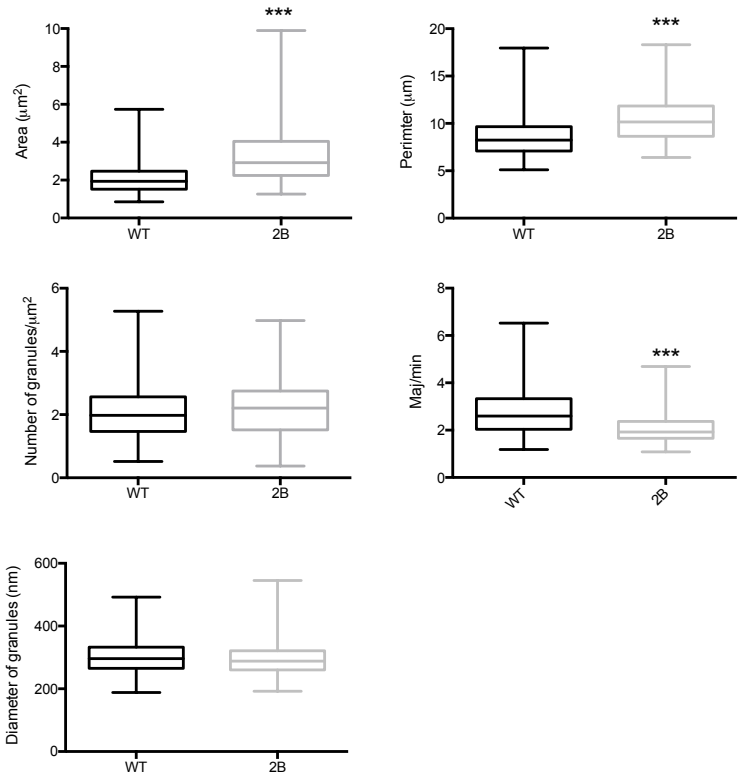
The activation level of integrin αIIbβ3 was evaluated by use of JON/A, a mAb specific for the activated conformation of mouse integrin αIIbβ3. Platelets were stimulated with ADP (10 μm) as agonist for 30 minutes and then incubated with PE-JON/A antibody. Because 2B mice present giant platelets increasing the amount of the integrin αIIb, the activation state of αIIbβ3 is calculated as a ratio (JON/A / αIIb total). CD41 or αIIb was measured with FITC anti-mouse CD41 antibody. Samples were analyzed in an Accuri C6 (Becton Dickinson) flow cytometer.

Legend to supplemental movies 1 and 2

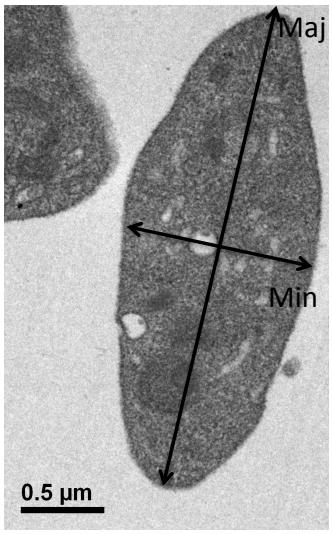
Mouse MKs were perfused at a shear rate of 18 dyn/cm² for 45 min on vWF-coated coverslips and translocating MKs were visualized on the vWF surface. Digital images were recorded at 1 image/s using “Replay” and “Histolab” software. For visualization, “Archimed” software (Microvision Instruments) was used to grab frames and record at a velocity of 5 images/s. Movies 1 and 2 are from WT and 2B mice, respectively. Bar is 20 μm. Arrows depict fragmentation into proplatelet, with a cytoskeletal extension devoid of a nucleus separating from the core of the MK.

Supplemental Figure 1

A



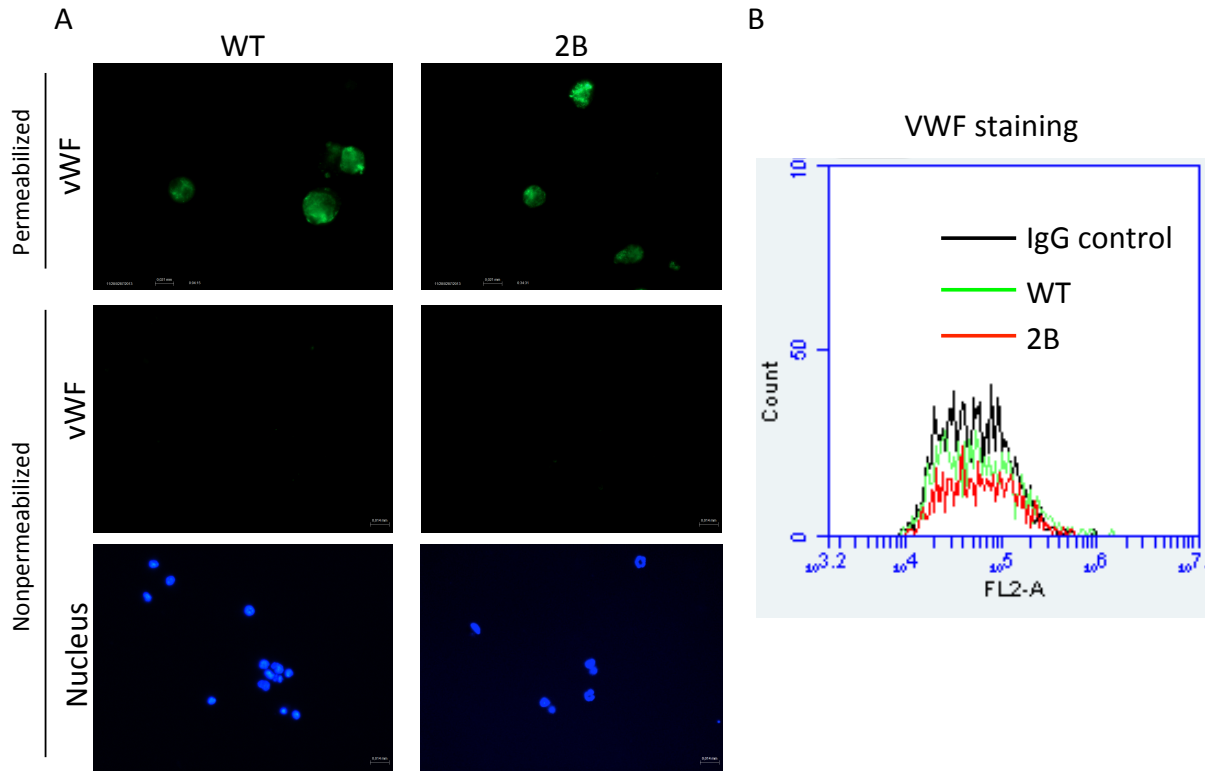
B



Supplemental Figure 1: (A) Platelet size measured by transmission electronic microscopy: area, perimeter, number of α-granules/μm^2, diameter of α-granules (nm) and the ratio Maj/min (major axis/minor axis) as illustrated in (B). Hundred platelets were analyzed in each genotype. Data are presented in a Box-and-whiskers plot and statistical significance was determined by Student's *t* test (*** $p<0.001$).

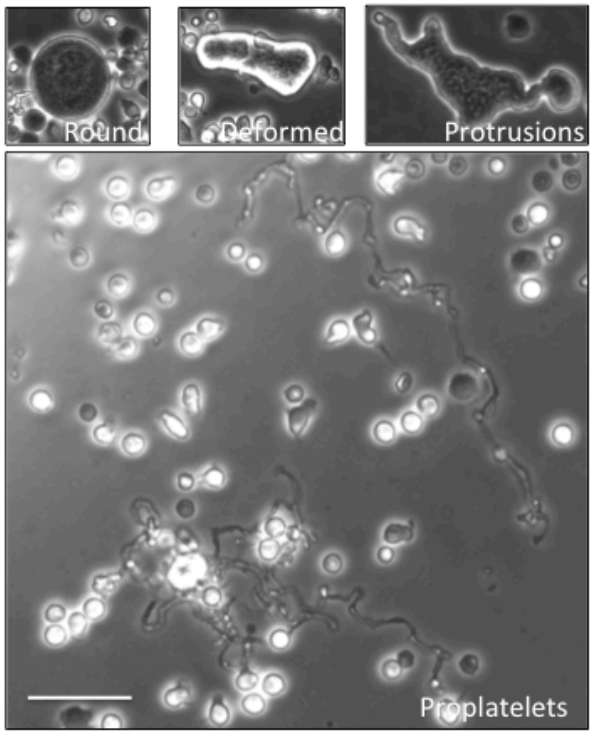
Supplemental Figure 2

As previously described (5), our data demonstrated that membrane-associated vWF was not detected on round mature MKs by immunofluorescence and flow cytometry.



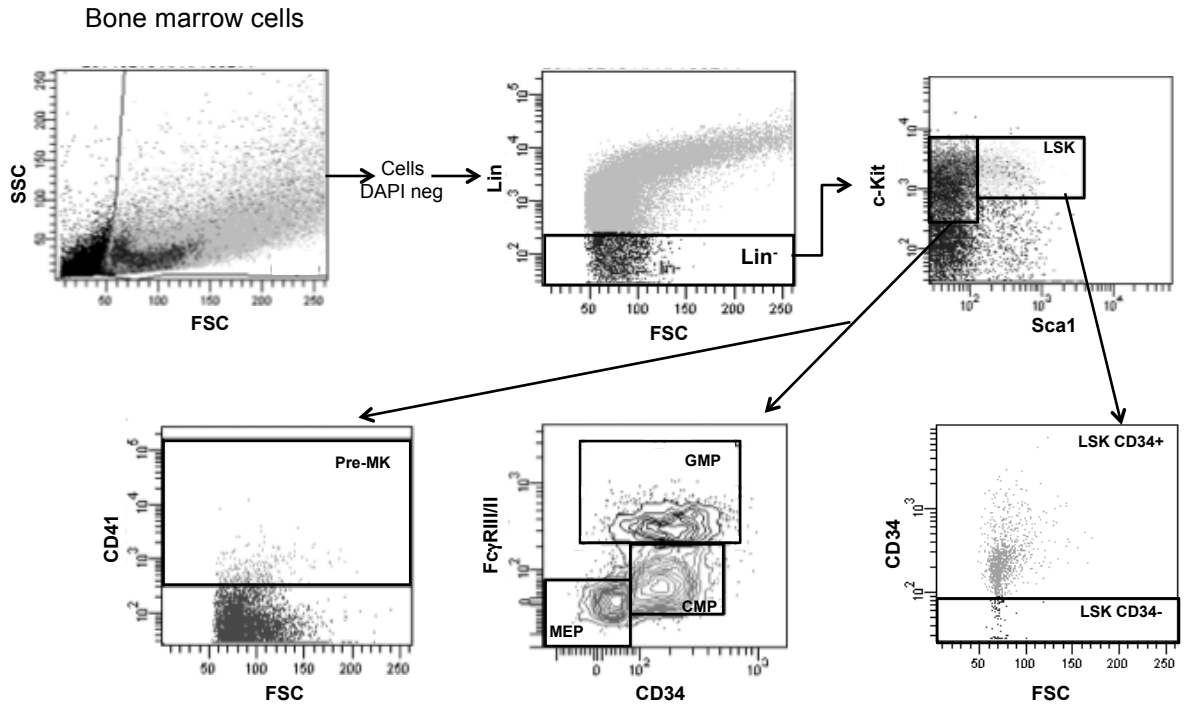
Supplemental Figure 2: WT vWF and p.V1316M vWF are synthetized by MK and are not expressed at the MK surface. (A) Immunofluorescence staining of the vWF in permeabilized and non permeabilized mature MKs. The nucleus is stained with DAPI. (B) Flow cytometry of the vWF at the surface of MKs in WT and 2B MKs.

Supplemental Figure 3



Supplemental Figure 3: Adult mouse bone marrow samples were bathed in a physiological buffer as previously described (2). In WT bone marrow (BM), some cells retained a spherical shape, others were deformed, formed protrusions or even developed thick extensions (proplatelets) and decreased size of the cell body. (Scale bar = 60 μm).

Supplemental Figure 4



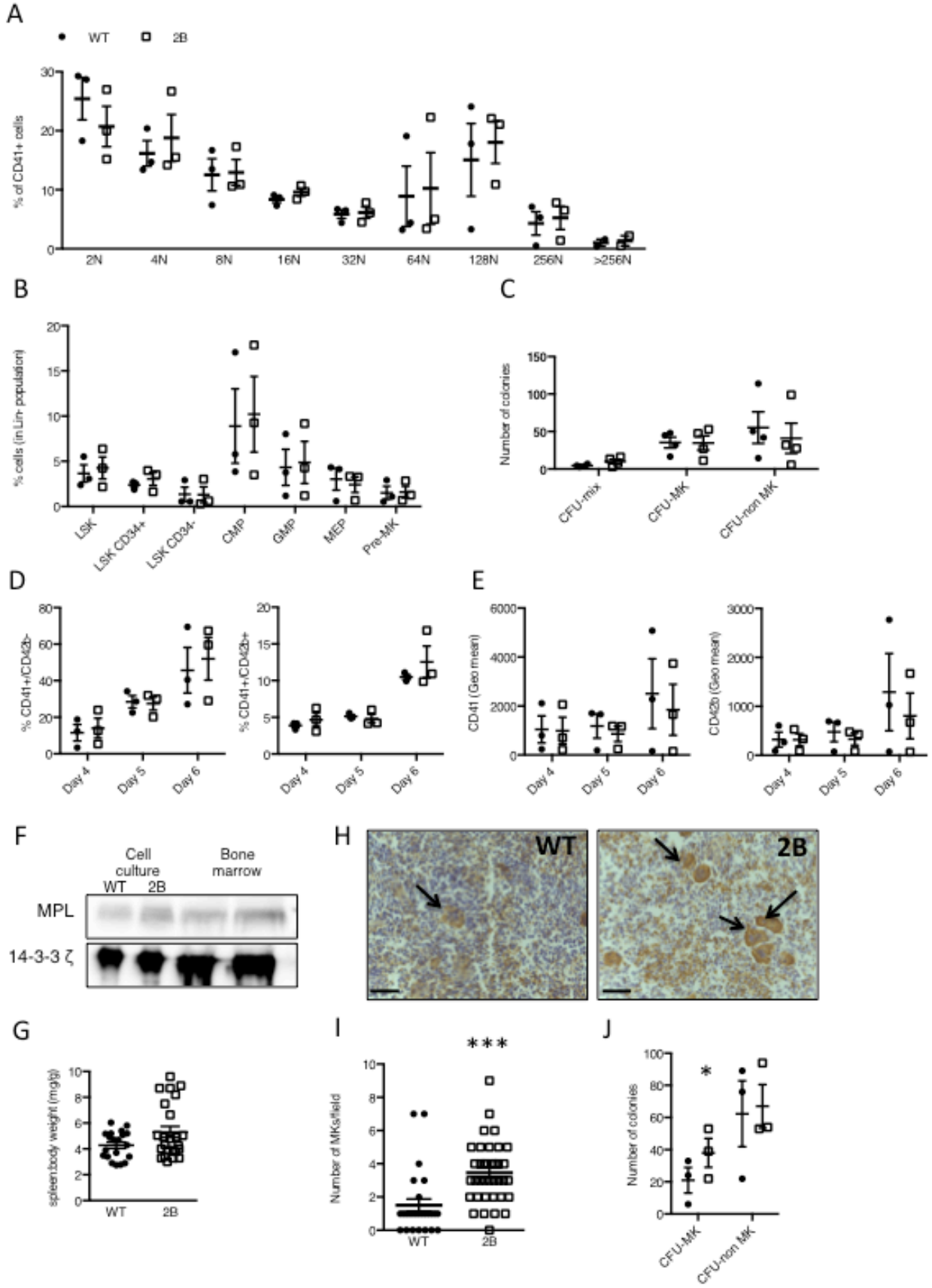
Supplemental Figure 4: Gating diagram describing the populations of mouse hematopoietic stem and progenitor cells in the bone marrow measured by flow cytometry. (Pre-MK: pre-megakaryocytes; MEP: megakaryocyte-erythrocyte progenitors; GMP: granulocyte-macrophage progenitor; CMP: common myeloid progenitor; LSK: $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$).

Supplemental Figure 5

The macrothombocytopenia does not result from a defect in MK differentiation.

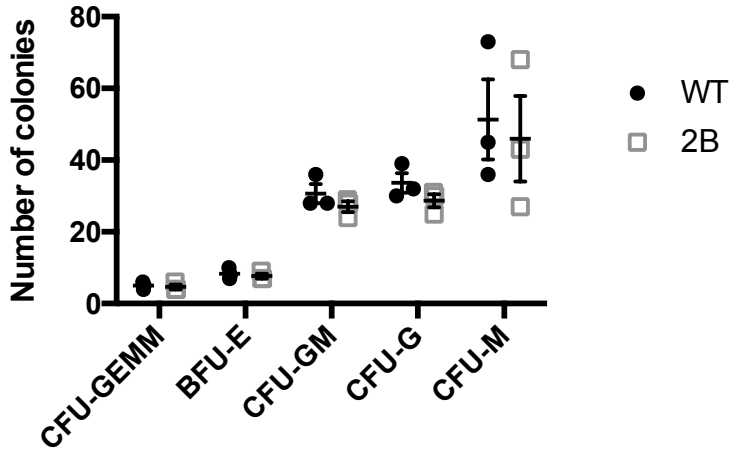
As a marker of MK differentiation, we examined DNA ploidy, and we also quantified the percentage of MK/erythroid cell fraction (MEP), as well as the percentage of MK progenitors (Pre-MK). No difference was observed in ploidy distribution of the BM $CD41^+$ cell population of WT and 2B mice, (Figure S5A). Also progenitor populations were similar in both mice (MEP: 2.5 ± 0.9 % *vs* 3 ± 1%; Pre-MK: 1.5 ± 0.7 *vs* 1.4 ± 0.9 % in 2B and WT mice, respectively, Figure S5B) and the percentage of $CD34^+$, $CD34^-$ cells, CMP (common myeloid progenitor) and GMP (granulocyte–monocyte progenitor) were similar as well (Figure S5B). After *in vitro* differentiation of Lin^- cells with thrombopoietin (TPO), we did not observe any difference in the % of MK precursors, mature MKs (Figure S5D) and expression of CD41, CD42b and MPL receptor (TPO receptor) (Figure S5E-F). Altogether, these data indicate that p.V1316M mutation does not modify the MK differentiation.

Because the spleen can also act as a site of hematopoiesis, we looked for evidence of spleen dysfunction. A higher spleen weight was observed in 29% of 2B mice (Figure S5G). Moreover, irrespective of the spleen weight, we observed clusters of MKs in the red pulp associated with a 2.3-fold increase of the number of MKs in 2B mice compared to WT mice ($p<0.001$) (Figure S5H-I). These results suggest that extramedullary hematopoiesis is taking place in 2B mice, as confirmed by the increase of the number of CFU-MK (Figure S5J). This could be associated with the TPO concentration in the circulation. Indeed, the level of TPO was significantly increased in the plasma of 2B mice (488 ± 24 pg/ml) compared to WT plasma (303 ± 19 pg/ml) (Table 1).


A
WT
2B
% of CD41+ cells
2N
4N
8N
16N
32N
64N
128N
256N
>256N
B
% cells (in Lin- population)
LSK
LSK CD34+
LSK CD34-
CMP
GMP
MEP
Pre-MK
C
Number of colonies
CFU-mix
CFU-MK
CFU-non MK
D
% CD41+/CD42b-
% CD41+/CD42b+
Day 4
Day 5
Day 6
E
CD41 (Geo mean)
CD42b (Geo mean)
F
Cell culture
Bone marrow
WT
2B
MPL
14-3-3 ζ
H
WT
2B
G
spleen:body weight (mg/g)
I
***
Number of MKs/field
J
*
Number of colonies
CFU-MK
CFU-non MK

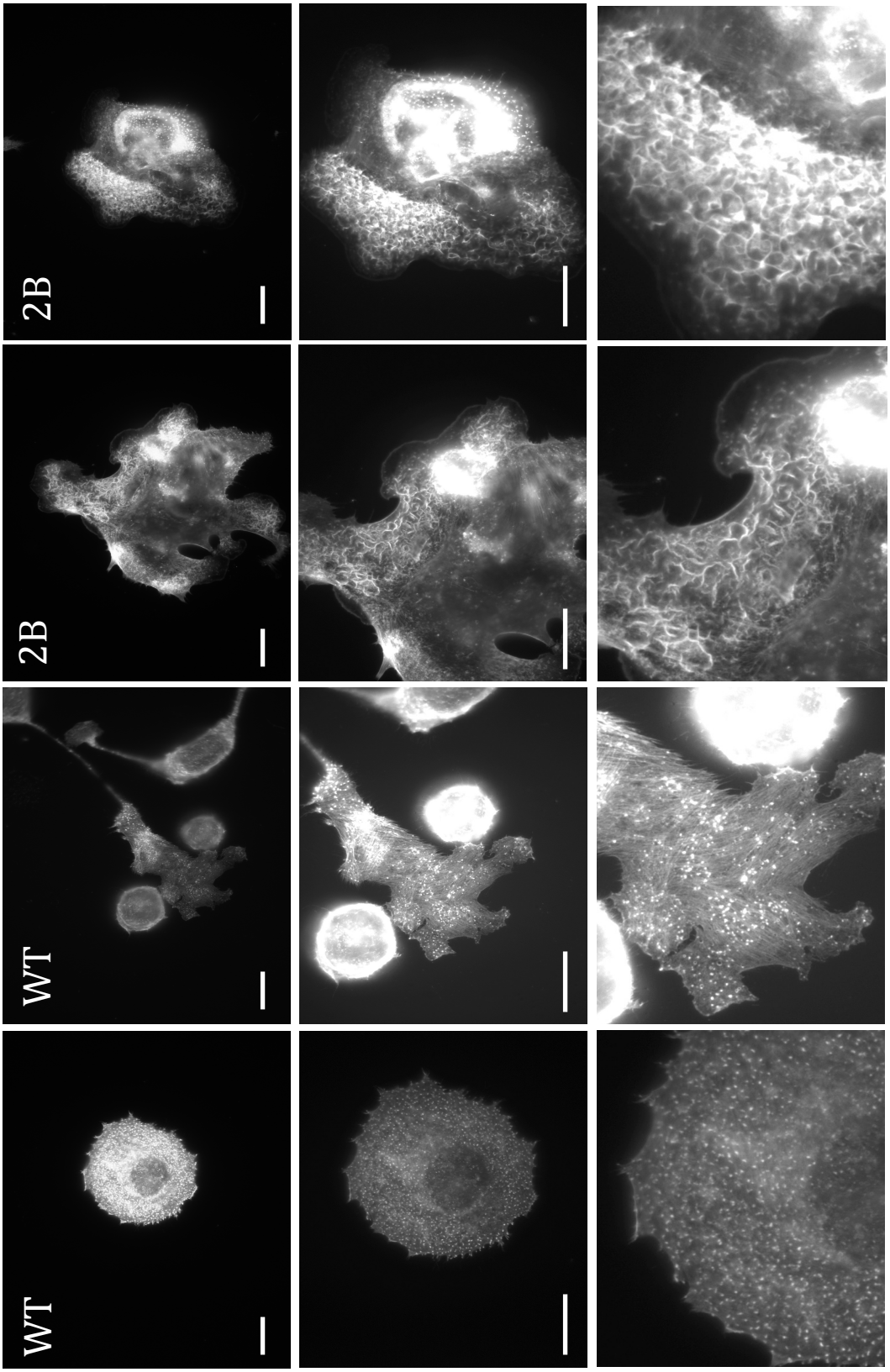
Supplemental Figure 5: MK ploidy, differentiation and spleen.
WT are represented as black circles and 2B as white squares. (A): Distribution of the ploidy in $CD41^+$ cells in the bone marrow. (B): Distribution of hematopoietic cells in the bone marrow expressed in % of cells in the lineage negative (Lin^-) population. LSK: $Lin^-Sca1^+c\text{-}Kit^+$; CMP: Common myeloid progenitors $Lin^-Sca1^-cKit^+CD34^+FcγRIII/II^-$; GMP: Granulocyte Macrophage Progenitors $Lin^-Sca1^-cKit^+CD34^+FcγRIII/II^+$; MEP: Megakaryocytic and Erythroid Progenitors $Lin^-Sca1^-cKit^+CD34^-FcγRIII/II^-$; Pre-MK: megakaryocyte progenitors $Lin^-Sca1^-cKit^+CD41^+$. (C): Number of colonies CFU-mix, CFU-MK and CFU-non MK. A-B-C) n=a pool of three mice of each genotype were analyzed three times. (D): Percentage of $CD41^+/CD42b^-$ cells (left) and $CD41^+/CD42b^+$ cells (right) at various times (day 4, 5 and 6) of the culture of Lin^- cells under TPO (10 ng/ml). The percentage of cells was measured in three separate experiments. (E): Expression of CD41 (left) and CD42b (right) in the $CD41^+/CD42b^+$ population. Values are expressed in Geo mean and were measured in three separate experiments. (F): Western blot of the expression of the MPL receptor and 14-3-3ζ as loading control from cultured MKs and MKs purified from the bone marrow. (G): Graph of the ratio spleen weight/ body weight (mg/g) measured at 10 weeks. n: WT=20 ; 2B=24. (H): Representative images of the spleen after vWF staining and hematoxylin-eosin staining. n=3 (Scale bars = 30 μm). MKs are vWF positive cells. Arrows indicate MKs. (I): Number of MKs/field in the spleen. n=3 for each genotype. (J): Colonies CFU-MK and CFU-non MK per $3.25x10^5$ unseparated spleen cells. n=a pool of three mice per genotype were analyzed in triplicate. Statistical significance was determined by student's t test (* $p<0.05$; *** $p<0.001$).

Supplemental Figure 6



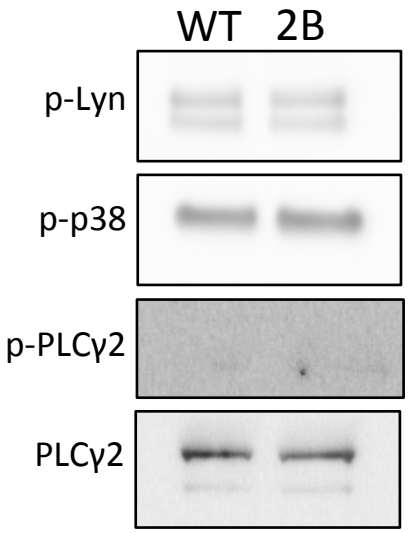
Supplemental Figure 6: CFU-C in WT and 2B mice. WT and 2B bone marrow cells were plated in a semi-solid medium and analyzed for the CFU-GEMM (multi-potential progenitors (colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte)), BFU-E (burst-forming unit-erythroid), CFU-GM (colony-forming unit-granulocyte, macrophage), CFU-G (colony-forming unit-granulocyte) and CFU-M (colony-forming unit-macrophage) count according to manufacturer's instructions (for mice; StemCell Technologies). A pool of three mice of each genotype, were analyzed three times.

Supplemental Figure 7



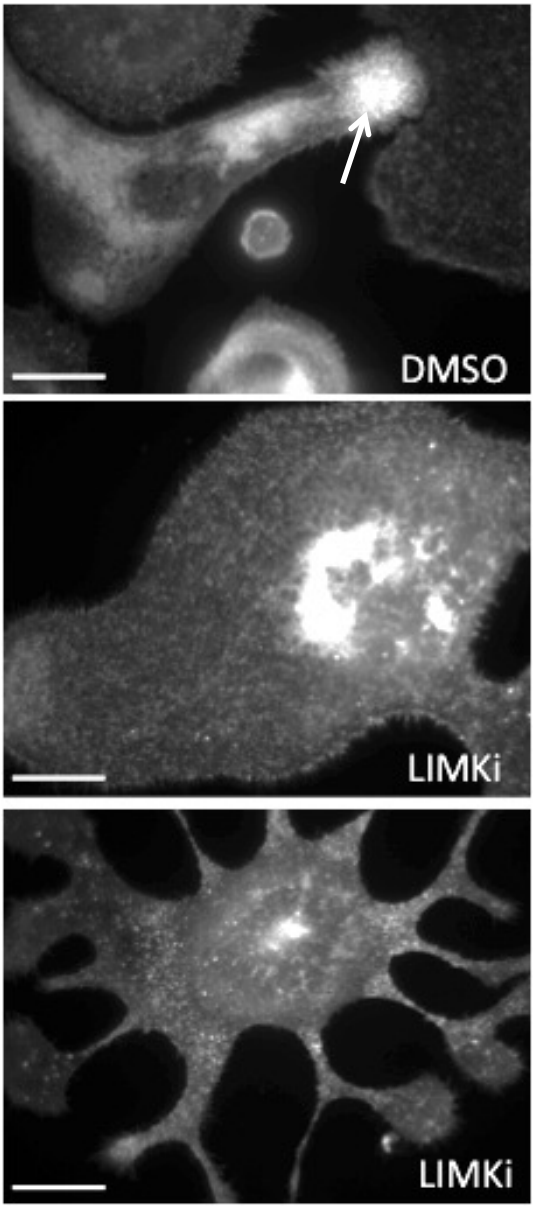
Supplemental Figure 7: Additional pictures of F-actin structure in mature WT and 2B MKs over a fibrinogen matrix after 3 hours, at three different magnifications. In 2B MKs, actin filaments behind the leading edge were destructured. Scale bars = 20 μm.

Supplemental Figure 8



Supplemental Figure 8: “Classical” platelet-GPIb/vWF signaling pathway was not dysregulated in 2B MKs.

Supplemental Figure 9



Supplemental Figure 9: Actin structure in 2B MKs after a 5-hour adhesion over a fibrinogen matrix and treated with LIMKi (10 μM) or DMSO as control. Scale bars = 20 μm. The accumulation of actin observed in control (white arrow) was almost abolished in the presence of LIMKi and lead to proplatelets formation with an actin structure in nodules similar to what is observed in WT cells (see supplement Figure 7).

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