Online Supplements:

Supplemental methods

Genotyping

Genotyping was done by direct PCR of lysed murine tissue. Primers: $IKK2^{fl/fl}$ forward: GTCATTTCCACAGCCCTGTGA, $IKK2^{fl/fl}$ reverse: CCTTGTCCTATAGAAGCACAAC, PF4-iCre forward: CCAAGTCCTACTGTTTCTCACTC, PF4-iCre reverse: TGCACAGTCAGCAGGTT.

Tail bleeding assay

WT and $IKK2^{\Delta Plt}$ mice were anesthetized with 100 mg/kg ketamine (Animedica) and 10 mg/kg xylazine (Animedica); and tail temperature equilibrated to 37°C to normalize vasoconstriction. 5 mm of the tail tip were amputated, and the tails immersed in 0.9 % NaCl solution at 37 °C. Bleeding was monitored for up to 20 minutes and time to bleeding cessation was recorded. Tail clips were weighed to exclude bleeding time differences caused by divergences of tail cutting.

Intravital microscopy of thrombus formation in $FeCl_3$ injured mesenteric arterioles

WT and $IKK2^{\Delta Plt}$ mice were anesthetized and platelets labelled *in vivo* with anti-GPIbβ-Dylight488 (X488, EMFRET Analytics). Platelet aggregation was induced in mesenteric arterioles by topical application of one drop of 1 M $FeCl_3$ (Sigma-Aldrich). Thrombus formation in arterioles was imaged for 40 minutes with an Olympus IX71 microscope (Visitron Systems GmbH) using a CAch N 10x/0,25 PhP FN22 UIS-2 objective (Olympus) and iXON Life (andor) camera, essentially as described by Haining et al[1].

Recruitment of peritoneal macrophages

1 ml 4 % Thioglycollate (BD bioscience) was injected in WT and $IKK2^{\Delta Plt}$ mice intraperitoneally. After three days, recruited macrophages were collected by peritoneal lavage and labeled with anti-mouse CD45-PerCP (30-F11, Biolegend) and anti-mouse F4/80-FITC (BM8, Biolegend), fixed in 1 % formaldehyde and analyzed using a CytoflexS flow cytometer with Cytexpert 2.2 software. Leukocytes were defined as $CD45^+$ and macrophages as $CD45^+$ $F4/80^+$ events.

Murine platelet isolation

500 µl heparinized (25 U/ml) blood, supplemented with 1/10 Citrate-dextrose solution (ACD, Sigma-Aldrich) was diluted with 300 µl Tyrode's HEPES buffer (TH) (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 16.63 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) and centrifuged for 5 minutes at 200 g. Platelet rich plasma and 2/3 of the erythrocyte fraction were taken and re-centrifuged for 6 minutes at 100 g. Platelets were taken and the centrifugation step was repeated after

addition of 300 µl Tyrode's HEPES buffer to the remaining red fraction to increase platelet yield. Platelets were centrifuged for 90 seconds at 1000 g in the presence of 1/25 volume ACD and 0.5 U/ml apyrase (Sigma-Aldrich) and resuspended in TH buffer.

Murine platelet activation and degranulation

Heparinized blood was diluted 1:5 with Tyrode's HEPES buffer supplemented with 2 mM Ca^{2+} and stimulated for 15 minutes with the indicated concentrations of ADP (Sigma-Aldrich), Protease Activated Receptor 4 agonist peptide AYPGKF-NH2 (PAR4-AP, Anaspec) or convulxin (CVX) (Santa Cruz). After labelling with anti-mouse CD41-BV421, anti-mouse CD62P-PE/Cy7 (both Biolegend) and JonA-PE (Emfret analytics), which labels activated glycoprotein (GP) IIb/IIIa for 15 minutes, cells were fixed in 1 % formaldehyde and analyzed using a CytoflexS flow cytometer with a Cytexpert 2.0 software (Beckman Coulter). JonA and CD62P median fluorescence intensities (MFI) of $CD41^{+}$ events were determined.

ATP measurement

Washed murine platelets were stimulated with 75 µM PAR4-AP or 100 ng/ml CVX for 10 minutes, followed by inhibition with 1 µg/ml PGI_2 (Sigma-Aldrich). Cells were centrifuged for 90 seconds at 3000 g, supernatant was taken and centrifuged again for 90 seconds at 4000 g to minimize cell contamination. ATP content of 1:10 diluted sample was measured with ENLITEN® rLuciferase/Luciferin Reagent (Promega) and an IVIS Lumina Series III (Caliper LifeSciences).

PF4 release

Heparinized blood was centrifuged for 5 minutes at 3 000 g, followed immediately by 1 minute at 13 000 g. Supernatant was taken and again centrifuged for 1 minute at 13 000 g. Platelet-free plasma (PFP) was stored at -80 °C. PF4 (CXCL4) content was measured from 1:300 diluted plasma with a mouse CXCL4/PF4 DuoSet ELISA (R&D Systems) according to manufacturer's protocol.

Platelet aggregation

400.000 murine platelets/µl were activated with 18 µg/ml collagen I (Bio/Data) or 165 mU/ml thrombin (Technoclone). Aggregation was measured by light transmission aggregometry (PAP-8, MöLab). Light transmission was determined between 0 and 100 %, defined with platelet suspension and Tyrode's HEPES buffer. Maximal aggregation was calculated by PAP-8 software.

Western blotting

Equal numbers of isolated murine platelets were lysed in Laemmli Buffer (50 mM Tris-HCl, 10 % Glycerol, 10 % SDS, 0.2 % bromphenolblue, 5 % β-mercaptoethanol) and samples denatured for 5 minutes at 95 °C. SDS-PAGE was performed with 10% polyacrylamide gels

and samples blotted onto PVDF membranes (Roth). For detection of IKK2-knockout constructs, 12 % polyacrylamide gels were used. Membranes were blocked with 5 % Skim Milk Powder (Sigma-Aldrich) in 0.1 % TBS-tween 20 and probed with the following antibodies overnight at 4 °C: anti-IKKβ (D30C6, 1:1000), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (D16H11, 1:1000) (both from Cell Signaling Technologies), anti-SNAP-23 (DS-19, Sigma-Aldrich) and anti-phospho-SNAP-23 Ser95 (from Makoto Itakura, Japan). This was followed by incubation with secondary antibody anti-rabbit IgG HRP linked (1:1000, Cell Signaling Technologies) for 1 hour at room temperature. For FLAG detection, Anti-DYKDDDDK antibody (D6W5B, 1:5000, Cell Signaling Technologies) was incubated for 1 hour at room temperature. Proteins were detected using Western Bright Chemiluminescence Substrate Sirius (Biozym) and visualized by a FluorChem HD2 Chemiluminescence Imager.

Cloning of plasmids mimicking the exon 3 and the exon 6/7-deletion of IKK2 and analysis in HeLa cells

IKK2 was amplified from murine cDNA by PCR and subsequently inserted in-frame into the pmCherry-C1 (Clontech) vector via XhoI and EcoRI (New England Biolabs), after replacing mCherry with an N terminal FLAG-tag sequence. pFLAG-IKK2(Δ3) and pFLAG-IKK2(Δ6,7) were generated by whole plasmid PCR using the Q5® High-Fidelity DNA polymerase (New England Biolabs) without amplifying exon 3 (Δ3) or exon 6 and 7, respectively. All constructs were verified by sequencing prior to experimental use. HeLa (ATTC) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, non-essential amino acids and antibiotics. All expression plasmids were transiently transfected using Turbofect (Thermo Fisher Scientific) according to product information. For Western blotting, cells were lysed one day after transfection in Laemmli Buffer. For luciferase assay, cells were transiently transfected with IKK2 exon deletion constructs, pNL3.2.NF-κB-RE Nanoluc reporter (Promega, N111A) and a vector constitutively expressing β-galactosidase for normalization purposes (driven by a ubiquitin promoter: PUB6/V5-His/LacZ from Thermo Fisher Scientific). Cells were lysed in passive lysis buffer (0.1 mM KH_2PO_4, 0.1 % Triton X-100) and Nanoluc luminescence was measured as according to manufacturer's protocol (Promega).

Treatment of platelets with IKK inhibitors

The compounds TPCA-1 and BMS-345541 (both from Sigma-Aldrich) were used as IKK-inhibitors. Fore murine samples, washed platelets were incubated with 0.4 µM TPCA-1, 4 µM BMS-345541 or respective DMSO controls for 30 min and activated with the indicated concentrations of PAR4-AP. Additionally, washed platelets were incubated with the indicated concentrations of TPCA 1, BMS-345541, or respective DMSO controls, followed by stimulation

with 40 µM PAR4-AP. Platelets were labelled with antibodies against activated GPIIb/IIIa or CD62P to measure integrin activation and degranulation by flow cytometry as described above. For human samples, citrated blood was centrifuged for 15 min at 120 g without break to generate platelet rich plasma (PRP). PRP was incubated with 0.4 µM TPCA-1, 4 µM BMS-345541 or respective DMSO control for 30 min and activated with 2.5 µM ADP, 5 µM Thrombin Receptor Activator Peptide 6 (TRAP-6) and 3 ng/ml CVX. Platelets were labelled with antibodies against activated GPIIb/IIIa (PAC-1, Biolegend) or CD62P (Biolegend) to measure integrin activation and degranulation by flow cytometry as described above.

PCR and quantitative PCR (qPCR)

Mature megakaryocytes were generated from WT and IKK2$^{\Delta Plt}$ mice as described in Salzmann et al.[2]. RNA and DNA from megakaryocytes and RNA from platelets were isolated according to manufacturer's protocol using QIAzol Lysis Reagent (Qiagen). qPCR was performed with SYBR Green reagent (Thermo Fisher Scientific) with an intron sequence of GAPDH as housekeeping gene for genomic qPCR and HPRT mRNA as a housekeeper for mRNA qPCR. The primers used for intron PCR and qPCR analyses are summarized in Table S1. Values are expressed as fold of control.

Transmission electron microscopy and quantification

Isolated WT and IKK2$^{\Delta Plt}$ platelets were fixed with 2.5% glutaraldehyde, sedimented and incubated in fixative for another 2 hours. After 3 washes with cacodylate buffer, cells were post-fixed in Palade's buffered osmium tetroxide. Dehydration and embedding in Epoxy resin followed standard protocols. Sections (70 nm) were cut with a Reichert UltracutS microtome and contrasted with lead citrate and uranyl acetate. Images were acquired with a FEI Tecnai20 electron microscope equipped with a 4K Eagle-CCD camera and processed with Adobe Photoshop. For quantification, images were taken at the same magnification and full cell profiles were used for counting mitochondria, αgranules and dense granules. 22 cross-sections of platelets were assessed for WT and 21 for IKK2$^{\Delta Plt}$ samples. Two researchers obtained the same averages independently

Megakaryocyte isolation and assays

Mature bone marrow derived MKs were generated and assays carried out as described previously[2].

Statistical analysis

Calculations were performed using GraphPad Prism 6.01 software. Comparison of two groups was done by unpaired t test or Mann-Whitney test if data was not distributed normally. Two or more groups were compared to the respective control group using One-Way ANOVA with Dunnett correction. Two groups with more than one condition were compared by Two-Way

ANOVA and Sidak correction. Statistical significances are depicted as: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Supplemental table:

	Forward	Reverse
PCR: recombined intron	GGACTTCTTTCATGGGGCCA	TAATTCTCGGAGTGCTCGCC
qPCR: genomic knockout	AGAACCCTCCTCTCCTACGT	ACCACCACGCCCACTTTATA
qPCR: GAPDH intron	CCCGAGGACAATAAGGCTCA	AGGAAATGAATGAACCGCCG
qPCR: IKK2 mRNA knockout	GCTCAGCCCAAAGAACAGAG	ACTGGTTCAAGTATCTTCGGAG
qPCR: HPRT	CGCAGTCCCAGCGTCGTG	CCATCTCCTTCATGACATCTCGAG

Table S1

Primer sequences used for PCR reactions.

Supplemental figures:

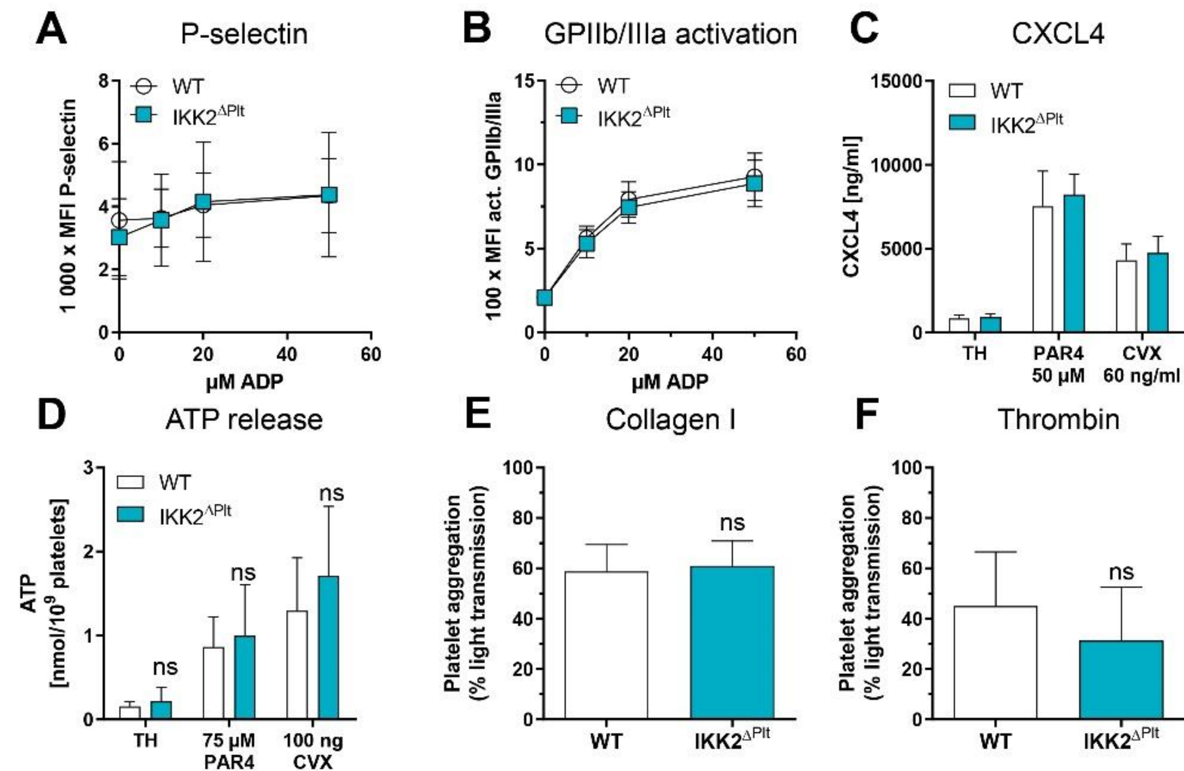

Figure S1:

(A) P-selectin and (B) activated glycoprotein (GP) IIb/IIIa median fluorescence intensity (MFI) of $CD41^+$ events in diluted whole blood, stimulated with ADP for 15 min. n = 12. (C) CXCL4/ PF4 release from αgranules of washed platelets stimulated with buffer control (Tyrode's HEPES buffer, TH) 50 µM PAR4 agonist peptide (PAR4-AP) or 60 ng/ml convulxin (CVX) for 10 minutes. n = 5-7. (D) ATP release from dense granules of washed platelets stimulated with TH (buffer control), 75 µM PAR4-AP or 100 ng/ml CVX for 10 minutes. n = 3-5. (E) Quantification of maximal aggregation of washed WT and $IKK2^{\Delta Plt}$ platelets, stimulated with 18 µg/ml collagen I (n = 10-12) or (F) 165 mU/ml thrombin (n = 7).

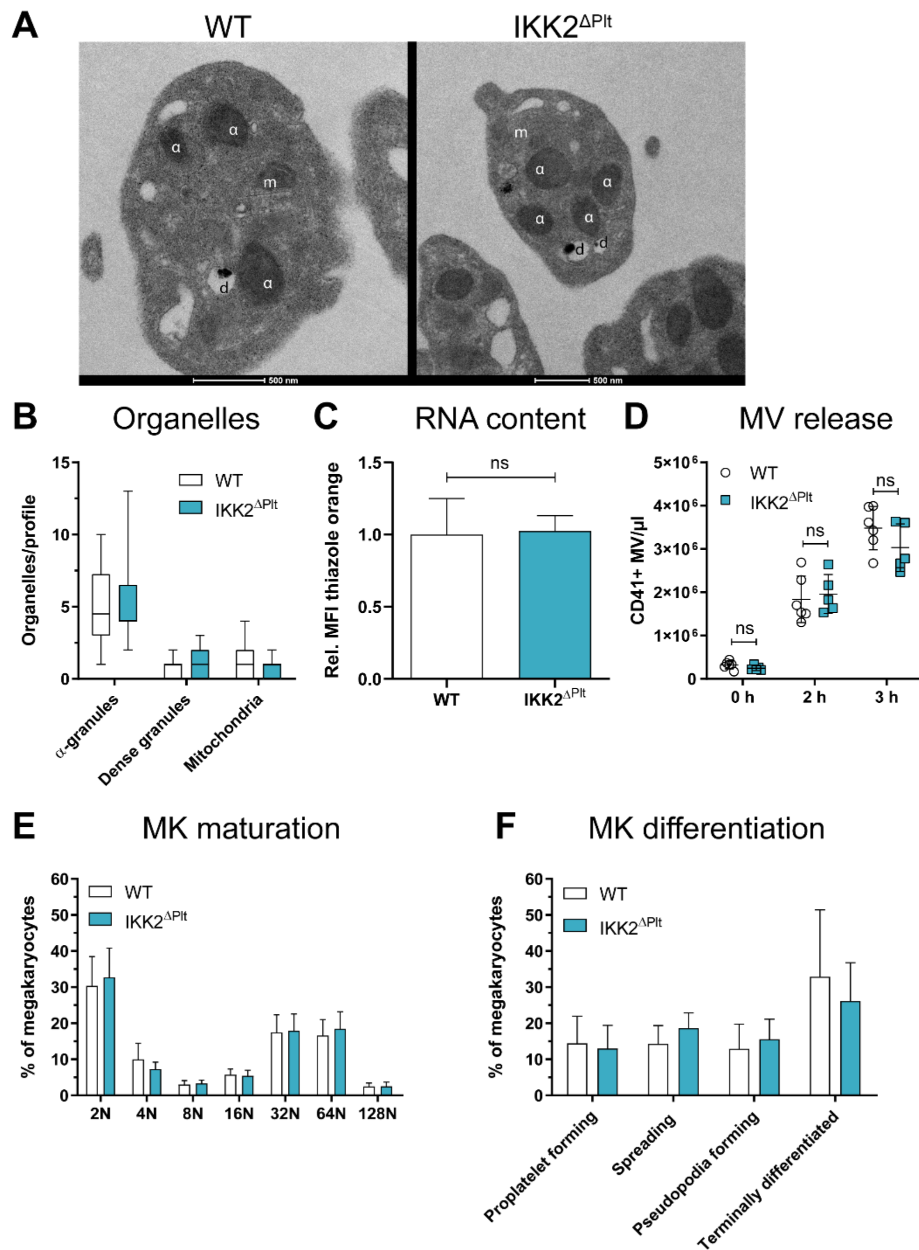

Figure S2:

(A) Representative images of platelet profiles acquired with electron microscopy. (B) Organelle numbers were determined from 22 WT and 21 IKK2$^{\Delta Plt}$ platelet profiles. (C) RNA content of platelets assessed by thiazole orange. (D) Microvesicle (MV) released after 3h hours incubation at 37 °C under light agitation. (E) Mature megakaryocytes were differentiated from WT and IKK2$^{\Delta Plt}$ bone marrow cells. DNA was stained with Sytox® AADvanced and cells analyzed with flow cytometry for determination of ploidy distribution. n = 8, mean ± SD (F) Mature megakaryocytes were incubated for 5 hours on fibrinogen at 37°C to allow proplatelet formation. Cells were fixed and stained with CD41 as megakaryocyte marker and Hoechst 33342 to stain the nucleus. Proplatelet formation was analyzed using Cellprofiler. n = 4, mean ± SD

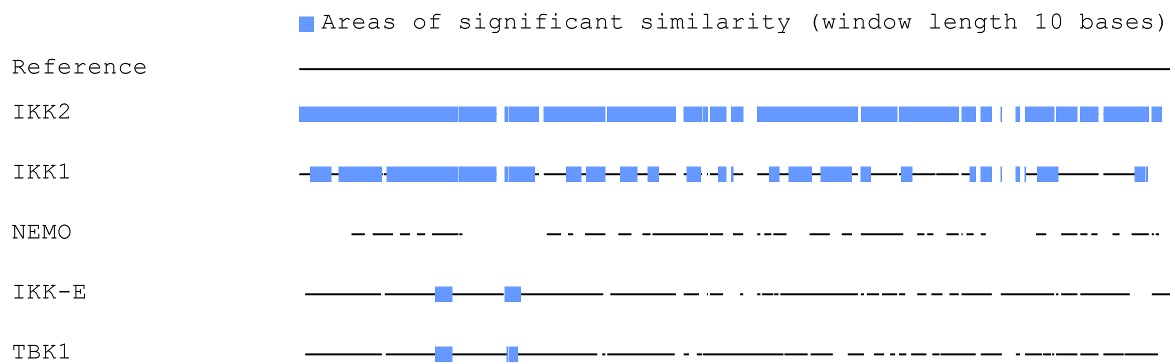

Figure S3:

Graphical sequence alignment of the components of the IKK-complex (IKK2, IKK1, NEMO) and two related kinases (IKK-epsilon, IKK-E; and TBK1). Areas with a similarity above 70% are shown as light blue boxes.

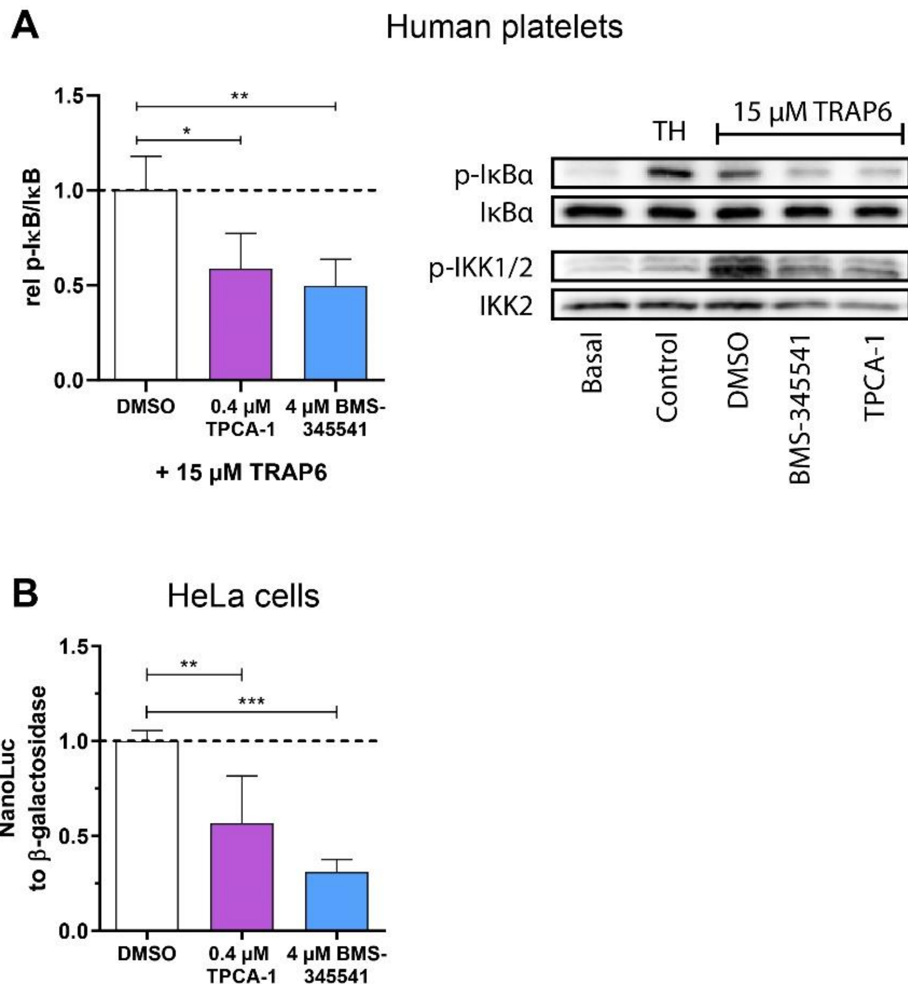

Figure S4:

(A) Ser32 IκBαphosphorylation of human platelets incubated with DMSO or IKK inhibitors for 30 minutes followed by stimulation with 15 µM TRAP6, a PAR1 agonist. Right panel shows a representative western blot including basal levels, stimulation control (Tyrode's HEPES buffer, TH) and IKK phosphorylation. (B) Basal NF-κB activity of HeLa cells after overnight incubation with IKK inhibitors. Nanoluc-activity normalized to constitutively expressed β-galactosidase activity is shown, relative to DMSO control. n = 4.

Supplementary References

1. Haining EJ, Cherpokova D, Wolf K, et al. CLEC-2 contributes to hemostasis independently of classical hemITAM signaling in mice. *Blood*. 2017;130(20):2224-2228.
2. Salzmann M, Hoesel B, Haase M, et al. A novel method for automated assessment of megakaryocyte differentiation and proplatelet formation. *Platelets*. 2018;29(4):357-364.