Supplemental Data

Supplemental Methods

<u>Generation of *Crlf3*$^{-/-}$ mice</u>

The generation of *Crlf3*$^{-/-}$ mice is illustrated in Supplemental Figure 7. The ES cell clone used was EPD0033_3_C11 and was selected based on neomycin resistance and β-galactosidase expression confirming *Crlf3*$^{tm1a(KOMP)Wtsi}$ allele expression. Mice were generated according to standard techniques and after germline transmission of the *Crlf3*$^{tm1a(KOMP)Wtsi}$ allele, exon 2 of *Crlf3* was excised by Cre-mediated recombination by crossing with *Hprt*$^{Tg(CMV\text{-}cre)Brd}$ mice, producing the mutant *Crlf3*$^{tm1b(KOMP)Wtsi}$ allele. After confirmation of allele conversion by PCR, chimeric mice carrying the *Crlf3*$^{tm1b(KOMP)Wtsi}$ allele were backcrossed to C57BL/6 mice.

<u>Blood and tissue analysis</u>

Blood smears were created by pushing whole venous blood across a microscope slide and leaving to air dry overnight. Blood smears were then stained with Rapid Romanowsky staining kit (TCS biosciences) following the manufacturer's instructions. For confocal microscopy, blood smears were washed with 50mM ammonium chloride to remove red cells, then remaining cells were permeabilised with PBS containing 0.1% w/v saponin and 0.2% w/v gelatin (both Sigma-Aldrich). Samples were incubated with primary antibodies against CD41 (133902; BioLegend) and vWF (A0082; DakoCytomation). Samples were washed before incubating with appropriate secondary antibodies (Alexa Fluor 488 Goat anti-rat IgG (A-11006) or Alexa Fluor 555 Goat anti-rabbit IgG (A-21428); both Invitrogen). Samples were again washed before glass coverslips were mounted using hydromount (National Diagnostics) and images were acquired using a Leica Sp5 inverted confocal microscope with the 63x immersion-oil objective and the Leica LAS 2.1 software.

Plasma thrombopoietin (TPO) levels were measured with the Quantikine Mouse TPO ELISA kit (R&D Systems).

For histologic analysis, mice were examined after sacrifice. Specimens were collected into 10% neutral buffered formalin solution (Sigma-Aldrich). Fixed tibia and spleens were embedded in paraffin and sections cut, deparaffinised and rehydrated. Sections were then stained with

1 Haematoxylin and Eosin stain or Gömöri's reticulin silver stain for reticulin fibres. After staining,
2 images were acquired using an Axiovert 40 CFL or Olympus microscope and AxioVision 40 version
3 4.8.2.0 software (both Zeiss) and MKs blindly quantified manually. A Pixera 600ES camera was used
4 for digital photomicroscopy of the blood and bone marrow.
5
6 Expression array and qRT-PCR
7
8 RNA was extracted from purified *in vitro* cultured MKs using RNeasy plus mini Kit (Qiagen).
9 Complementary (c) DNA was made using SuperScript III First Strand Kit (Invitrogen) starting from
10 100ng total RNA and using oligo-dT as reverse transcription primers. Expression levels were
11 quantified using Illumina Mus 6v2 expression chip. Data processing and analysis were performed as
12 described elsewhere[1]. Quantitative polymerase chain reaction (qPCR) of the murine genes *Crlf3* and
13 *Gapdh* was carried out using specific primers that had previously been tested for optimal efficiency,
14 Brilliant II SYBR green chemistry (Agilent Technologies) and Mx3000P Real-Time PCR system
15 (Stratagene). The analysis of gene expression was performed by the ΔCt method, where:
16 ΔCt = Ct target gene (*Crlf3*) – Ct housekeeping gene (*Gapdh*)
17 Relative gene expression was expressed as $2^{-\Delta Ct}$.
18
19 *Crlf3* specific primers:
20 Forward: 5'-GGGTGTTCTCTACTCCAGCG-3'
21 Reverse: 5'-GCCATTCTGCCTTTCTGCAC-3'
22
23 *Gapdh* specific primers:
24 Forward: 5'-CCCTTAAGAGGGATGCTGCC-3'
25 Reverse: 5'- TACGGCCAAATCCGTTCACA-3'
26
27 Western blot
28
29 Total cellular lysates of washed platelets, iPSCs and iPSC-MKs were prepared by direct cellular lysis
30 with RIPA buffer (150mM sodium chloride, 60mM Tris pH 8.0, 0.6% w/v sodium deoxycholate, 0.1%
31 w/v sodium dodecyl sulfate, 1% v/v NP-40) supplemented with protease (Roche) and phosphatase
32 (Sigma-Aldrich) inhibitors, incubation on ice for 30 min and followed by centrifugation at 13,000*g* at
33 4°C for 5 min. Fractionated platelet samples were prepared as previously described[2]. For
34 immunoprecipitation (IP) samples, iPSCs were harvested at D20 of MK forward programming. iPSC-

MKs were centrifuged for 8 min at 120 g, washed three times in ice cold PBS and snap frozen on dry ice. Samples were then resuspended in 10 mM Tris-HCl pH 8, 150 mM NCl, 0.5 % NP-40 and protein content was quantified by BCA assay (Thermo). 50 µL Protein G Dynabeads (Invitrogen) were washed x3 in PBS 0.01% Tween-20. Samples were resuspended in 50 µL PBS 0.01% Tween-20 and incubated for 40 min at RT on an orbital rotator with 1 µg of antibody. The beads were washed x3 in PBS 0.01% Tween-20 and added to cell lysate at 1 ml/mL. Samples were rotated at 225 rpm at 4°C overnight. Supernatants were stored and beads were washed x3 in PBS 0.01% Tween-20 with 1X protease inhibitors (cOmplele mini EDTA free, Roche) and 1X PhosSTOP (Roche). They were transferred to a new eppendorf on the last wash and resuspended in 30 µL MPER (Thermo) 1:4 NuPAGE sample buffer (Invitrogen) with 1:10 reducing agent (Invitrogen). Denaturation was carried out for 10 min at 70°C. Supernatant was collected from beads and loaded on a Western Blot as below.

Samples were diluted 1:1 with 2x Laemmli sample buffer (125mM Tris pH 6.8, 20% v/v glycerol, 4% w/v sodium dodecyl sulfate, 10% v/v β-mercaptoethanol, 0.002% w/v bromophenol blue) and incubated at 95°C for 5 min to denature proteins. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and transferred to PVDF membrane (Millipore) and probed by the following antibodies. Membranes were imaged with Odyssey Fc OFC-1095 version 1.0.36 imaging system. Expression was quantified using Image Studio Lite.

The following primary antibodies were used: anti-CRLF3 (HPA007596; Atlas antibodies), anti-GAPDH (2118; Cell Signalling Technology), anti-α-tubulin (T5168; Sigma-Aldrich and GTX102078; GeneTex), anti-tyrosine tubulin (T9028; Sigma-Aldrich), anti-glutamylated α-tubulin (AG-20B-0020 and AG-25B-0030; AdipoGen Life Sciences), anti-acetylated α-tubulin (sc23950; Santa Cruz Biotechnology), anti-FLAG (F1804; Sigma-Aldrich), anti-β-actin (A1978; Sigma-Aldrich), anti- STK38 (ab56977; Abcam), anti-pSTK38 (SPC-1035; StressMarq), anti-MOB1A (ab221988; Abcam), anti-pMOB1 (8699S, Cell Signalling Technology) and anti-human thrombospondin (ab1823; Abcam). Where α-tubulin or modified α-tubulin blots presented as a double band in MK samples, analysis was conducted on the band corresponding to the band seen in platelet samples.

For the measurement of the post-translational modification of tubulin, we first measured the polyglutamylated/acetylated/tyrosinated tubulin content. Following this, the membranes were stripped and total tubulin and GAPDH (loading control) content were measured concurrently. Before re-probing, stripped membranes were checked to be blank across all lanes. This method allowed us

to measure total tubulin content (vs GAPDH) as well as the ratio of modified tubulin vs total tubulin on the same samples. In some blots we measured one type of tubulin modification, then another after stripping the membrane and finally total tubulin and GAPDH.

In vitro proplatelet formation

Mature MKs were resuspended in culture medium supplemented with 100U/mL heparin sodium (Wockhardt UK Ltd) and seeded onto glass coverslips coated with 20ng/mL fibrinogen (Enzyme Research). Cells were incubated at 37°C under 5% CO_2 for either 3 or 5 hours. Attached MKs were fixed with 10% neutral buffered formalin solution and washed in PBS. Fixed MKs were stained with antibodies against CD41 (133902; BioLegend or sc-19963; Santa Cruz Biotechnology), F-actin (Alexa Flour 555 Phalloidin (A34055); Invitrogen), polyglutamylated tubulin (AG-20B-0020 or AG-25B-0030; AdipoGen Life Sciences), MOB1A (ab221988, Abcam), α-tubulin (T5168, Sigma-Aldrich), β -tubulin (ab21057; Abcam), and the nuclear stain DAPI (Sigma-Aldrich). Images were acquired using a Leica Sp5 inverted confocal microscope with the 20x objective and the Leica LAS 2.1 software or a Zeiss LSM 880 MP Airyscan inverted confocal microscope with the 20x objective and Zeiss Zen software. For proplatelet dynamics, proplatelet forming MKs were counted against the total number of MKs to calculate the percentage of proplatelet forming MKs. At least 460 MKs were counted for each condition.

Bone marrow transplantation

Recipients were sub-lethally irradiated with a split dose of 950 cGY, separated by >4 hours. 5×10^6 WT or *Crlf3*$^{-/-}$ bone marrow (BM) cells were injected into WT or *Crlf3*$^{-/-}$ recipient mice via the tail vein. Peripheral venous blood was obtained to determine platelet counts using a Woodley ABC blood counter (Woodley). Donor chimaerism was determined by *Crlf3* expression by qRT-PCR of *in vitro* cultured MKs derived from recipient BM cells taken at termination of the experiment.

Blood loss after tail transection

Experiments were performed as per Evans et al, 2021[3]

Electron Microscopy

For transmission electron microscopy (TEM), washed platelets fixed with 2.5% glutaraldehyde in 50mM cacodylate buffer (pH 7.2). After embedding in epon 812, ultra-thin sections were generated and stained with 2% uranyl acetate and lead citrate. Samples were visualised with a Zeiss EM900 microscope.

For scanning electron microscopy (SEM), washed platelets were resuspended in 0.85% sodium chloride, diluted 1:1 with 0.4% glutaraldehyde in 0.1M sodium cacodylate pH 7.4 and incubated at room temperature. Platelets were then pelleted at 1,000*g* and resuspended in fixation buffer (2% glutaraldehyde, 2% formaldehyde, 0.05M sodium cacodylate, pH 7.4). Platelets were rinsed in deionised water (DIW) and incubated in 1% osmium ferricyanide for 4 days, rinsed again in DIW and incubated in 2% uranyl acetate in 0.05M Maleate buffer (pH 5.5) for 4 days at 4°C. Platelets were resuspended in DIW and attached to poly-L-lysine coated glass coverslips. After unattached platelets were removed, samples were quench frozen in melting propane cooled in liquid nitrogen, then dried overnight in a Quorum K775 freeze dryer. The dry coverslips were attached to 13mm Cambridge SEM stubs with colloidal silver DAG sputter coated with 10 nm of iridium and viewed in a FEI Verios 460L using a secondary electron detector.

Tandem Affinity Purification (TAP)-tagging CRLF3 in induced pluripotent stem cells (iPSCs)

Tandem Affinity Purification (TAP)-tagging of CRLF3 at the C-terminus was performed by homology directed repair (HDR) by CRISPR/Cas9. CRISPR ID 1146192702 was picked from www.sanger.ac.uk/htgt/wge/ [4]. Single-guide RNA (sgRNA) expression constructs were generated using the plasmid-based procedure as described by Ran, *et al.*[5] and cloned into the pSpCas9(BB)-2A-GFP plasmid (Addgene).

A donor vector containing 1Kb homology arms surrounding the TAP-tag[6] in a pBS KS vector backbone (Addgene) was created by Gibson Assembly following manufacturer's instructions (Gibson Assembly Cloning Kit; New England Biosciences).

sgRNA/Cas9-GFP plasmid and TAP-tag donor vector DNA were introduced into Qolg (HipSci line HPSI1113i-qolg_3 (http://www.hipsci.org/)) induced pluripotent stem cells (iPSCs) in a single cell solution by nucleofection using Amaxa Human Stem Cell Nucleofector Kit 2 and an Amaxa Nucleofector II (both Lonza), following manufacturer's instructions. 48 hours after nucleofection, cells were sorted based on GFP expression by flow assisted cell sorting. GFP positive iPSCs were

1 seeded individually to laminin 521 (Biolamina) coated 96 well tissue culture plates and maintained in
2 Essential 8 (E8) medium (Gibco) supplemented with 100U/ml pen/strep (PAA Laboratories) for 5
3 days then maintained as colonies in E8 medium on truncated vitronectin (VTN-N; ThermoFisher
4 Scientific) coated tissue culture plastic plates (Corning) until needed.
5
6 Mass Spectrometry and LC-MS/MS analysis
7
8 50×10^6 TAP-tagged or untagged iPSC-MKs were dissociated to a single cell solution, washed 3 times
9 in PBS then snap frozen and stored at -80°C until needed. Frozen pellets were then lysed in lysis
10 buffer (10mM Tris hydrochloride, 150mM sodium chloride, 0.1% NP-40), centrifuged at 13,000rpm
11 for 15 minutes and lysates collected. Proteins interacting with TAP-tagged CRLF3 were then
12 collected by incubating the lysates with magnetic beads (Dynal protein G; Invitrogen) coated with
13 anti-FLAG antibody (anti-FLAG M2; Sigma-Aldrich) before collecting the beads. Beads were then
14 washed 3 times in lysis buffer and twice in TEV buffer (10mM Tris hydrochloride, 150mM sodium
15 chloride, 0.1% NP-40, 0.5mM EDTA, 1mM Dithiothreitol) to remove unbound proteins. Bead bound
16 proteins were dissociated by incubation with 100U AcTEV protease (Invitrogen) in TEV buffer before
17 collection of the eluates. The eluates were then diluted in calmodulin binding buffer (CBB; 10mM
18 Tris-HCl, 150mM sodium chloride, 1mM magnesium acetate, 1mM Imidazole, 2mM calcium chloride,
19 10mM β-mercaptoethanol, 0.1% NP-40) before interacting proteins were further purified by
20 incubation of the diluted eluates with calmodulin binding peptide (CBP) affinity resin (Agilent),
21 pelleting and removal of the supernatant. CBP resin was then washed 3 times with CBB before
22 incubation in calmodulin elution buffer (10mM Tris-HCl, 150mM sodium chloride, 0.02% NP-40,
23 1mM magnesium acetate, 1mM Imidazole, 20mM EGTA, 10mM β-mercaptoethanol) to dissociate
24 bound proteins, which were collected in the eluates after pelleting of the CBP resin. The eluates
25 were then snap frozen and stored at -80°C until mass spectrometry was performed.
26
27 Samples were mixed with SDS loading buffer and run on 12% SDS PAGE gel to remove MS
28 incompatible detergents. Samples were only run till a dye front was about 2cm below the well. Gel
29 was stained with Coomassie blue colloidal stain and each lane cut into 4 equal pieces. Each piece
30 was finely chopped and transferred to 0.5ml Eppendorf tube and was treated as individual MS
31 sample. The gel pieces were distained by washing with 50% acetonitrile, 50% ammonium
32 bicarbonate followed by wash with HPLC water. Distained samples were then treated with 100%
33 acetonitrile, air dried and reduced with 10mM DTT in 100mM ammonium bicarbonate for 1 hour at
34 37°C. The DTT solution was removed and replaced with 55mM iodoacetamide in 100mM

1 ammonium bicarbonate and incubated for 45 minutes at RT in darkness. Samples were then washed
2 with 50% ammonium bicarbonate, 50% acetonitrile followed by addition of 100% acetonitrile.
3 Acetonitrile was removed and samples dried again, before resuspending in a 1:200 solution of
4 trypsin in 50mM ammonium bicarbonate and incubating overnight at 37°C.
5 LC-MS/MS analysis
6 Post-trypsin digestion, the supernatant was pipetted into a sample vial and loaded onto an
7 autosampler for automated LC-MS/MS analysis.
8
9 All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC system
10 and a Q Exactive Orbitrap mass spectrometer (both Thermo Fisher Scientific). Separation of peptides
11 was performed by reverse-phase chromatography at a flow rate of 300nL/min and a Thermo
12 Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2µm particle size,
13 100Å pore size, 75nm i.d. x 50cm length). Peptides were loaded onto a pre-column (Thermo
14 Scientific PepMap 100 C18, 5µm particle size, 100Å pore size, 300nm i.d. x 5mm length) from the
15 Ultimate 3000 autosampler with 0.1% formic acid for 3 minutes at a flow rate of 10nL/min. After this
16 period, the column valve was switched to allow elution of peptides from the pre-column onto the
17 analytical column. Solvent A was water + 0.1% formic acid and solvent B was 80% acetonitrile, 20%
18 water + 0.1% formic acid. The linear gradient employed was 2-40% B in 30 minutes.
19
20 The LC eluent was sprayed into the mass spectrometer by means of an Easy-Spray source
21 (ThermoFisher Scientific). All *m/z* values of eluting ions were measured in an Orbitrap mass analyzer,
22 set at a resolution of 70000 and was scanned between *m/z* 380-1500. Data dependent scans (Top
23 20) were employed to automatically isolate and generate fragment ions by higher energy collisional
24 dissociation (HCD, NCE:25%) in the HCD collision cell and measurement of the resulting fragment
25 ions was performed in the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and
26 ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic
27 exclusion window of 20 seconds was employed.
28
29 Post-run, the data was processed using Protein Discoverer (version 2.1., Thermo Scientific). Briefly,
30 all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search
31 algorithm (Matrix Science) and searched against the UniProt human database
32 (UniProt_Human201701 201701, 70956 sequences; 23911858 residues) and a common contaminant
33 sequences (115 sequences, 38274 residues). Variable modifications of oxidation (M), deamidation
34 (NQ) and carbamidomethyl were applied. The peptide and fragment mass tolerances were set to

5ppm and 0.1 Da, respectively. A significance threshold value of $p<0.05$, false discovery rate (FDR) was set at 5% for identification of both proteins and peptides and a peptide cut-off score of 20 were also applied. Data is presented as unique peptide counts and normalised total spectra, which normalises the spectral count for each peptide in each sample to the average total spectral count across all samples.

Cloning, production, purification and structural solution of amino acid 174-442 of CRLF3

Crlf3 amino acid 174-442 was amplified from murine *in vitro* cultured MK cDNA by PCR using specific primers that include the BamHI or NotI restriction sites and Platinum High Fidelity Taq Polymerase (Invitrogen) following manufacturer's instructions. Purified PCR products were ligated into BamHI and NotI digested pGEX-6P-2 (GE Healthcare) using T4 Ligase (New England Biolabs) following manufacturer's instructions. pGEX vectors containing *Crlf3* constructs were transformed into BL21-CodonPlus™-(DE3)-RP competent *E.coli* cells (Stratagene) following manufacturer's instructions. Glutathione-*S*-transferase (GST) fusion protein from *Crlf3* constructs were produced as previously reported[55]. GST was removed from CRLF3 constructs by affinity chromatography using GSTrap FF 5mL columns and PreScission Protease (both GE Healthcare), following manufacturer's instructions. Remaining free GST was removed by ion exchange chromatography using HiTrap Q FF columns (GE Healthcare) following manufacturer's instructions. Protein was concentrated under centrifugation using VivaSpin 2 columns with 10kDa cut off (Sartorius Stedim Biotech) following manufacturer's instructions. Crystallisation was screened by the vapour diffusion method in 96-well sitting drop plates set up with a Nanodrop Screenmaker 96+8 (Innovadyne Technologies), using drops containing 200nL protein and 200nL screening solution equilibrated with 70µL screening solution. Crystals from Screen plates Structure Screen 1 and 2, PACT Premier, Stura Footprint Screen, MacroSol and JCSG-Plus (all Molecular Dimensions), Wizard 1, 2, 3 and 4 and Cryo I and II (all Emerald BioSystems) and PEG/Ion Screen and PEG/Ion 2 Screen (both Hampton Research) were used. Screen plates were kept at 20°C and imaged using a Rock Imager crystal screening system (Formulatrix). Crystals from a range of conditions were picked for X-ray diffraction and cryoprotected for data collection at 100 K by adding 25% ethylene glycol (Hampton Research) or (in the case of the native data) coating the crystal in perfluoropolyether oil. A number of crystals underwent heavy atom soaking before cryoprotection. Diffraction data were recorded at Diamond Light Source (Didcot, UK), using beamline I04 for native and Hg-SAD data and beamline I02 for long-wavelength native. Diffraction data were indexed, integrated, and scaled using Mosflm[7] and Aimless[8] via the xia2 automated data processing pipeline[9]. Long-wavelength (1.7001 Å) native data were collected to a resolution of 1.74

1 Å from a crystal grown in 12 % PEG 3350, 0.1M sodium acetate, but attempts to solve the structure
2 of CRLF3 construct 3 by S-SAD phasing failed, perhaps because of significant radiation damage. The
3 structure was determined by Hg-single anomalous diffraction (SAD) using the AutoSol-wizard[10] of
4 the PHENIX suite[11] with a dataset collected with a wavelength of 1.006 Å from a crystal grown in 20%
5 PEG 3350, 0.2M sodium formate, pH 7.0, soaked with 10mM thimerosal (Sigma-Aldrich) for 16
6 hours. Native data were collected with a wavelength of 0.9795 Å from a crystal grown in 25% PEG
7 3350, 0.2 M ammonium acetate, 0.1 M Bis-Tris, pH 7.5. The structure was refined to a resolution of
8 1.61 Å using Phenix.refine[11] and by manual building in Coot[12]. The final model has good statistics,
9 with 96% of the chain in the Ramachandran favoured region and no Ramachandran outliers. Other
10 crystallographic statistics are presented in Supplemental Table 3.
11

1 **Supplemental Tables (Tables 2, 4, 5 and 7 are found in an additional excel file)**
2
3 **Supplemental Table 1: CRLF3 deficient mice (*Crlf3*$^{-/-}$) have an isolated reduction in platelet count**

	Male		Female	
	WT	*Crlf3*$^{-/-}$	WT	*Crlf3*$^{-/-}$
Young				
Plt (10^3/µL)	1424 (251)	875 (170)***	1117 (261)	686 (105)***
MPV (fL)	5.01 (0.38)	5.19 (0.37)	4.96 (0.09)	5.08 (0.15)
Hgb (g/dL)	14.51 (1.16)	13.51 (1.42)	15.72 (2.33)	15.02 (1.58)
RBC (10^6/µL)	10.32 (1.38)	9.46 (1.48)	10.21 (1.70)	9.68 (1.23)
WBC (10^3/µL)	11.15 (2.64)	13.56 (5.71)	7.80 (1.44)	7.40 (3.46)
Age (weeks)	15.34 (2.93)	15.55 (3.38)	16.83 (2.90)	14.83 (2.39)
n	10	7	5	5
Middle aged				
Plt (10^3/µL)	1485 (205)	1079 (203)***	1035 (133)	771 (134)***
MPV (fL)	4.78 (0.23)	5.02 (0.22)	4.96 (0.09)	5.13 (0.14)
Hgb (g/dL)	13.39 (1.88)	13.08 (1.23)	13.42 (0.97)	13.83 (1.03)
RBC (10^6/µL)	9.92 (1.56)	9.17 (0.80)	8.71 (0.75)	9.04 (0.60)
WBC (10^3/µL)	9.71 (2.70)	8.38 (1.06)	6.86 (1.34)	5.70 (1.38)
Age (weeks)	31.17 (5.27)	27.31 (4.15)	27.14 (4.30)	25.86 (4.69)
n	10	5	5	6
Old				
Plt (10^3/µL)	1514 (444)	1132 (384)***	1324 (242)	772 (162)***
MPV (fL)	5.30 (0.42)	5.21 (0.60)	5.50 (0.64)	5.42 (0.39)
Hgb (g/dL)	13.86 (0.75)	12.43 (1.44)	13.47 (1.73)	13.24 (1.33)
RBC (10^6/µL)	10.34 (1.03)	9.02 (1.55	9.57 (1.65)	8.95 (1.36)
WBC (10^3/µL)	10.53 (3.18)	12.30 (3.36)	6.18 (0.95)	7.96 (3.47)
Age (weeks)	65.39 (13.21)	64.94 (14.43)	69.01 (14.56)	65.55 (15.40)
n	12	23	11	14

4
5 Full blood counts determined by an automated haemocytometer on EDTA anticoagulated venous
6 blood taken from male and female young (12-20 weeks), middle aged (21-40 weeks) and old (>48
7 weeks) mice. Data represents mean (SD). Unpaired 2-tailed Student's *t* test with correction for
8 multiple comparisons using the Holm-Sidak method. *** denotes $p<0.005$. Plt - platelet count; MPV -
9 mean platelet volume; HgB - haemoglobin concentration; RBC - red blood cell count; WBC - white
10 blood cell count.
11

1 **Supplemental Table 3: Data collection and refinement statistics**

	Hg-SAD	Native 1	S-SAD
Data collection			
Wavelength (Å)	1.006	0.9795	1.7001
Space group	C2	C2	C2
Cell dimensions			
a, *b*, *c* (Å)	87.25, 40.94, 76.29	88.03, 40.38, 76.53	87.80, 40.60, 76.66
α, β, γ (°)	90, 98.74, 90	90, 99.94, 90	90, 99.80, 90
Resolution (Å)	27.3-1.97 (2.02-1.97)*	37.7-1.61 (1.65-1.61)	32.1-1.74 (1.79-1.74)
R_{meas}	0.095 (0.861)	0.066 (1.100)	0.067 (0.930)
$I / \sigma I$	16.6 (2.1)	13.3 (1.4)	20.0 (2.1)
Completeness (%)	99.7 (96.0)	96.7 (94.9)	88.4 (49.2)
Redundancy	6.7 (6.7)	3.4 (3.6)	9.3 (6.4)
$CC_{1/2}$	0.999 (0.829)	0.999 (0.504)	0.999 (0.492)
CC_{anom}	0.689 (0.039)	—	0.323 (0.040)
Refinement			
Resolution (Å)	27.3-1.97	37.7-1.61	32.1-1.74
No. refl. work / free	19150 / 907	31712 / 1613	23106 / 1204
R_{work} / R_{free}	0.186 / 0.234	0.177 / 0.201	0.177 / 0.200
No. atoms			
Protein	1951	2000	2011
Ligand/ion	11	0	0
Water	93	108	166
B-factors			
Protein (Å^2)	39.5	36.8	38.8
Water (Å^2)	41.8	32.1	42.3
R.m.s. deviations			
Bond lengths (Å)	0.012	0.009	0.011
Bond angles (°)	1.134	1.066	1.001
PDB ID	6RPY	6RPX	6RPZ

2 *Values in parentheses are for highest-resolution shell.
3

1 **Supplementary Table 6: Full blood counts of *Crlf3*$^{-/-}$ x JAK2V617F ET animals**

	Plt ($10^3/\mu L$)	MPV (fL)	Hgb (g/dL)	RBC ($10^6/\mu L$)	WBC ($10^3/\mu L$)	Age (weeks)	*n*
Young							
1 - WT control	875 (306)	5.62 (0.26)	14.52 (0.75)	10.52 (0.54)	10.37 (4.24)	10.22 (3.46)	6
2 - Crlf3$^{-/-}$	686 (222)	5.71 (0.32)	13.63 (1.48)	9.98 (1.22)	8.90 (2.93)	9.49 (3.38)	7
3 - JAK2V617F ET	1489 (255)	5.64 (0.36)	14.79 (0.94)	10.68 (0.63)	10.16 (2.27)	12.53 (4.79)	7
4 - Crlf3$^{-/-}$ JAK2V617F	879 (229)	5.83 (0.53)	14.78 (1.54)	10.93 (1.27)	11.42 (2.35)	12.31 (4.24)	6
Statistical testing							
1 v 2	***	ns	ns	ns	ns	ns	
1 v 3	***	ns	ns	ns	ns	ns	
1 v 4	ns	ns	ns	ns	ns	ns	
2 v 3	***	ns	ns	ns	ns	ns	
2 v 4	***	ns	ns	ns	ns	ns	
3 v 4	***	ns	ns	ns	ns	ns	
Old							
1 - WT control	1030 (426)	5.46 (0.52)	13.90 (1.19)	10.24 (0.41)	12.42 (4.13)	57.86 (7.77)	5
2 - Crlf3$^{-/-}$	712 (227)	5.31 (0.59)	13.54 (1.77)	9.55 (1.42)	8.00 (2.06)	65.74 (8.17)	7
3 - JAK2V617F ET	1446 (276)	5.30 (0.47)	13.58 (1.33)	10.23 (1.05)	9.27 (3.01)	68.06 (11.05)	9
4 - Crlf3$^{-/-}$ JAK2V617F	890 (137)	5.12 (0.34)	14.97 (2.23)	11.13 (1.66)	8.72 (0.68)	71.95 (16.10)	6
Statistical testing							
1 v 2	***	ns	ns	ns	ns	ns	
1 v 3	***	ns	ns	ns	ns	ns	
1 v 4	ns	ns	ns	ns	ns	ns	
2 v 3	***	ns	ns	ns	ns	ns	
2 v 4	*	ns	ns	ns	ns	ns	
3 v 4	***	ns	ns	ns	ns	ns	

2

3 Full blood counts determined using an automated haemocytometer on EDTA anticoagulated venous
4 blood withdrawn from young (≤20-week-old) and old (≥48-week-old) female WT control, Crlf3$^{-/-}$,
5 JAK2V617F ET and Crlf3$^{-/-}$ JAK2V617F mice. Data represents mean (SD). Two-way ANOVA with
6 correction for multiple comparisons using the Holm-Sidak method. *, *** and ns denote p<0.05,
7 p<0.005 and non-significant, respectively. Plt - platelet count; MPV - mean platelet volume; HgB -
8 haemoglobin concentration; RBC - red blood cell count; WBC - white blood cell count.

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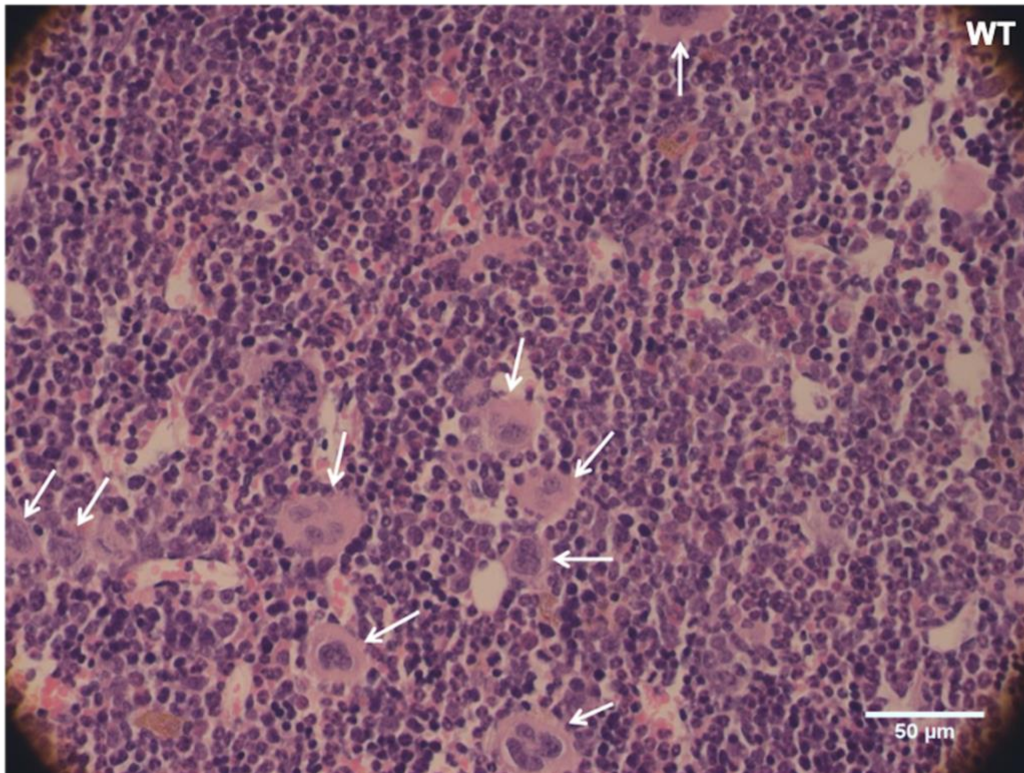
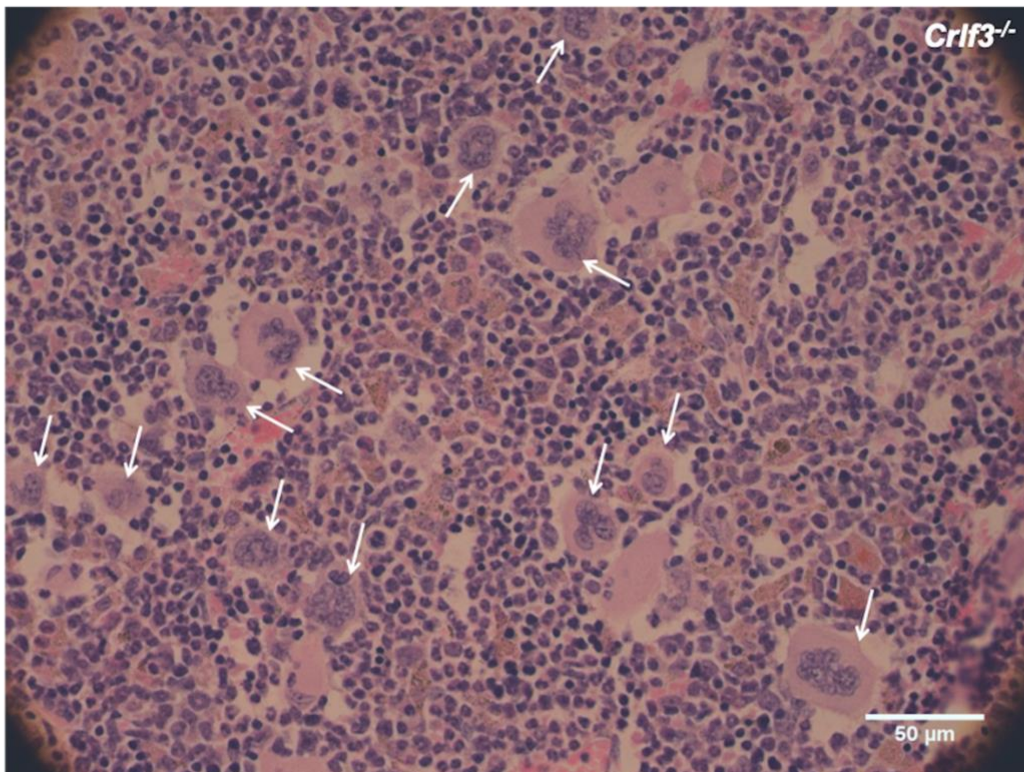
1 **Supplemental Figures**





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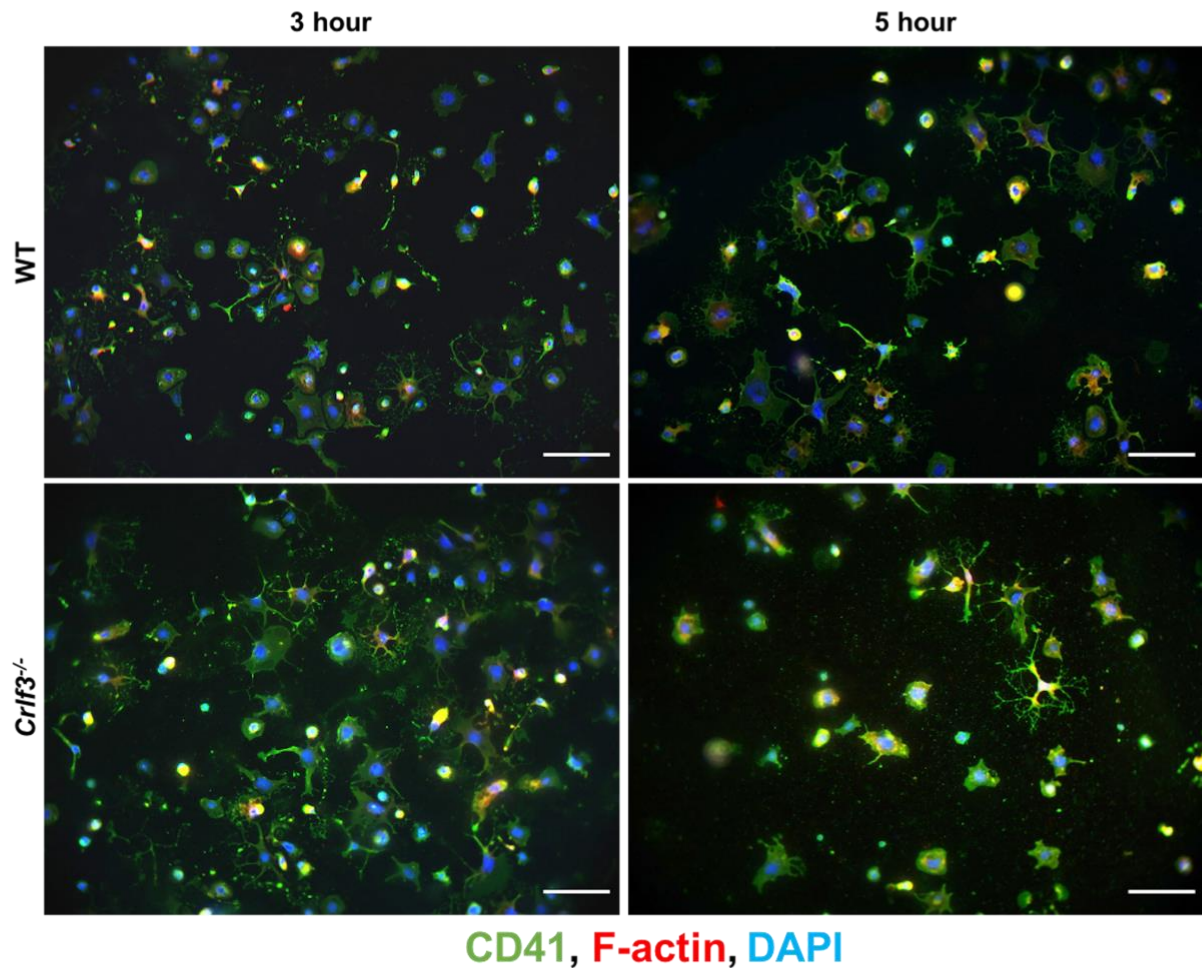
3 **Supplemental Figure 1: Quantification of bone marrow residing MKs**

4 Control (WT) and *Crlf3*$^{-/-}$ tibia were fixed, sectioned, and stained with Haematoxylin and eosin (H&E).

5 MKs were quantified after imaging by light microscopy. Images are representative of each genotype

6 and arrows point to MKs. Scale bars are 50µm.



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2 **Supplemental Figure 2: CRLF3 deficiency increases speed of proplatelet formation**

3 *In vitro* cultured MKs were seeded onto fibrinogen coated coverslips and incubated at 37°C for 3 or 5

4 hours to induce proplatelet formation. Samples were fixed, stained with CD41 (green), F-actin (red)

5 and DAPI (blue), and imaged by confocal microscopy. Images are representative of each genotype at

6 each time point. Scale bars are 50µm.

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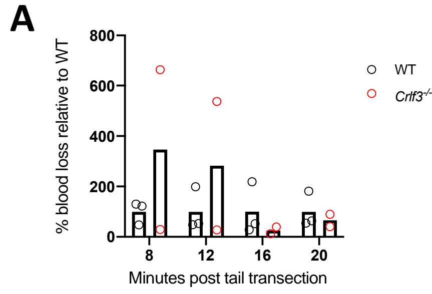

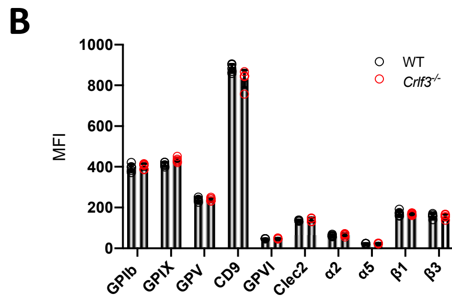

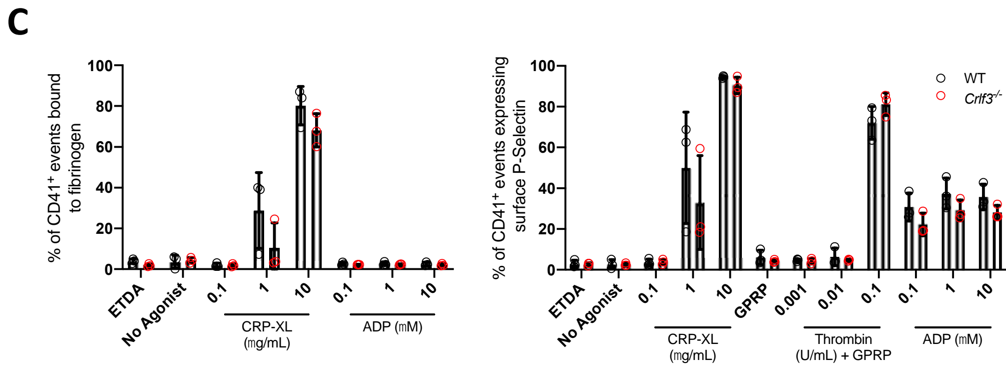

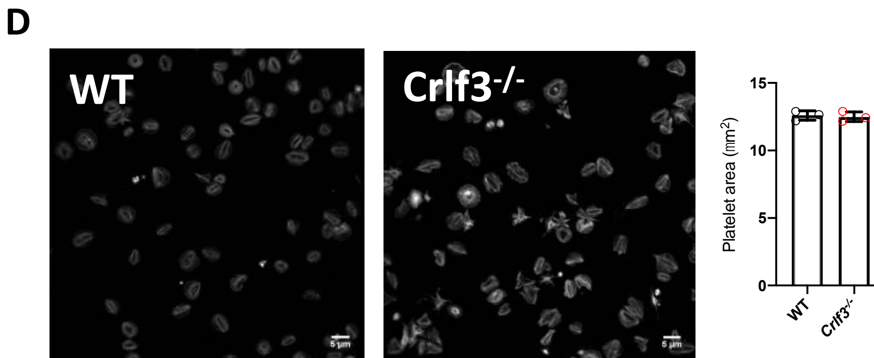

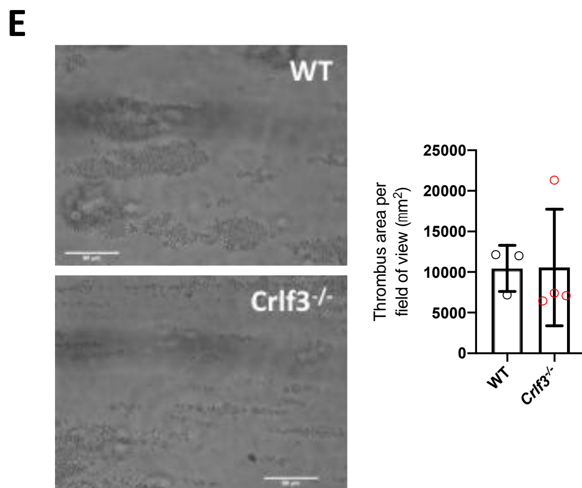

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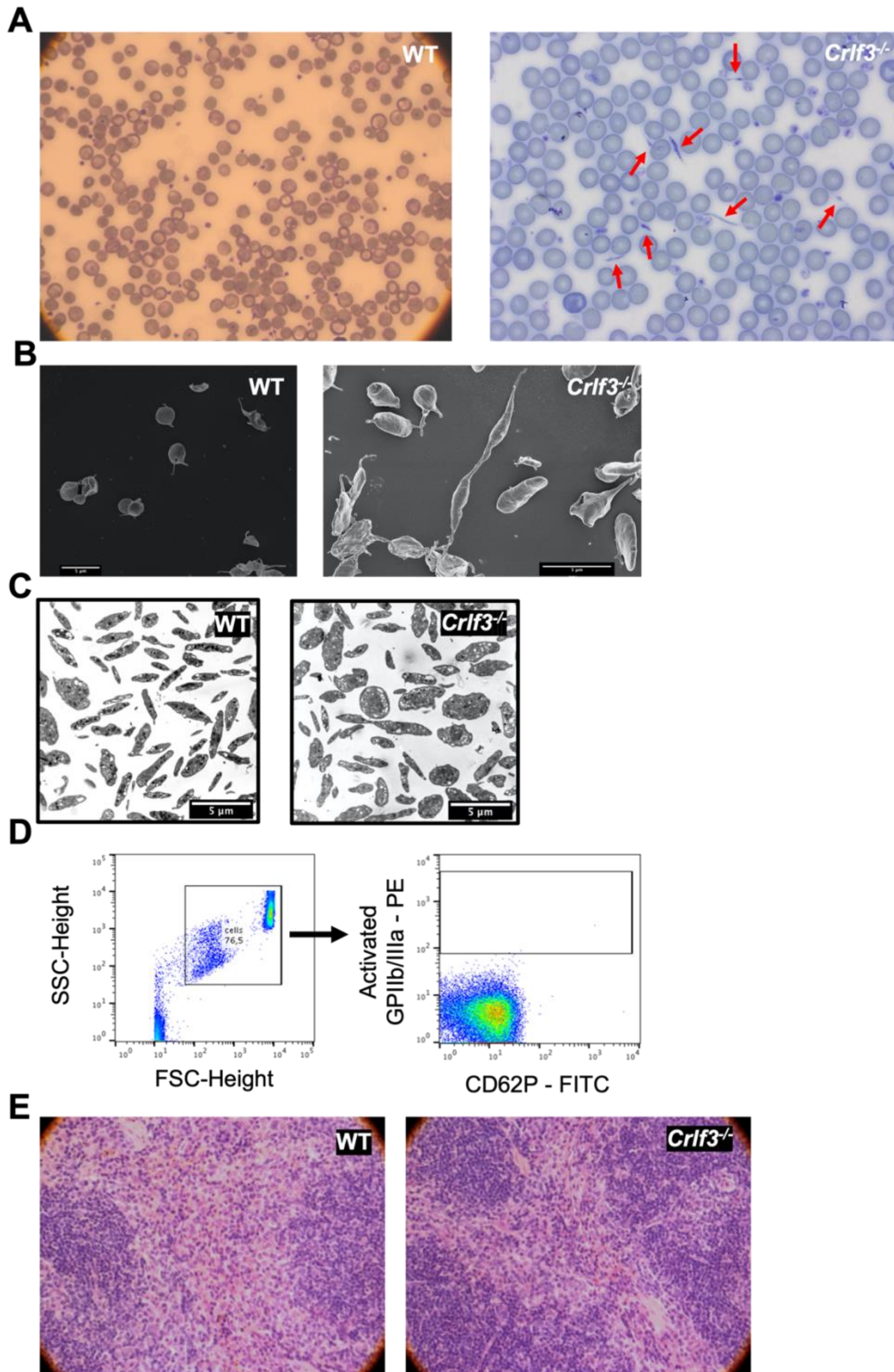
Supplemental Figure 3: CRLF3 deficiency does not affect platelet function

(**A**) Control (WT, black) and *Crlf3*$^{-/-}$ (red) blood loss after removal of tail tip. (**B**) Control (WT, black) and *Crlf3*$^{-/-}$ (red) whole blood incubated with antibodies against the membrane proteins GPIb, GPIX, GPV, CD9, GPVI, Clec2, α2, α5, β1 and β3 and assessed by flow cytometry, gating on platelet sized events (n=5). (**C**) Control (WT, black) and *Crlf3*$^{-/-}$ (red) whole blood incubated with specific antibodies against CD41, P-Selectin and fibrinogen with or without addition of agonists. Percentage of CD41^{+} events bound to fibrinogen (left panel) or expressing surface P-selectin (right panel) was determined by flow cytometry (n=3). (**D**) Control (WT, black) and *Crlf3*$^{-/-}$ (red) washed platelets seeded onto fibrinogen coated coverslips and incubated for 30 mins at 37°C, fixed, stained for F-actin and imaged by fluorescence microscopy. Platelet area determined by thresholding images on F-actin staining using ImageJ (n=3). (**E**) Heparinised whole blood flowed at arterial shear rate (1,000 s^{-1}) over a surface coated with fibrillar collagen and imaged by light microscopy. Thrombus area for control (WT, black) and *Crlf3*$^{-/-}$ (red) mice determined by manually measuring the size of each thrombi using ImageJ (n=3 WT/4 *Crlf3*$^{-/-}$). Data represents mean ± SD. Unpaired 2-tailed Student's t test (**D** and **E**) with correction for multiple comparisons using the Holm-Sidak method (**B** and **C**). MFI = mean fluorescent intensity.



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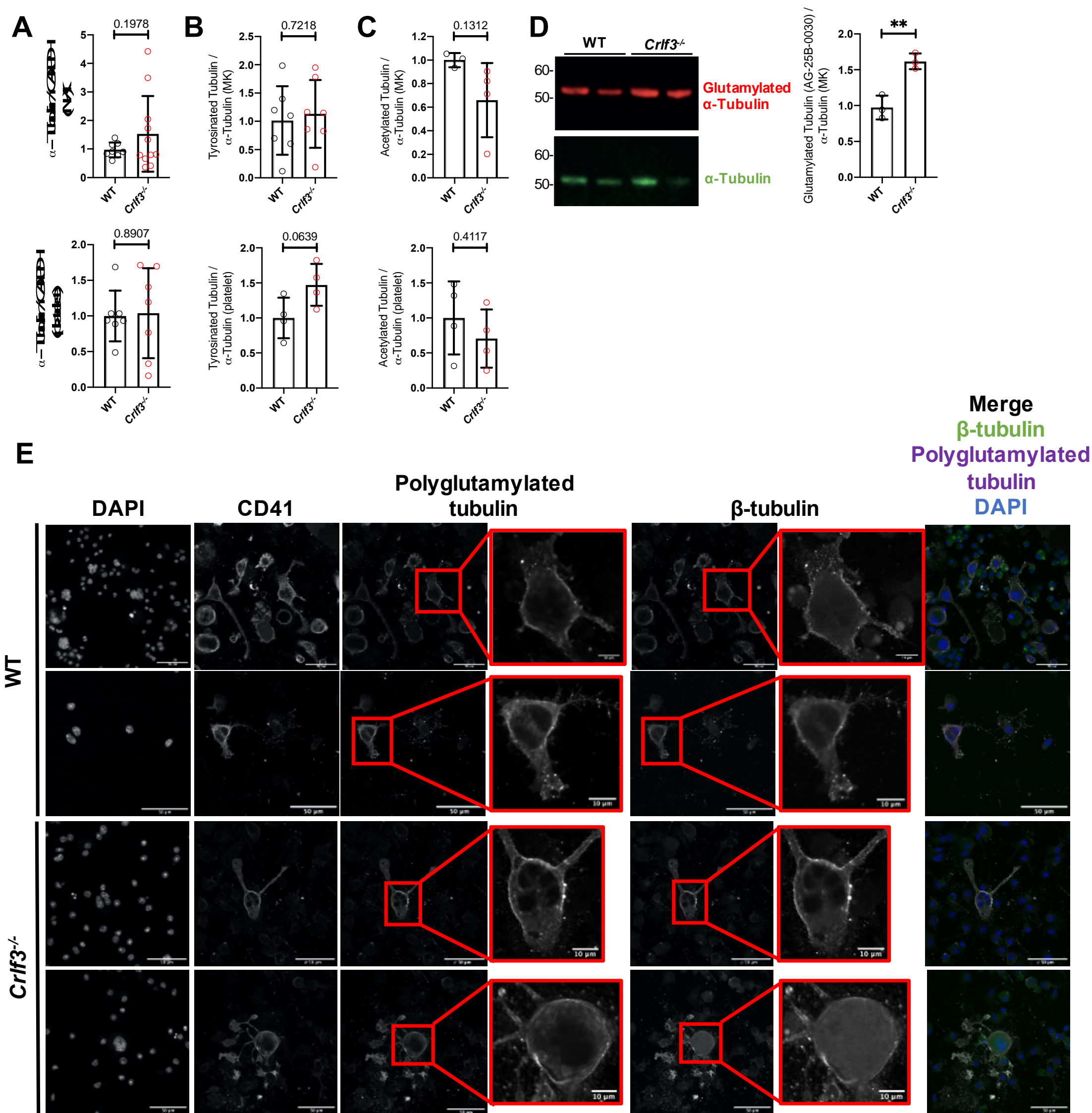
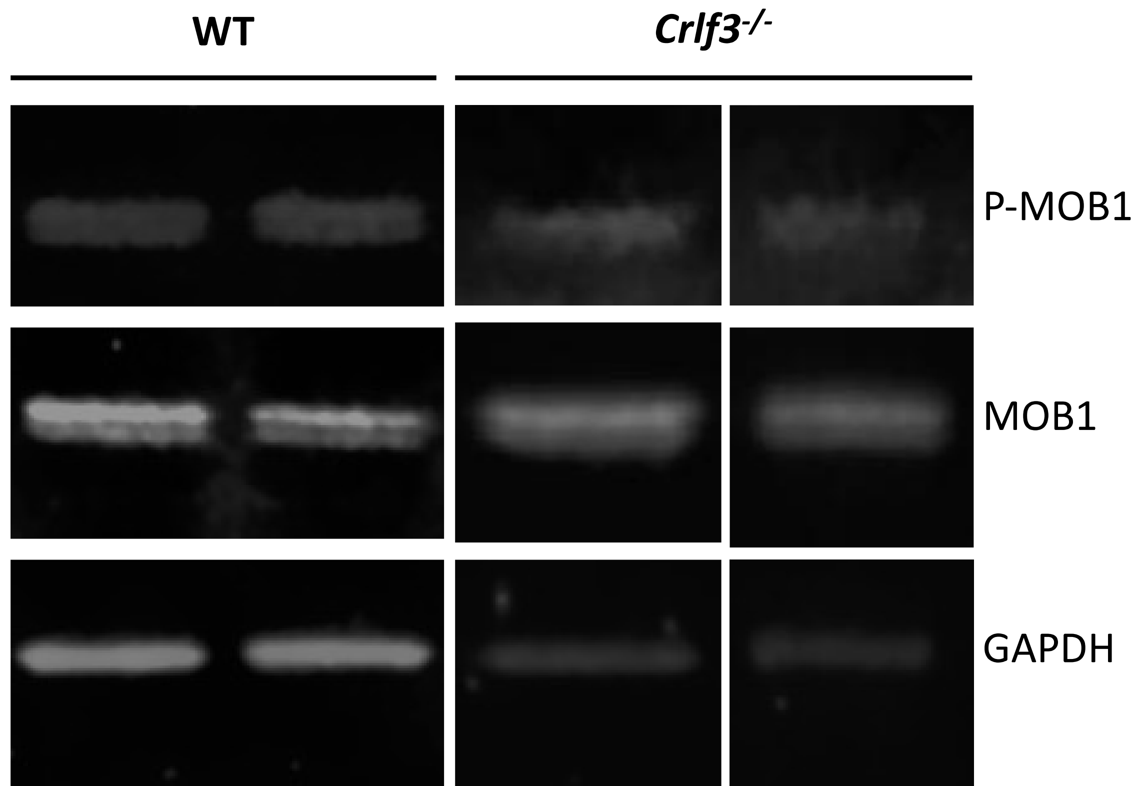
2 **Supplemental Figure 4: CRLF3 deficiency causes ineffective thrombopoiesis**

3 (**A**) Blood smears from control (WT) and *Crlf3*$^{-/-}$ whole blood taken at 63x and 100x magnification
4 under light microscopy, respectively. Red arrows point to elongated spindle form preplatelet
5 structures. (**B**) Control (WT) and *Crlf3*$^{-/-}$ washed platelets were fixed and prepared for scanning and
6 (**C**) transmission electron microscopy. Scale bars are 5µm. (**D**) To rule out the possibility that
7 preplatelets are platelet microaggregates, heparinised venous blood was incubated with specific
8 antibodies against activated GPIIb/IIIa and CD62P and assessed by flow cytometry. (**E**) Hematoxylin
9 and eosin-stained spleen sections from control (WT) and *Crlf3*$^{-/-}$ mice imaged at 20x magnification
10 under light microscopy.



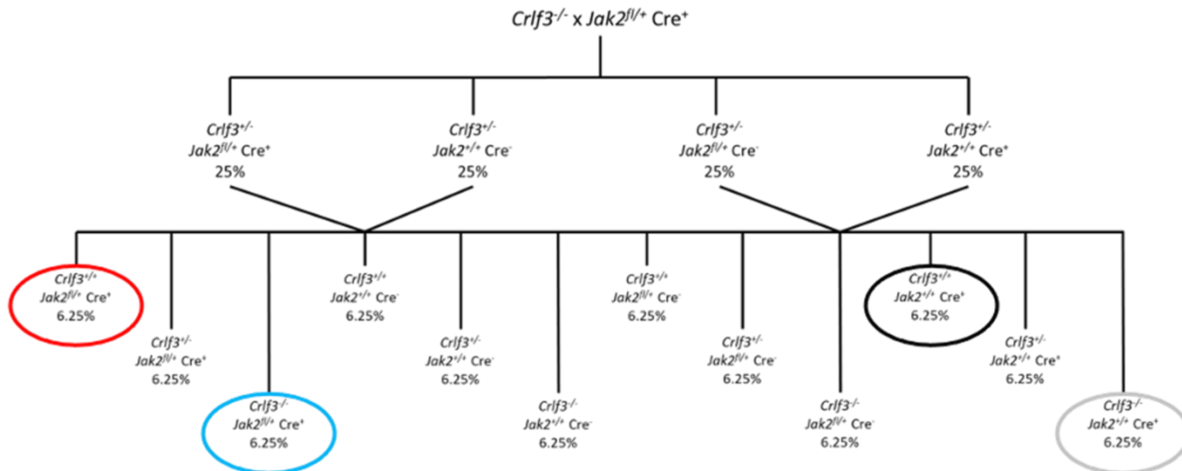
Supplemental Figure 5: Tubulin modifications in MKs and platelets

(**A**) Densitometry quantification of α-Tubulin relative to GAPDH (*n*=7 platelet; *n*=8 WT/11 *Crlf3*$^{-/-}$ MK), (**B**) tyrosine α-Tubulin relative to α-Tubulin (*n*=4 platelet; *n*=7 MK), (**C**) acetylated α-Tubulin relative to α-Tubulin (*n*=4 platelet; *n*=3 WT/4 *Crlf3*$^{-/-}$ MK) and (**D**) polyglutamylated tubulin (AG-25B-0030, AdipoGen) relative to α-Tubulin (n=3 MK) from western blots of *in vitro* cultured MK (top panels) and platelet (bottom panels) lysates from control (WT, back) and *Crlf3*$^{-/-}$ (red) animals probed with specific antibodies. (**E**) *In vitro* cultured MKs were seeded onto fibrinogen coated coverslips and incubated at 37°C for 5 hours to induce proplatelet formation. Samples were fixed, stained for CD41, polyglutamylated α-Tubulin (AG-25B-0030; magenta), β-tubulin (green) and DAPI (blue) imaged by fluorescence microscopy. Images are representative for *Crlf3*$^{-/-}$ and control (WT) proplatelet forming MKs. Scale bars are 50µm and 10µm in the boxed zoomed images. Data represents mean or mean± SD. Unpaired 2-tailed Student's or Welch's (A - MK only) t test.



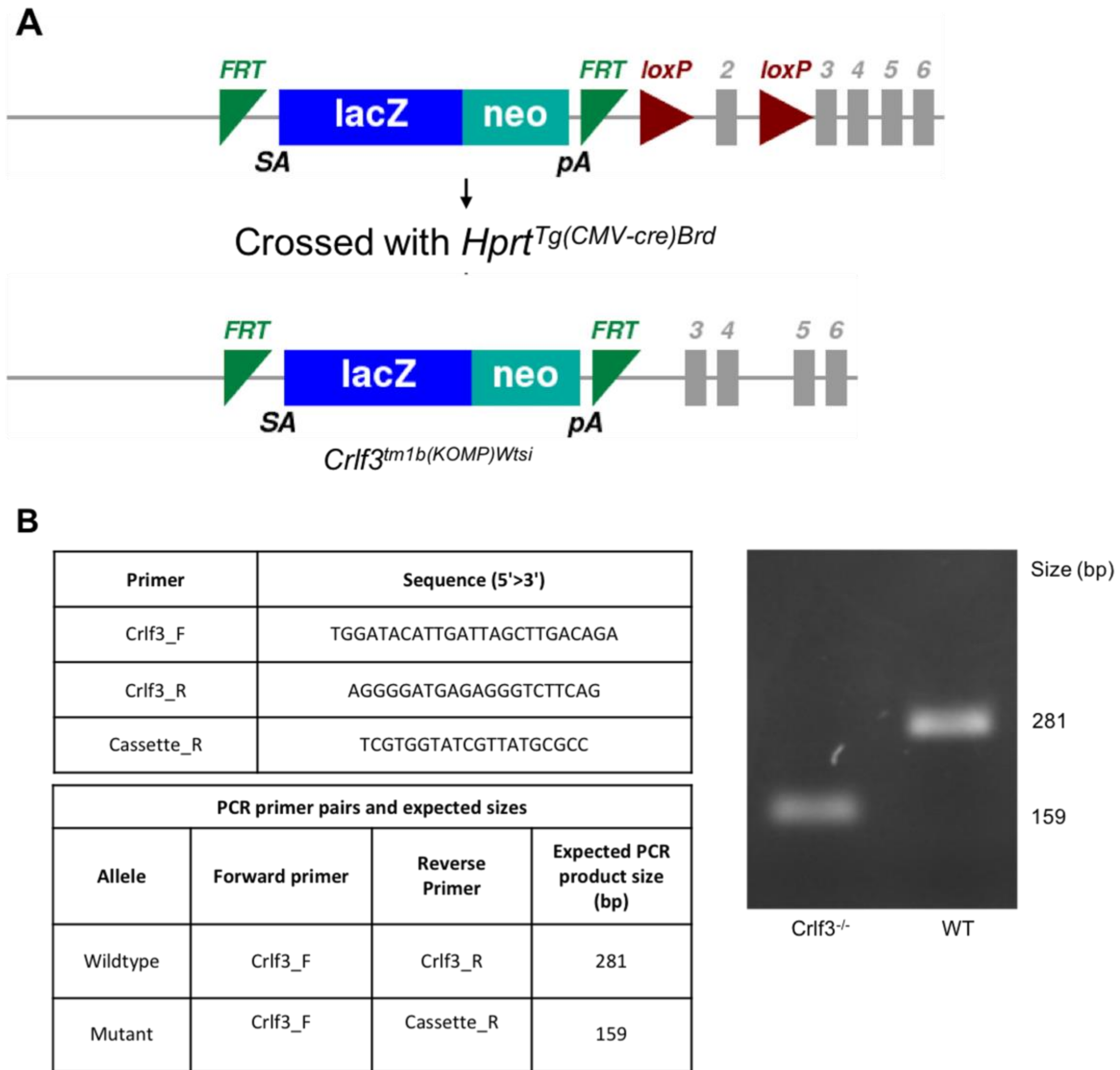
Supplemental Figure 6: Mob1 phosphorylation in control and *Crlf3$^{-/-}$* MK

Western blot of WT (control) and *Crlf3$^{-/-}$ in vitro* cultured MKs probed with specific antibodies against phosphorylated MOB1 (P-MOB1), MOB1 and GAPDH.



Supplemental Figure 7: Breeding strategy to study effect of *Crlf3* ablation on essential thrombocythaemia

Crlf3$^{-/-}$ mice were crossbred with JAK2V617F Essential Thrombocythaemia (ET) mice[13] generating *Crlf3* heterozygous intermediates, which were cross bred again to generate lines of interest. Genotypes of interest are circled (black = WT control; grey = *Crlf3$^{-/-}$*; red = *JAK2V617F* ET and green = *Crlf3$^{-/-}$ JAK2V617F*).



Primer	Sequence (5'>3')
Crlf3_F	TGGATACATTGATTAGCTTGACAGA
Crlf3_R	AGGGGATGAGAGGGTCTTCAG
Cassette_R	TCGTGGTATCGTTATGCGCC

PCR primer pairs and expected sizes			
Allele	Forward primer	Reverse Primer	Expected PCR product size (bp)
Wildtype	Crlf3_F	Crlf3_R	281
Mutant	Crlf3_F	Cassette_R	159

Supplemental Figure 7: Generation of *$Crlf3^{tm1b(KOMP)Wtsi}$* (*$Crlf3^{-/-}$*) mice (**A**) The allele structure for *Crlf3*. Exon 2 was excised by cre-mediated recombination. (**B**) Primers used for mouse genotyping, expected band size and representative image of a genotyping assay.

1 **Videos**

2

3 **Video 1 and 2: Proplatelet formation is not impaired in *Crlf3$^{-/-}$* mice**

4 Anesthetised Control (WT; **Video 1**) and *Crlf3$^{-/-}$* (**Video 2**) mice injected with tetramethylrhodamine

5 dextran and anti-CD105 (both red) to label bone vasculature and anti-GPIX (green) to label platelets

6 and MKs. Fronto-parietal skull exposed, and images acquired every 5 seconds by fluorescence

7 microscopy and videos created using ImageJ. Scale bars are 50µm.

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