Supplementary Information

Supplemental Methods

Animals

All animal protocols were approved by the UCSD Institutional Animal Care and Use Committee (IACUC). Mice were housed in standard conditions with up to 5 mice per cage, fed standard chow, and monitored in accordance with IACUC guidelines. C57BL/6J (stock # 000664) mice were obtained from The Jackson Lab and maintained in our animal facility for more than 10 generations. Vav1-Cre (stock # 008610)[68] and Mx1-Cre (stock # 003556)[69] transgenic mice were obtained from The Jackson Lab and maintained as hemizygotes in our animal facility for more than 10 generations. *Stk4*- and *Stk3*- floxed mice (stock # 017635)[70] were obtained from The Jackson Lab and maintained as homozygotes for floxed alleles in our animal facility. *Stk4*- and *Stk3*-floxed mice were crossed into the various Cre strains as described in main text. To control for any potential genetic background effects, all mice (or mouse-derived hematopoietic cells used for transplantations) were analyzed in groups with littermates of the same generation of backcross into respective Cre strains. For use in all described competitive bone marrow transplant assays, the C57BL/6 congenic strain carrying the differential CD45.1 leukocyte receptor, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (stock # 002014) were obtained from The Jackson Lab and maintained in our animal facility for more than 10 generations. Genotyping for floxed alleles and Cre transgenes was conducted as described on The Jackson Lab website for each respective strain.

Antibodies

<u>Western blotting:</u>

MST1 (Cell Signaling, #3682)

MST2 (Cell Signaling, #3952)

Phospho-MOB1 (Thr35) (Cell Signaling, #8699)

Phospho-IκBα (Ser 32) (Cell Signaling, #2859)

IκBα (L35A5) (Cell Signaling, #4814)

Phospho-NF-κB p65 (Ser536) (93H1) (Cell Signaling, #3033)

Phospho-IRAK1 (T209) (Abcam, #ab218130)

IRAK1 (D51G7) (Cell Signaling, #4505)

Monoclonal Anti-Flag M2 (Sigma, #F3165)

Monoclonal Anti-HA (HA-7) (Sigma, #H9658)

β-Actin clone AC-15 (Millipore Sigma, #A1978)

IRDye 800CW anti-mouse IgG (Li-Cor, #925-32210)

IRDye 680LT anti-rabbit IgG (Li-Cor, #925-68021)

Flow cytometry:

FITC anti-mouse CD45.1 (eBioscience, #11-0453-85)

FITC anti-mouse CD45.2 (Biolegend, #109806)

FITC anti-mouse Ter-119 (eBioscience, #11-5921-81)

FITC anti-mouse Ly-6G (Biolegend, #127606)

FITC anti-mouse B220 (Biolegend, #103206)

PE anti-mouse CD71 (eBioscience, #12-071-82)

PE anti-mouse CD48 (Biolegend, #103406)

PerCP/Cy5.5 anti-mouse CD3ε (Biolegend, #100328) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse CD4 (Biolegend, #100540) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse CD8α (eBioscience, #45-0081-82) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse CD11b (Biolegend, #101228) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse Gr-1 (Biolegend, #108428) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse Ter-119 (Biolegend, #116228) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse B220 (Biolegend, #103236) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse CD19 (eBioscience, #45-0193-82) [Lineage cocktail]

PerCP/Cy5.5 anti-mouse CD127 (eBioscience, #45-1271-82) [Lineage cocktail]

PE/Cy7 anti-mouse CD117 (Biolegend, #105814)

APC anti-mouse CD3ε (eBioscience, #17-0031-82)

APC anti-mouse CD11b (Biolegend, #101212)

APC anti-mouse B220 (Biolegend, #103212)

APC anti-mouse Sca-1 (Biolegend, #108112)

Biotin anti-mouse CD150 (eBioscience, #13-1501-82)

APC/AlexaFlour750 Streptavidin (Molecular Probes, #SA1027)

Bone Marrow Transplantation

C57BL/6J (stock # 000664) transplant recipient mice were obtained from The Jackson Lab and maintained in our animal facility for more than 10 generations. For use in all described competitive bone marrow transplant assays, the C57BL/6 congenic strain carrying the differential CD45.1 leukocyte receptor, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (stock # 002014) were used as recipients. These were obtained from The Jackson Lab and maintained in our animal facility for more than 10 generations. Recipient mice were between 8-12 weeks of age at time of transplantation and contained equal distributions of males and females. Recipient mice were randomly allocated amongst experimental groups while maintaining a matched distribution based on both age and sex. All lethal irradiations were performed as a single administration of a dosage of 9.5 Gy approximately 6-8 hours prior to transplantation of cells.

For non-competitive transplantation experiments, total bone marrow from three mice each of indicated genotypes was harvested as described in the 'Hematopoietic cell isolation and collection' section and pooled together based on genotype. These total bone marrow cells were then immediately used for transplantations via resuspension in PBS at a density of 2×10^7 cells/mL and 100 µl of cell suspension was administered per mouse (i.e. 2×10^6 cells per recipient mouse) via intravenous tail-vein injection. No additional helper cells were used during the transplantation experiments, and therefore the post-irradiation bone marrow of recipient mice is derived from the transplant donor cell genotype.

For competitive bone marrow transplantation experiments, total bone marrow from three mice each of indicated genotypes (in CD45.2 leukocyte receptor background) was harvested as described in the 'Hematopoietic cell isolation and collection' section and pooled together based on genotype. Total bone marrow was harvested in parallel from three mice of C57BL/6 congenic strain carrying the differential CD45.1 leukocyte receptor and pooled. CD45.2 ('donor') bone marrow cells were mixed at a 1:1 ratio with CD45.1 ('recipient') cells, and these cells were then immediately used for transplantations via resuspension in PBS at a

density of 2 x 10^7 cells/mL and 100 µl of cell suspension was administered per mouse (i.e. 2 x 10^6 cells per recipient mouse) via intravenous tail-vein injection in B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1) recipient mice. For analysis, donor-derived (CD45.2) frequencies were then measured in the peripheral blood beginning four weeks post-transplantation.

For the JAK2-V617F MPN model, hematopoietic stem cell-enriched (via 5-FU administration) bone marrow cells were harvested as described in the 'Hematopoietic cell isolation and collection' section and transduced as described in the 'Viral Transduction' section. The total population of cells (of which ~2% are transduced with JAK2-V617F) were resuspended in PBS at a density of 0.5 x 10^7 cells/mL and 100 µl of cell suspension was administered per mouse (i.e. 0.5 x 10^6 total cells per recipient mouse) via intraveneous tail-vein injection. No additional helper cells were used during the transplantation experiments, only the untransduced bone marrow cell population of the indicated genotype, and therefore the post-irradiation bone marrow of recipient mice is derived from the transplant donor cell genotype (i.e. $Stk4^{+/+}Stk3^{+/+}$ or $Stk4^{+/-}Stk3^{+/-}$), of which ~2% are transduced with JAK2-V617F (GFP+) at initial time of transplantation. Successful engraftment of transduced donor bone marrow cells were confirmed via measuring GFP+ cell frequency in the peripheral blood four weeks post-transplantation, and any mice showing undetectable GFP+ cell frequency are excluded from further analysis in the model.

Cell Lines

HEK293T cells were acquired from ATCC. Cells were cultured in DMEM (HyClone, #SH30022.01) supplemented with 10% bovine calf serum and 1% penicillin/streptomycin at standard conditions of 5% CO_2 at 37°C. THP-1 cells were acquired from ATCC. Cells were cultured in RPMI supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at standard conditions of 5% CO_2 at 37°C.

Chemical and Inhibitor Treatments

IRAK1/4 inhibitor was purchased from Tocris Bioscience (#5665-50). IRAK1/4 inhibitor was resuspended in DMSO at a concentration of 10 mM and aliquoted prior to use. For injection into mice working solutions were prepared by dilution of stock inhibitor solution 1:20 into 0.22 µM filtered PBS. Working solutions were then heated to 55 °C for approximately 15 minutes and subjected to rocking at room temperature for 4 – 6 hours to

aid in resuspension. Where applicable, an equal volume of 5% DMSO solutions were injected as controls (Mock treatment). For *in vivo* IRAK1/4 inhibitor experiments, 4 mg/kg intraperitoneal (I.P.) injections were performed once every three days throughout the total course of treatment (4 weeks). For cell culture experiments working solutions were prepared via dilution in cell culture media (supplemented RPMI), heated to 37 °C, and similarly rocked at room temperature to aid in resuspension, prior to administering to cells. THP-1 cells were cultured overnight in media containing the indicated concentrations of inhibitor, or mock control media (0.1% DMSO), prior to, and during, stimulation with LPS (100 ng/mL) for two hours the next morning. LPS (List Biological Laboratories, #201) stock solutions were prepared by resuspension in sterile H_20 at a concentration of 5 mg/mL. Working solutions were prepared by subsequent serial dilution in cell culture media, and administration to cells was performed at a final concentration of 100 ng/mL for the times indicated in the manuscript. Polyinosinic-polycytidylic acid (pIpC) (Sigma, #P0913) was resuspended in sterile H_2O at a concentration of 1 mg/mL. Prior to injection, pIpC was temporarily heated to 65°C, and then cooled to room temperature. To induce gene inactivation *in vivo* using the Mx1-Cre system, 500 µg per mouse was injected intraperitoneally (I.P.) every other day for a total of 6 doses. 5-Fluorouracil (5-FU) (Sigma, #F6627) was resuspended in sterile H_2O at a concentration of 15 mg/mL. Prior to injection, 5-FU was temporarily heated to 70°C to assist with resuspension, and then cooled to room temperature. 5-FU was administered to mice as a single dose of 150 mg/kg by intraperitoneal (I.P.) injection. Bone marrow from 5-FU treated mice for use in transplantation experiments was harvested 5 days following injections. Any mice that did not show myeloablation in the bone marrow at time of cell harvest were excluded from use for any downstream experiments.

Flow Cytometry and Cell Sorting

Flow cytometric analysis was conducted using a BD FACSCanto instrument equipped with standard lasers (488 nm, 640 nm) and filters. Data collection was performed using BD FACSDiva software. Compensation was set up using appropriate single-stained controls and positive-staining gates were established using appropriate FMO controls. Post-acquisition data analysis was performed using FlowJo software (FlowJo, LLC). Flow sorting of bone-marrow mononuclear cells was performed on a FACSAriaII equipped with standard lasers (405 nm, 488 nm, 640 nm) and filters, and using a nozzle size of 85 µm. Stainings were conducted in PBS

supplemented with 0.1% bovine serum albumin. Antibody validation and optimal antibody concentrations for stainings were pre-determined via titration.

MDS Gene Expression Analysis

For gene expression analysis in CD34+ bone marrow cells comparing del(20q) MDS patients with healthy control individuals, we utilized a previously published MDS patient gene expression dataset (GSE58831). Individual Affymetrix CEL files from 6 del(20q) MDS patients (MDS097, MDS113, MDS129, MDS158, MDS187, MDS202) and 10 normal controls (NBM01-NBM10) were downloaded from NCBI GEO repository. Array normalization was performed using the RMA method. Differential gene expression analysis was performed using the limma R package from Bioconductor, with adjusted P-values calculated by a standard Benjamini & Hochberg (False Discovery Rate) method for multiple comparisons. Differentially expressed gene probes are ranked based on adjusted p-value (see Supplemental Table 1). For display purposes, a row-normalized gene expression heatmap of the 25 most significantly downregulated was generated using the 'HeatMapImage' (V 6.0) module on the Gene Pattern server (https://genepattern.broadinstitute.org).

Hematopoietic Cell Collection and Isolation

Bone marrow cells were harvested by flushing two femurs and two tibias per mouse with ice cold PBS using disposable syringes with 21-gauge needles. Splenocytes were harvested by physical tissue disruption and repeated pipetting in ice cold PBS. Red cell lysis was performed by resuspending cell mixture in ice cold ACK buffer (0.1 mM Na_2EDTA, 10 mM $KHCO_3$, 150 mM NH_4Cl) for 5 minutes, followed by washing with ice cold PBS and passage through a 40 μM cell strainer to eliminate large tissue/cell clumps and debris. For short-term culture and retroviral transduction/transplantation experiments, hematopoietic cells were resuspended in IMDM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, recombinant mIL-3 (10 ng/mL), hIL-6 (10 ng/mL), and mSCF (20 ng/mL). Where applicable, mouse lineage-negative (Lin^-) cells were isolated according to manufacturer's instructions using mouse Lineage Cell Depletion Kit (Miltenyi Biotec, #130-090-858), and enrichment efficiency was verified via flow cytometry.

Luciferase Assay

HEK293T cells seeded in 24-well plates were transfected with a pNFκB_Luciferase reporter vector (100 ng) containing 4X NF-κB responsive elements upstream of a minimal TATA promoter, pRL-TK *Renilla* luciferase control vector (25 ng), MIP-empty or MIP-MST1 (80 ng), and pcDNA3-Flag-TRAF6 (10 ng). 48 hours following transfections, luciferase activity was measured on a BD Monolight 3010 luminometer using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. pcDNA3-Flag-TRAF6 was first confirmed to activate pNFκB_Luciferase activity relative to an empty vector control.

Oligos

SalI-Kozak-JAK2-F:

5'-TTTTTGTCGACGCCACCATGGGAATGGCCTGCCTT-3'

SalI-JAK2-R:

5'-TTTTTGTCGACTCATCCAGCCATGTTATCCCT-3'

m*Stk4*-qPCR-F:

5'-ACTTACAGATACCATGGCAAAGA-3'

m*Stk4*-qPCR-R:

5'-CTGCCACACAGTTGTACCCA-3'

m*Stk3*-qPCR-F:

5'-GGTCCATTCGGCCCTTCTTT-3'

m*Stk3*-qPCR-R:

5'-CACTTCCATAAGACCCTTCTCC-3'

m*Gapdh*-qPCR-F:

5'-GGTGCTGAGTATGTCGTGGAGTCTA-3'

m*Gapdh*-qPCR-R:

5'-AAAGTTGTCATGGATGACCTTGG-3'

h*STK4*-qPCR-F:

5'-GACGGTACAGCTGAGGAACC-3'

h*STK4*-qPCR-R:

5'-TGCCATAGGACCCTTCTCCA-3'

h*IL6*-qPCR-F:

5’-ACCCCCAATAAATATAGGACTGGA-3’

h*IL6*-qPCR-R:

5’-CGAAGGCGCTTGTGGAGAA-3’

h*IL1B*-qPCR-F:

5’-CAGAAGTACCTGAGCTCGCC-3’

h*IL1B*-qPCR-R:

5’-CCTGGAAGGAGCACTTCATCT-3’

h*IL15*-qPCR-F:

5’-GTGATGTTCACCCCAGTTGC-3’

h*IL15*-qPCR-R:

5’-TGCATCTCCGGACTCAAGTG-3’

h*GAPDH*-qPCR-F:

5’-GAAGGTGAAGGTCGGAGTC-3’

h*GAPDH*-qPCR-R:

5’-GAAGATGGTGATGGGATTTC-3’

h*POLR2A*-qPCR-F:

5’-GCACCACGTCCAATGACAT-3’

h*POLR2A*-qPCR-R:

5’-GTGCGGCTGCTTCCATAA-3’

Peripheral Blood Analysis

Peripheral blood samples (approximately 100 µl per mouse) were collected in EDTA-coated microvettes (Fisher Scientific, # NC9299309) by submandibular venipuncture using 5 mm animal lancets (Braintree Scientific, #GR5MM). Analysis of differential blood cell counts and parameters was performed using a Scil Vet abc Plus+ instrument (Henry Schein Animal Health). Instrument was regularly calibrated and maintained according to manufacturer’s instructions. GFP+ cell frequencies in peripheral blood samples were measured by flow cytometry via dilution of peripheral blood samples in ACK buffer for 5 minutes, followed by washing once in 1 mL PBS, and resuspension in 350 µl of PBS prior to flow cytometric analysis. Non-viable cells were excluded from analysis via addition of propidium iodide (PI). Differential blood cell frequencies were

secondarily verified by flow cytometry (for example, CD11b+/Gr-1+ staining for measuring granulocyte frequencies).

Principal Component Analysis and Hierarchical Clustering

Principal component analysis (PCA) and hierarchical clustering of differentially abundant serum proteins were performed using ClustVis.[71] PCA is performed by singular value decomposition (SVD) method with unit variance scaling for row (individual serum proteins) normalization. Hierarchical clustering is performed using correlation distance and average linkage with unit variance scaling for row (individual serum proteins) normalization.

qPCR Analysis

Cell lysis and RNA isolation were performed using Trizol reagent (ThermoFisher Scientific, #15596026) according to manufacturer's instructions. cDNA was prepared from 0.5 – 1 µg RNA using qScript cDNA Supermix (Quanta, #95048) according to manufacturer's protocol. Quantitative PCR was performed using KAPA SYBR Fast 2X Master Mix (KAPA Biosystems, #KK4618) according to manufacturer's protocol, in 20 µl reactions, each performed in technical duplicates. qPCR reactions were performed using a BioRad CFX Connect instrument. Data analysis was performed using a standard delta-delta Ct method relative to the geometric-mean of two reference genes, *GAPDH* and *POLR2A* (human cell experiments), or relative to the mean of *Gapdh* (murine experiments).

Serum Protein Profiling and IL-1β ELISA

For Quantibody array based serum protein profiling approximately 0.5 - 1 mL peripheral blood was collected in non-EDTA coated vacutainer tubes by submandibular venipuncture during terminal analysis of mice at 36 weeks post-transplantation from three mice per experimental group. Whole blood was incubated at room temperature for 20 minutes followed by centrifugation at 1,800x *g* for 10 minutes at 4 °C. Serum was then collected, aliquoted, and stored at -80°C prior to shipping samples. Abundance of 200 mouse serum proteins/cytokines were analyzed by multiplexed sandwich ELISA-based quantitative array platform (Mouse Quantibody 4000 Array, Ray Biotech). Serum protein analysis and quantification was performed by Ray Biotech (https://www.raybiotech.com/). Post-quality control, a total of 153 serum proteins were effectively

quantified by this analysis (see Supplemental Table 2).

For non-terminal serum assessment of cytokine abundance, 200 µl of peripheral blood was collected in non-EDTA coated vacutainer tubes by submandibular venipuncture. Whole blood was incubated at room temperature for 20 minutes followed by centrifugation at 1,800x *g* for 10 minutes at 4 °C. Serum was then collected, diluted 1:4 in reagent diluent solution (1% BSA in 0.22 µm filtered PBS), and stored at -80 °C. Mouse IL-1 beta / IL-1F2 Duoset ELISA was purchased from R & D Systems (DY401-05). ELISA-based measurement of serum IL-1β in the JAK2-V617F model was performed according to manufacturer's instructions. For IRAK1/4 inhibitor-related experiment, serum was analyzed from a total of 12 age-matched wild-type control mice, seven JAK2-V617F;$Stk4^{+/+}Stk3^{+/+}$ (measured pre- and post-IRAK1/4 inhibitor treatment), and seven JAK2-V617F;$Stk4^{+/-}Stk3^{+/-}$ (measured pre- and post-IRAK1/4 inhibitor treatment). Recombinant IL-1β standards used for establishment of standard curve quantification (working range: 15.6 pg/mL – 1000 pg/mL) were measured in technical triplicate, and experimental serum samples were measured in technical duplicates. Absorbance measurements were performed using a microplate reader at 450 nm wavelength with background correction for individual wells performed by subtraction of absorbance measured at 570 nm.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism (V 7.0) software. Individual statistical tests used for data analysis are indicated in figure legends. All data are displayed as mean (bar graph, horizontal line, or point) with error bars always representing S.E.M. All student's t-tests are conducted as two-tailed tests. Statistical significance in figures is displayed as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. In some cases, where p values are close to reaching a statistical significance threshold, exact values are displayed in figure panels. Where applicable, mouse sample sizes for measurement of various blood parameters were determined based on a minimal meaningful effect size of one standard deviation from a distribution of healthy wild-type control mice, assuming power = 0.9 and $\alpha = 0.05$.

Tissue Fixation and Histology

Femurs and tibias were decalcified and fixed for 72 hours at room temperature in Cal-Ex II (Fisher Scientific, #CS511-1D). Spleens were fixed for 72 hours at room temperature in 4% formaldehyde (Fisher Scientific,

#BP531-500). All tissue processing and staining was performed by the UCSD Moores Cancer Center Tissue Technology Shared Resource. Multiple sections per paraffin block were stained with hematoxylin and eosin (H&E), reticulin silver stain for reticulin fiber deposition, and trichrome stain for collagen deposition. For grading myelofibrosis in mouse bone marrow sections a scale was established based on both criteria established in human MPN patients and in previous mouse models of JAK2(V617F)-driven MPN, using experimentally unrelated wild-type mice as negative controls:

0 – near absence of reticulin fibers, no more reticulin fibers than non-experimental wild-type C57BL/6 mice

1 – low/medium density of reticulin fibers throughout sections without a high-degree of interconnectedness between fibers or only showing intense staining and interconnectedness in focal areas

2 – high density and staining intensity of reticulin fibers throughout sections with consistently high degree of interconnectedness

3 – high density and staining intensity of reticulin fibers throughout sections with consistently high degree of interconnectedness and significant collagen deposition as detected by trichome stain

Vectors

JAK2 (V617F)-pcw107-v5 was a gift from David Sabatini and Kris Wood (Addgene plasmid # 64610). To generate the MSCV-JAK2(V617F)-IRES-GFP vector used in the current study a JAK2(V617F) PCR fragment with added SalI sites was digested and subsequently ligated into XhoI-digested MSCV-IRES-GFP vector. pcDNA3-Flag-TRAF6 was a gift from Michael Karin (Addgene Plasmid # 66929). pJ3M-MST1 (Addgene Plasmid # 12203) and pJ3M-MST1-K59R (Addgene Plasmid # 12204) were gifts from Jonathan Chernoff. MST1 and MST1-K59R were subcloned into MSCV-Ires-Puro$_R$ (MIP) vector backbones for use in experiments described here. pcDNA3-HA-Ubiquitin (HA-Ub) was generated by sub-cloning processed wild-type ubiquitin cDNA into the pCDNA3.1-HA vector backbone. pNF-κB_Luc plasmid (Agilent Technologies) consists of 4X NF-κB responsive elements upstream of a minimal TATA promoter and firefly luciferase. pRL-TK *Renilla* luciferase control reporter vector was purchased from Promega. Vector cDNA sequences were confirmed by Sanger sequencing (Eton Bioscience, San Diego, CA). pLKO.1-based lentiviral shRNA vectors are from the RNAi Consortium (TRC), clones used in this manuscript are SHC002 (shCTRL), TRCN0000001623 (shSTK4_1), and TRCN0000001624 (shSTK4_2).

Viral Transduction

For retroviral transduction of JAK2-V617F: Transfections of HEK293T cells were conducted by combining 5 µg of MSCV-IRES-GFP or MSCV-JAK2(V617F)-IRES-GFP retroviral expression vector, 5 µg of ecotropic packaging vector (plK6.1-MCV.ecopac.UTd), and 40 µl of polyethylenimine (PEI) in 1 mL of Opti-MEM reduced serum medium (Gibco, #31985-070). Approximately 16 hours post-transfection, media was aspirated, cells were washed once in PBS, and 6 mL fresh IMDM (Gibco, #12440-053) supplemented with 10% fetal bovine serum was added to each plate. 24 hours following media change, IMDM media containing retroviral particles was collected, passed through a 0.45 µm syringe filter, pooled, and supplemented with recombinant cytokines mIL-3 (10 ng/mL), hIL-6 (10 ng/mL), and mSCF (20 ng/mL), and polybrene (4 µg/mL). For retroviral transduction, HSC-enriched bone-marrow mononuclear cells harvested from mice were resuspended in this supplemented retroviral supernatant at densities of 2-3 x 10^6 cells/mL and centrifuged (2,000x *g*) in 6-well plates at 32°C for 3 hours (Allegra X-12R centrifuge, Beckman Coulter); followed by overnight culture at 37°C. Two consecutive retroviral transductions were performed in this manner on subsequent days. Transduction efficiency was measured (GFP+ frequency) by flow cytometry the morning following the second centrifugation (immediately prior to transplantation into recipient mice). Low retroviral titers were utilized, which were typically measured at ~1.5-2% GFP+ following two rounds of transduction.

For lentiviral transduction of shRNA constructs: Transfections of HEK293T cells were conducted by combining 5 µg of psPAX2, 2.5 µg of pMD2.G, and 3 µg of respective pLKO.1-based shRNA vector, and 44 µl of polyethylenimine (PEI) in 1 mL of Opti-MEM reduced serum medium (Gibco, #31985-070). Approximately 16 hours post-transfection, media was aspirated, cells were washed once in PBS, and 10 mL fresh RPMI supplemented with 10% fetal bovine serum was added to each plate. 24 hours following media change, RPMI media containing lentiviral particles was collected, passed through a 0.45 µm syringe filter, pooled, and supplemented with polybrene (final working concentration: 4 µg/mL). For lentiviral transduction, THP-1 cells were resuspended in this supplemented retroviral supernatant at densities of ~ 0.5 x 10^6 cells/mL and centrifuged (2,000x *g*) in 6-well plates at 32°C for 3 hours (Allegra X-12R centrifuge, Beckman Coulter); followed by overnight culture at 37°C. Two consecutive transductions were performed in this manner on subsequent days. 24 hours following second transduction cells were resuspended in 1.0 µg/mL puromycin,

and selected for 72 hours, at which point viable drug-resistant cells were enriched via FICOLL gradient. Post-selection cells were maintained in growth media supplemented with 0.5 µg/mL puromycin.

Western Blotting and Co-Immunoprecipitation

Samples were lysed in ice-cold NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitor (Roche, #11873580001) and phosphatase inhibitor (Roche, #4906845001). Lysates were cleared by centrifugation at 11,000 xg for 10 minutes at 4°C, and denatured in 2x loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue). For co-immunoprecipitation experiments approximately 500 µg lysate was combined with 40 µl of anti-Flag M2 agarose beads (Sigma, #M8823) and incubated at 4°C with rotating overnight. The following morning beads were washed 4x with ice-cold NP-40 lysis buffer via centrifugation at 11,000 xg. Washed beads were then resuspended in 60 µl 2x loading buffer and denatured prior to loading. Immunoblotting was performed using the Li-Cor Odyssey infrared imaging instrument. Post-acquisition image analysis and cropping was performed using Li-Cor Image Studio Lite (V 5.2.5) software.

For TRAF6 auto-ubiqutin chain formation co-immunoprecipitation experiments, transfections of HEK293T cells are performed in 10 cm plates using pcDNA3-empty or pcDNA3-Flag-TRAF6 (2.5 µg), MIP-empty or MIP-MST1 or MIP-MST1-K59R (5 µg), pcDNA3-HA-Ub (6 µg), and 55 µl polyethylenimine (PEI). Approximately 16 hours post-transfection, media was aspirated, cells were washed once in PBS, and 10 mL fresh media was added. 48 hours following initial transfection cells are harvested and lysed for subsequent western blotting and co-immunoprecipitation experiments as described above.

Data Availability

Plasmids, resources, and data generated in the current study are provided as Supplemental Tables, or will be made available upon request to the corresponding investigator. The published datasets used for analysis during the current study are available in the NCBI GEO repository.

Supplemental References

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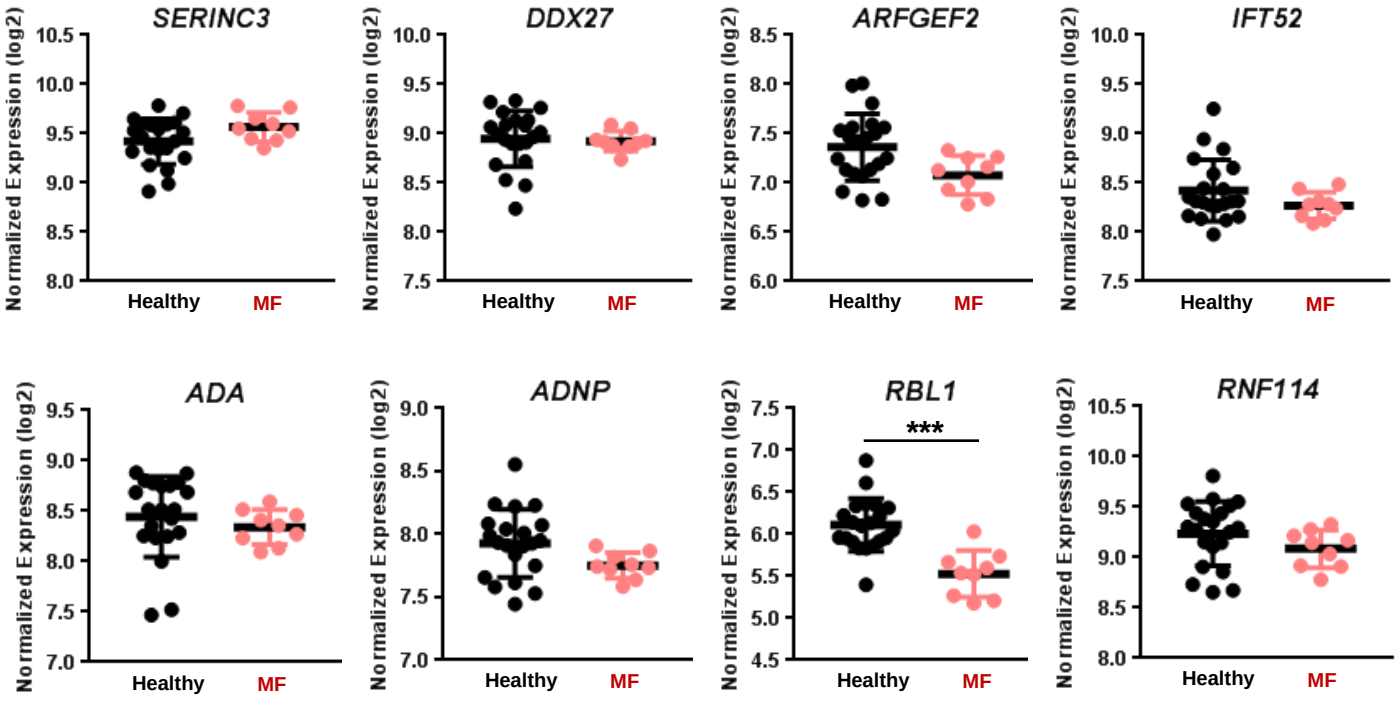
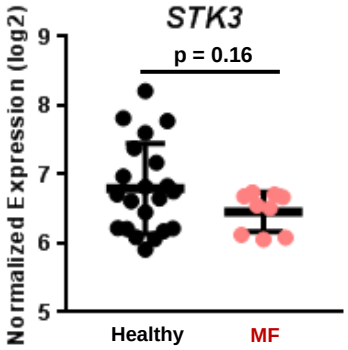


Supplemental Figure 1. Assessment of 20q gene expression in myelofibrosis patients. 20q gene expression measured by microarray in total peripheral blood from healthy controls (black) and MPN patients with Primary Myelofibrosis (MF, red). Data are from GSE26049. Statistical significance is determined by two-tailed student's t-test, *** $p < 0.001$.

A



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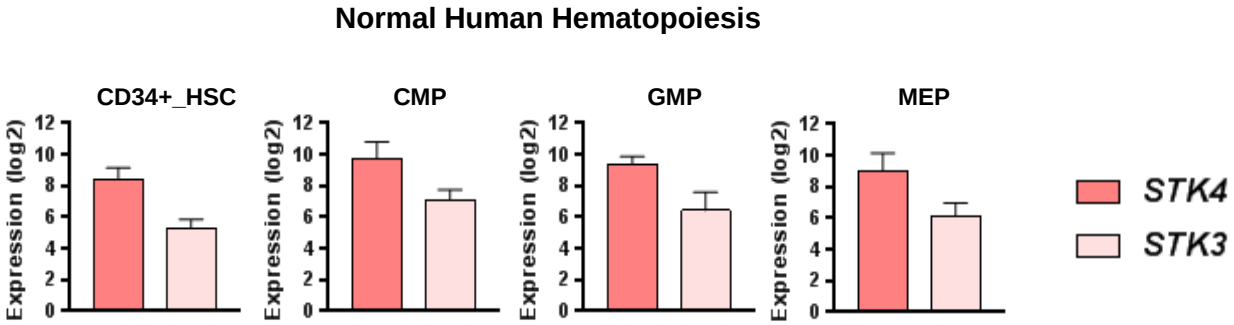
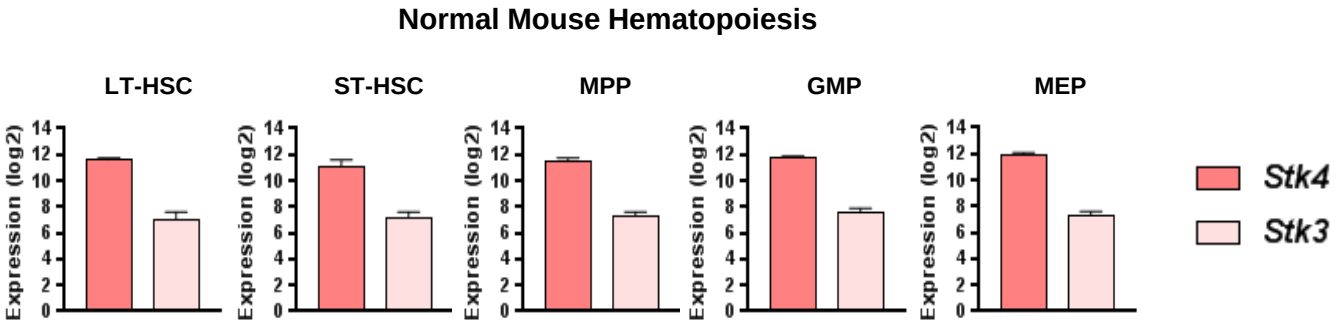


C

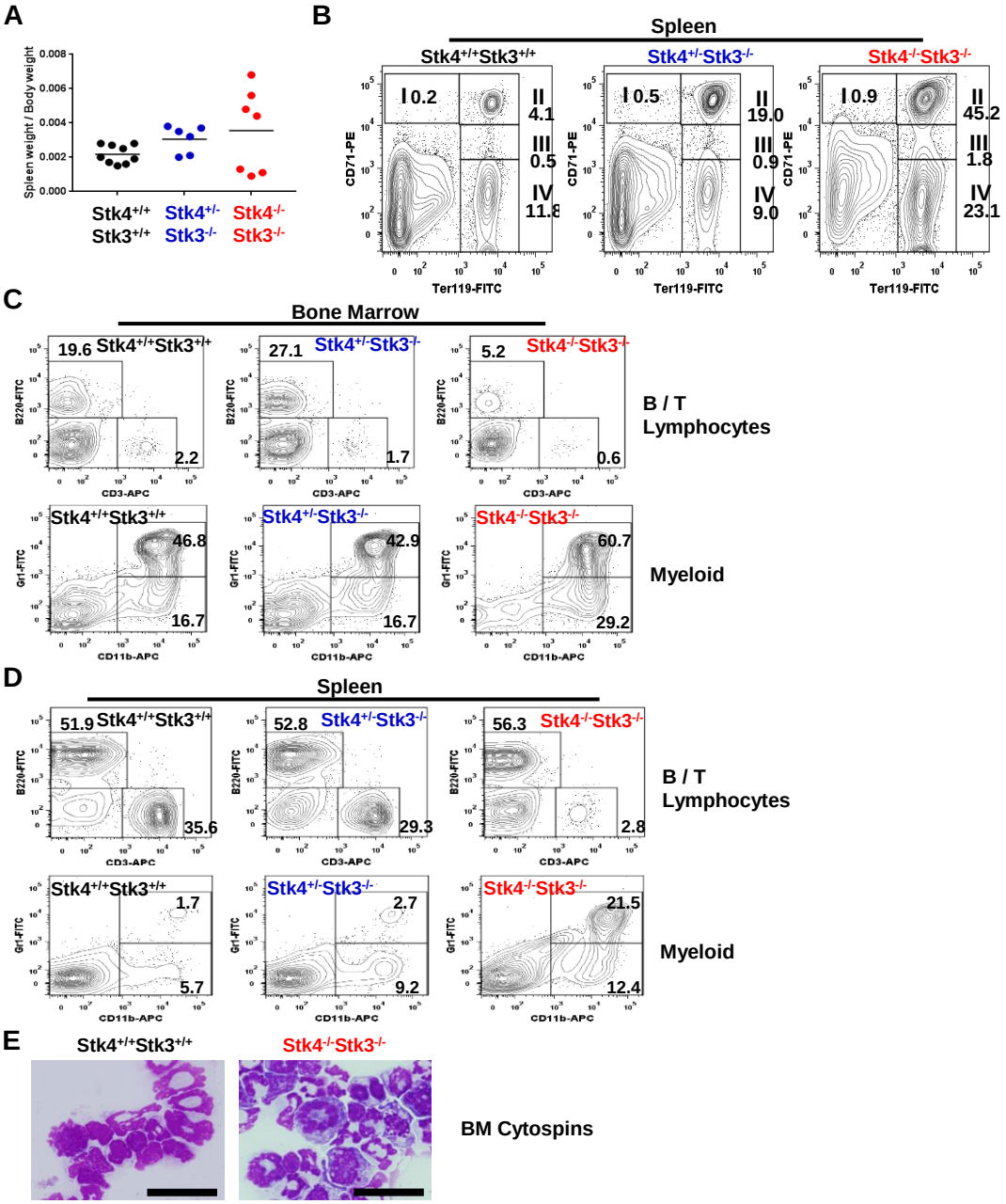


Supplemental Figure 2. Assessment of *Stk4* and *Stk3* expression in normal and malignant hematopoiesis.

A) *STK3* gene expression measured by microarray in total peripheral blood from healthy controls (black) and MPN patients with Primary Myelofibrosis (MF, red). Data are from GSE26049.

B) *STK4* and *STK3* gene expression measured by microarray in indicated human hematopoietic stem and progenitor cell populations. HSC, hematopoietic stem cell, CMP, common myeloid progenitor, GMP, granulocyte-macrophage progenitor, MEP, megakaryocyte-erythroid progenitor. Data are from GSE24759

C) *Stk4* and *Stk3* gene expression measured by microarray in indicated murine hematopoietic stem and progenitor cell populations. LT-HSC, long-term hematopoietic stem cell, ST-HSC, short-term hematopoietic stem cell, MPP, multi-potent progenitor, GMP, granulocyte-macrophage progenitor, MEP, megakaryocyte-erythroid progenitor. Data are from GSE14833.



Supplemental Figure 3. Frequent extramedullary erythropoiesis and myelo-granulocytic skewing in Stk4$^{-/-}$Stk3$^{-/-}$ mice.

Genotypes analyzed for this figure include: *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Vav1-Cre^{+} (Stk4$^{-/-}$Stk3$^{-/-}$), *Stk4*$^{f/+}$*Stk3*$^{f/f}$; Vav1-Cre^{+} (Stk4$^{+/-}$Stk3$^{-/-}$), *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Vav1-Cre^{-} or *Stk4*$^{f/+}$*Stk3*$^{f/f}$;Vav1-Cre^{-} (Stk4$^{+/+}$Stk3$^{+/+}$). Unless otherwise indicated, all data are derived from mice of 6 - 9 weeks in age.

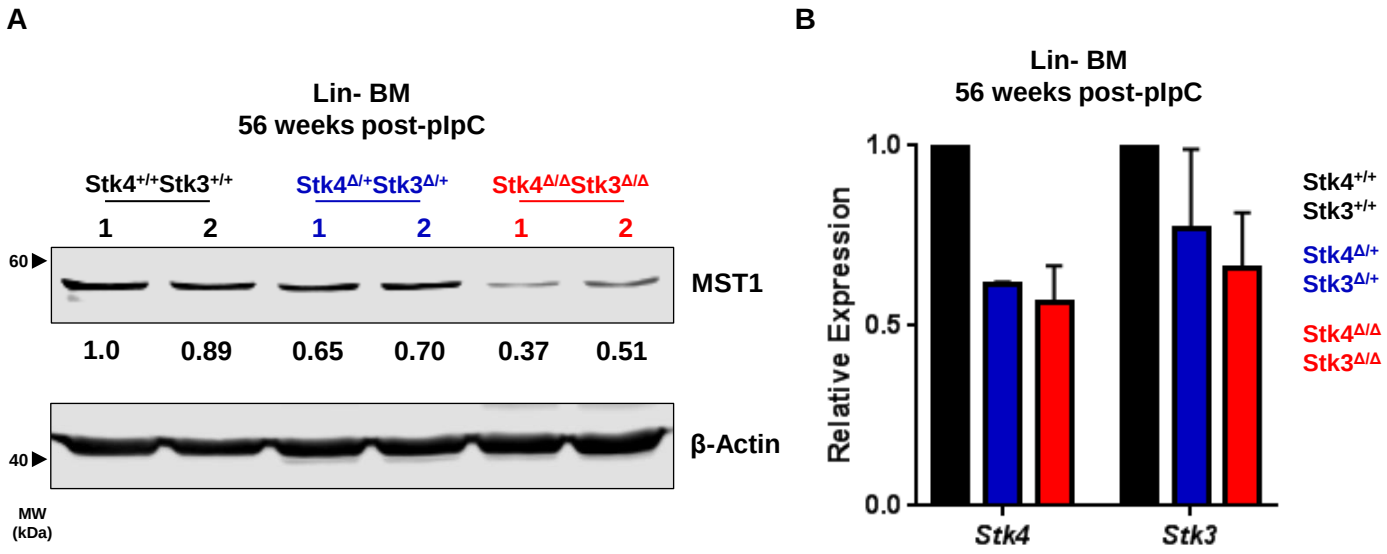
A) Measurement of spleen weight (grams) divided by total body weight (grams) for mice of indicated genotypes. Line indicates mean and individual data points represent individual mice.

B) Representative erythroid progenitor (CD71/Ter-119) flow cytometry staining within total splenocytes from mice of the indicated genotypes. The four stages of erythroblast development (I-IV) are indicated.

C) Representative lymphoid (CD3/B220, top) and myeloid (CD11b/Gr-1, bottom) flow cytometry plots of mononuclear bone marrow cell populations for mice of indicated genotypes.

D) Representative lymphoid (CD3/B220, top) and myeloid (CD11b/Gr-1, bottom) flow cytometry plots of mononuclear spleen populations for mice of indicated genotypes.

E) Representative bone marrow cytospins of mice of the indicated genotypes. Scale bar = 50 μM.

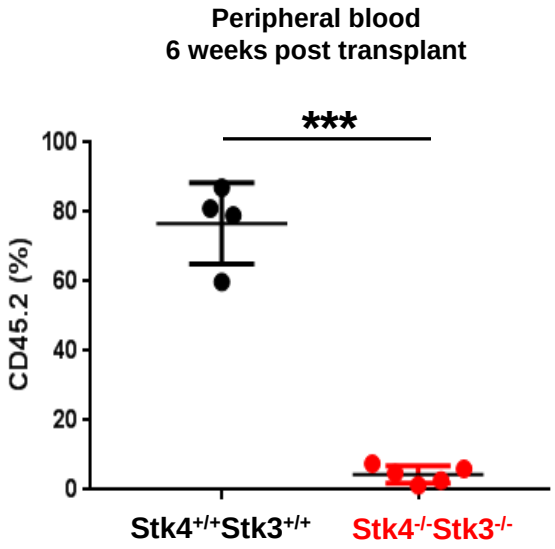


Supplemental Figure 4. Selection pressure towards non-excised alleles upon aging in Mx1-Cre model.

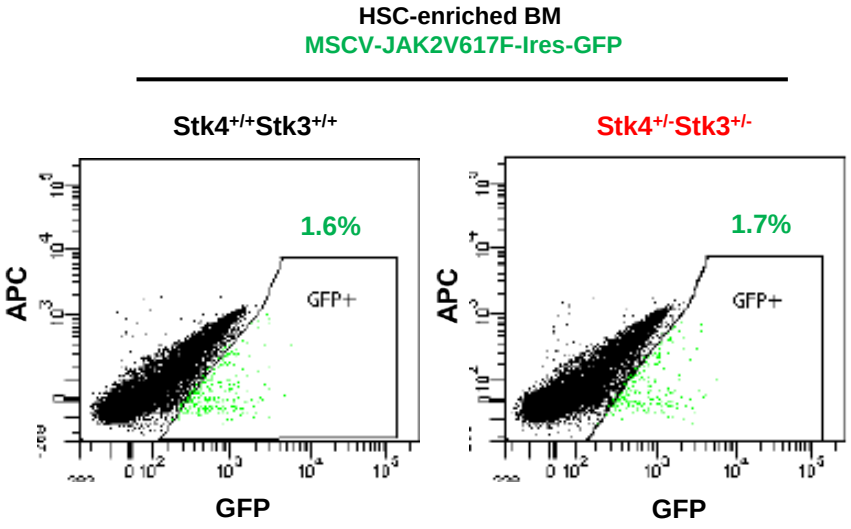
Mice used in this Figure are of the following genotypes: *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Mx1-Cre^{+} (Stk4$^{\Delta/\Delta}$Stk3$^{\Delta/\Delta}$), *Stk4*$^{f/+}$*Stk3*$^{f/+}$;Mx1-Cre^{+} (Stk4$^{\Delta/+}$Stk3$^{\Delta/+}$), and *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Mx1-Cre^{-} (Stk4$^{+/+}$Stk3$^{+/+}$)

A) Western blot (from two mice per genotype) depicting indicated proteins in murine hematopoietic progenitor (Lin-) bone marrow cells, 56 weeks post-treatment with pIpC to induce gene deletion.

B) RT-qPCR analysis showing relative expression for indicated genes (from two mice per genotype) in murine hematopoietic progenitor (Lin-) bone marrow cells, 56 weeks post-treatment with pIpC to induce gene deletion.

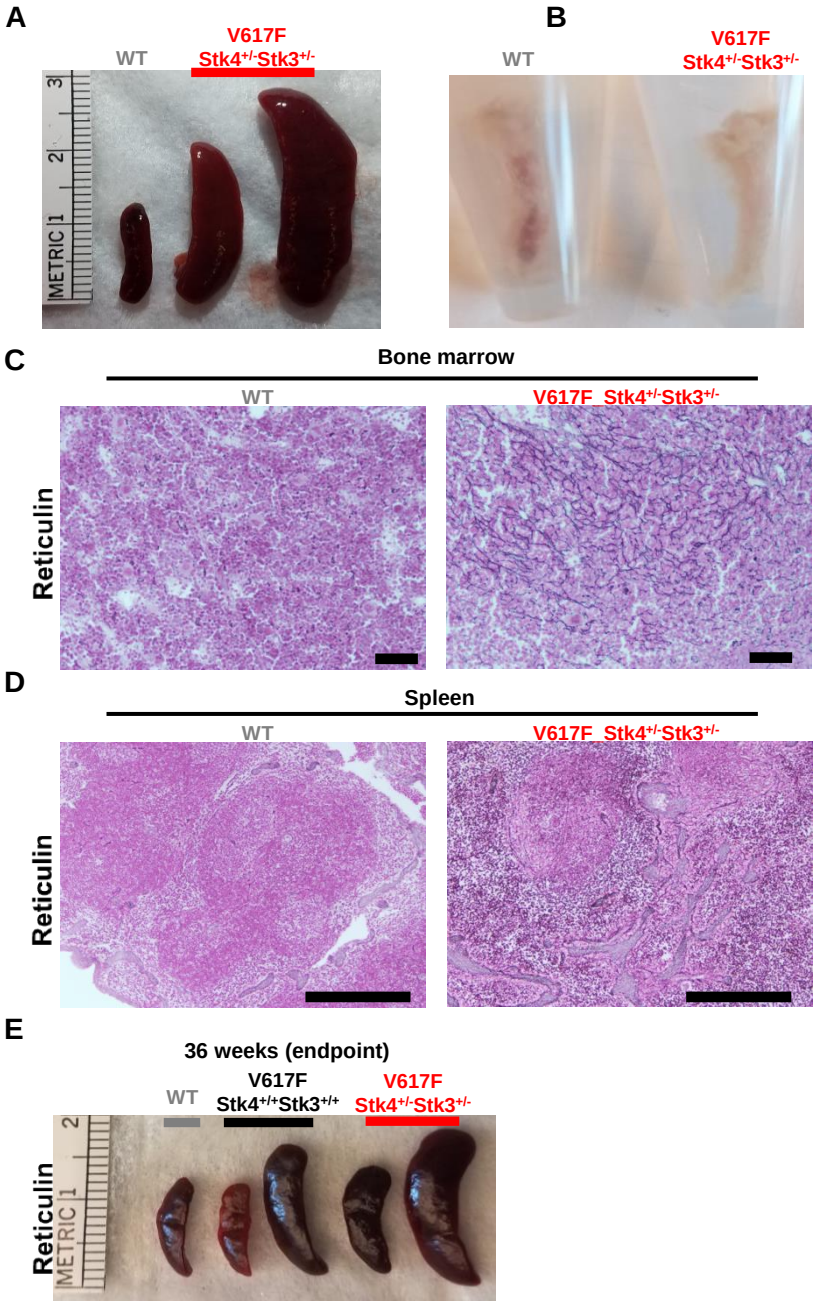


Supplemental Figure 5. Hippo kinase-deficient hematopoietic cells fail to properly engraft in bone marrow.
Genotypes analyzed for this figure include: *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Vav1-Cre^{+} (Stk4$^{-/-}$Stk3$^{-/-}$) and *Stk4*$^{f/f}$*Stk3*$^{f/f}$;Vav1-Cre^{-} or *Stk4*$^{f/+}$*Stk3*$^{f/f}$;Vav1-Cre^{-} (Stk4$^{+/+}$Stk3$^{+/+}$). CD45.2 donor-derived frequencies in the peripheral blood of lethally irradiated CD45.1 recipient mice, 6 weeks following non-competitive transplantation of bone marrow-derived cells are shown. Statistical significance is determined using student's t-test, *** = $p < 0.001$.



Supplemental Figure 6. Equal retroviral transduction efficiency in JAK2-V617F MPN model.

Flow cytometry plot, gated on viable cells (PI-), measuring GFP+ percentages in transduced bone marrow cells from 5-FU treated mice approximately 24 hours post-transduction.



Supplemental Figure 7. Heterozygous Hippo kinase inactivation cooperates with JAK2-V617F to promote myelofibrosis in mice.

Genotypes used in this model are: $Stk4^{f/+}Stk3^{f/+}$;$Vav1\text{-}Cre^{+}$ ($Stk4^{+/-}Stk3^{+/-}$) and $Stk4^{f/+}Stk3^{f/+}$; $Vav1\text{-}Cre^{-}$ ($Stk4^{+/+}Stk3^{+/+}$), with or without transduction with JAK2-V617F (V617F)

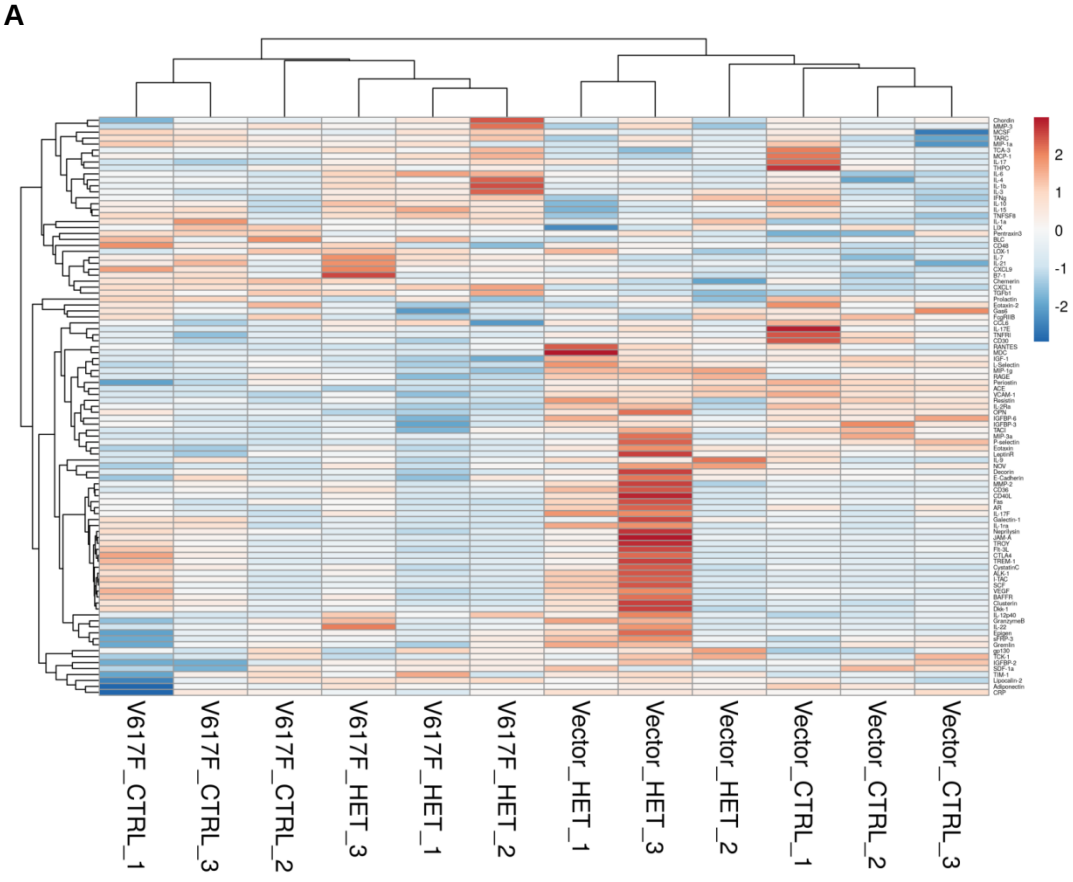
A) Representative splenomegaly from mice that were euthanized due to progression to MF (right side) are compared to a representative wild-type control spleen (left side). Ruler is shown for scale.

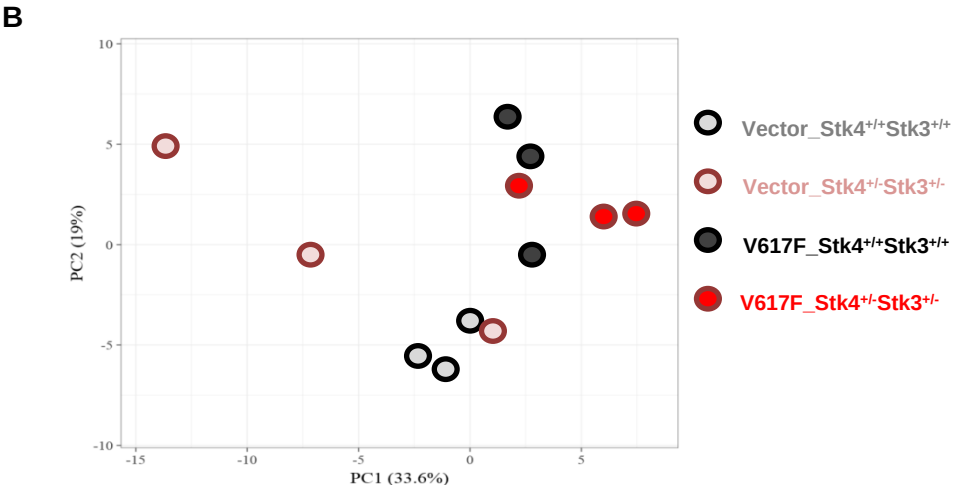
B) Image shows representative femur (inside Eppendorf tube) from a wild-type control (left) and moribund V617F-$Stk4^{+/-}Stk3^{+/-}$ (right) mouse demonstrating visible anemia in the bone marrow.

C) Reticulin-stained bone marrow section from wild-type (left) or moribund V617F-$Stk4^{+/-}Stk3^{+/-}$ mouse (right) with post-Polycythemia vera myelofibrosis. Scale bar = 100 μM.

D) Reticulin-stained spleen section from wild-type (left) or moribund V617F-$Stk4^{+/-}Stk3^{+/-}$ mouse (right) with post-Polycythemia vera myelofibrosis. Scale bar = 500 μM.

E) Representative spleens from mice of the indicated groups and genotypes at the experimental endpoint of 36 weeks. Ruler is shown for scale.

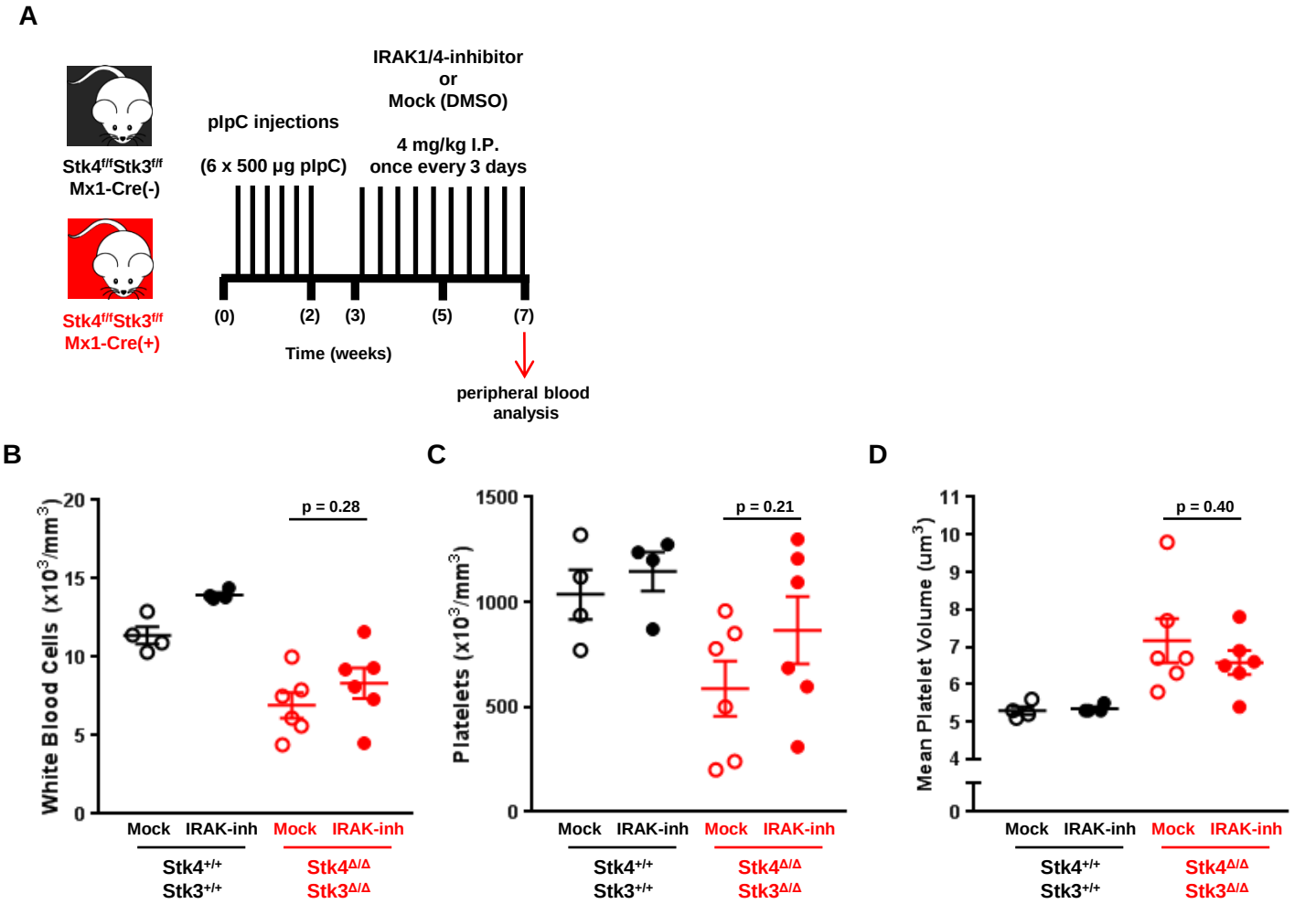




Supplemental Figure 8. JAK2-V617F expression has a greater effect on serum cytokine variance than does genotype.

A) Hierarchical clustering analysis of serum protein abundance in three mice per experimental group is performed for both columns (individual mice) and rows (individual serum proteins) by using correlation distance and average linkage. Unit variance scaling is applied for row normalization.

B) Principal component analysis (PCA) of serum protein abundance in three mice per experimental group is performed by singular value decomposition (SVD) with imputation method. Unit variance scaling is applied for row normalization. Principal component 1 (PC1) and principal component 2 (PC2) explain 33.6% and 19% of total variance, respectively. Both panels are generated using ClustVis (https://biit.cs.ut.ee/clustvis/).



Supplemental Figure 9. IRAK1 inhibition does not rescue all hematopoietic defects associated with Hippo kinase inactivation.

A) Experimental schematic depicting strategy for generation inducible gene inactivation with pIpC followed by IRAK1/4-inhibitor treatment, 4 mg/kg by intraperitoneal (I.P.) injection once every three days for four weeks. N = 4 ($Stk4^{+/+}Stk3^{+/+}$, mock treated), 4 ($Stk4^{+/+}Stk3^{+/+}$, IRAK1/4-inhibitor treated), N = 6 ($Stk4^{\Delta/\Delta}Stk3^{\Delta/\Delta}$, mock treated), N = 6 ($Stk4^{\Delta/\Delta}Stk3^{\Delta/\Delta}$, IRAK1/4-inhibitor treated).

B) Peripheral white blood cell counts for mice of the indicated genotypes, following four weeks of mock (5% DMSO) or IRAK1/4-inhibitor treatment, as described in (B). Statistical significance between mock and IRAK-inh treated groups in $Stk4^{\Delta/\Delta}Stk3^{\Delta/\Delta}$ genotype is determined by two-tailed student's t-test.

C) As (B), but for platelet counts.

D) As (B), but for mean platelet volume (MPV).