Supplemental methods

Mice Maintenance

All mice (CD45.2) were backcrossed over ten generations to the C57BL/6 background. Littermates or age- and gender-matched mutant and control mice at the age of six weeks received intraperitoneal injections of poly (I:C) (GE Healthcare Life Sciences) at a dose of 0.5μg/gram body mass every other day for six times. Experiments were conducted at two weeks after poly (I:C) injections.

Sick mice examination

Disease development was followed up to 600 days since the born date. Complete blood cell counts were analyzed in moribund mice at the time of sacrifice and in surviving mice at 600 days. Bone marrow (BM) and spleen cells were stained for myeloid ($Mac1^+Gr1^+$), B ($B220^+$) and T ($CD3^+$) (Biolegend) lineages, and analyzed by flow cytometry. Tissue sections of formalin-fixed solid organs were prepared for H&E staining by the Unit for Laboratory Animal Medicine at the University of Michigan.

Semi-quantitative PCR

Semi-quantitative PCR was performed as previously described.[1] In brief, genomic DNA was purified using a Qiagen AllPrep DNA/RNA Mini Kit. A total of 200ng of DNA was used as a template. Reduce PCR cycle to 25 cycles to amplify both WT and mutant *Nras* allele.

Bone marrow transplantation

For the BM competitive repopulation assay, BM cells from donor mutant and control mice (CD45.2) were flushed out of one femur and tibia and filtered through a 70μm

strainer into FACS buffer (HBSS buffer containing 2% fetal calf serum). $0.5x10^6$ donor BM cells were transplanted along with $1.5x10^6$ competing BM cells (CD45.1) into lethally irradiated (2x550 Rad) CD45.1 recipient mice by retro-orbital injections. Serial transplantation was conducted by transplanting $3x10^6$ BM cells from primary recipients into lethally irradiated secondary recipients (CD45.1). In HSC and MPP transplantation, 15 FACS-sorted donor-HSCs ($CD150^+CD48^-LSK$) or MPPs ($CD150^-CD48^-LSK$) were transplanted along with $3x10^5$ competing BM cells (CD45.1) into lethally irradiated recipient mice (CD45.1). Donor engraftment was monitored for 20 weeks after transplantation with blood staining. At 20 weeks after transplantation, mice were sacrificed and BM cells were stained with a combination of antibodies for HSCs and MPPs as described previously.[2] Donor and recipient cells were detected by incubating with directly conjugated antibodies against CD45.2 and CD45.1 (Biolegend).

***In vivo* BrdU incorporation assay**

BrdU (Sigma) was administered as a single dose of 200mg per kg body mass by intraperitoneal injection followed by 1 mg/ml BrdU in the drinking water. At 24 hours after the injection, BM cells were incubated first with the anti-c-Kit antibody conjugated to biotin (Biolegend). After washing, cells were incubated with anti-biotin conjugated to paramagnetic microbeads (Miltenyi Biotec). The microbead bound ($c\text{-}Kit^+$) cells were then enriched using LS columns (Miltenyi Biotec). Enriched cells were then incubated with antibodies to lineage (Lin) markers including Gr-1, CD2, CD3, CD5, CD8, B220 and Ter119 that were conjugated to FITC, anti-CD150 conjugated to PE, anti-Sca1 conjugated to PerCP/Cy5.5, anti-CD48 conjugated to PE/Cy7 (Biolegend) and streptavidin conjugated to Alexa Fluor 700 (Invitrogen). BrdU incorporation was measured in HSCs by flow cytometry using the APC BrdU Flow Kit (Fisher Scientific).

H2B-GFP label retention assay

Doxycycline was added to the water at a concentration of 2g/L along with 1% sucrose (Fisher Scientific). After 6 weeks, doxycycline water was removed and mice were chased for 6 or 18 weeks. BM cells were stained with antibodies to lineage markers that were conjugated to PE, anti-CD150 conjugated to PE/Cy7, anti-Sca1 conjugated to PerCP/Cy5.5, anti-CD48 conjugated to Alexa Fluor 700 and anti-cKit conjugated to APC (Biolegend). After washing, cells were incubated with anti-APC conjugated to paramagnetic microbeads (Miltenyi Biotec). The microbead bound (c-Kit^{+}) cells were then enriched using LS columns (Miltenyi Biotec). GFP signal was analyzed in HSCs, MPPs and LSKs by flow cytometry.

RNA-seq

~10,000 SLAM (CD150^{+}CD48^{-}LSKs) HSCs were FACS-sorted from 10-week old *Nras*$^{G12D/+}$ (n=3), *Tet2*$^{+/-}$ (n=3), *Nras*$^{G12D/+}$;*Tet2*$^{+/-}$ (n=3) and control WT (n=3) littermates directly into TRIzol (Ambion). RNA was isolated with the AllPrep-DNA/RNA micro Kit (Qiagen). Ribosomal RNA (rRNA) and mitochondrial RNA (mtRNA) sequences were then removed with the RiboGone-Mammalian Kit (Takara). RNA-seq libraries were prepared using the SMARTer Stranded RNA-seq Kits (Takara) according to the manufacturer's instructions. Six separate samples were multiplexed into each lane and sequenced on an Illumina HiSeq 2500 with a paired-end sequencing length of 200bp. The quality of reads obtained was evaluated using FastQC (University of Michigan Bioinformatic core). The sequenced libraries were aligned to the mouse genome (mm10) using TopHat2.[3] The gene annotation files include RefSeq (NCBI). Differential expression was performed with edgeR.[4] Genes with an absolute fold change greater than 1.5 and a FDR <0.1 were reported. GSEA analysis was performed using GSEA

v.2.2.0 software.[5] RNA-seq data have been deposited in the Gene Expression Omnibus, accession number GSE97640.

Quantitative real-time RT-PCR

Total RNA was purified using a Qiagen AllPrep Mini Kit. Up to 2μg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (AppliedBiosystems). Quantitative real-time PCR reactions were carried out using SYBR Green MasterMix on an Applied Biosciences qPCR cycler. Relative expression was determined by the Δ/ΔCT method and normalized to the internal control beta-actin.

***In vitro* HSC liquid culture**

100 HSCs were sorted into the SF-O3 medium (Iwai North America Inc.) supplemented with 1% bovine serum albumin (BSA) (MP Biomedicals), stem cell factor (SCF) (Peprotech) and thrombopoietin (TPO) (Peprotech) at different doses, and cultured *in vitro* at 37°C for 10 days. 500 HSCs were sorted into the SF-O3 medium supplemented with 1% BSA and TPO (20ng/ml), and cultured *in vitro* at 37°C for 6 days. Total viable cells were enumerated in PBS with trypan blue staining.

Flow cytometry analysis of phospho-STAT5

BM cells were starved in IMDM containing 1%BSA at 37°C for 30 minutes. Cells were then stimulated with TPO at a concentration of 6.25ng/ml. Cells were then fixed with paraformaldehyde at a final concentration of 2% (Electron Microscopy Sciences) at room temperature for 10 minutes. Wash cells in PBS twice. Cells were then incubated with anti-Sca1 conjugated to FITC (BD Biosciences) for 30 minutes at room temperature. Cells were permeabilized in ice-cold 95% methanol (Electron Microscopy Sciences) while vortexing, and then stored at -20°C overnight. The next day, cells were washed 3

times with PBS and incubated in FACS buffer for 2 hours at 4°C. Cells were then pelleted and incubated with anti-phospho STAT5 antibody conjugated to Alexa Fluor 647 (BD Biosciences) and lineage markers conjugated to PE (Biolegend) for 1 hour at room temperature. Samples were washed in FACS buffer and analyzed on an LSRFortessa cell analyzer. Data were collected using DIVA software and analyzed using Flowjo.

References

1. Xu J, Haigis KM, Firestone AJ, et al. Dominant role of oncogene dosage and absence of tumor suppressor activity in Nras-driven hematopoietic transformation. *Cancer Discov*. 2013;3(9):993-1001.

2. Li Q, Bohin N, Wen T, et al. Oncogenic Nras has bimodal effects on stem cells that sustainably increase competitiveness. *Nature*. 2013;504(7478):143-147.

3. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol*. 2013;14(4):R36.

4. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.

5. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550.

Supplemental Table 1. Summary of immuno-staining analysis

	Bone marrow			Spleen		
Genotype	$Mac1^+Gr1^+$ (%)	$B220^+$ (%)	$CD3^+$ (%)	$Mac1^+Gr1^+$ (%)	$B220^+$ (%)	$CD3^+$ (%)
WT	46.7±7.5	18.7±7.9	2.7±1.3	3.4±0.6	59.7±2.9	23.9±2.1
$Tet2^{+/-}$	44.3±4.3	10.2±3.4	5.9±4.8	4.6±1.2	44±21.4	20.3±3.2
$Nras^{G12D/+}$	57.8±6.7*	4.5±4.5**	3.4±2.4	20.7±11.7*	23.5±18.3**	14.9±8.9
$Nras^{G12D/+}$; $Tet2^{+/-}$	58.5±8.4*	4.8±2.3**	3.3±1.4	18.2±14.5	28.2±16.5**	14.4±7.4*

*$p<0.05$ **$p<0.01$ (mutant versus control)

Supplemental Table 2. Summary of peripheral blood analysis

Genotype	WBC (K/ul)	MO (%)	HB (g/dL)	PLT (K/ul)	RBC (M/ul)	Spleen (g)	Liver (g)
WT	7.5±5.3	1.9±1.4	14±1.6	1527.5±186.6	10.03±0.54	0.17±0.09	1.58±0.12
$Tet2^{+/-}$	9.2±5.3	4.7±1*	12.6±0.3	1770.6±847.4	8.0±0.42*	0.38±0.54	2.12±0.77
$Nras^{G12D/+}$	40.7±31.7*	6.4±3.6*	11±3.4	848.3±560.5*	7.6±2.33	0.76±0.69	2.73±1.79
$Nras^{G12D/+}$; $Tet2^{+/-}$	17.6±4.68*	4±3	13.5±4.1	1557±28.6	9.81±2.3	0.87±0.78*	2.16±0.95

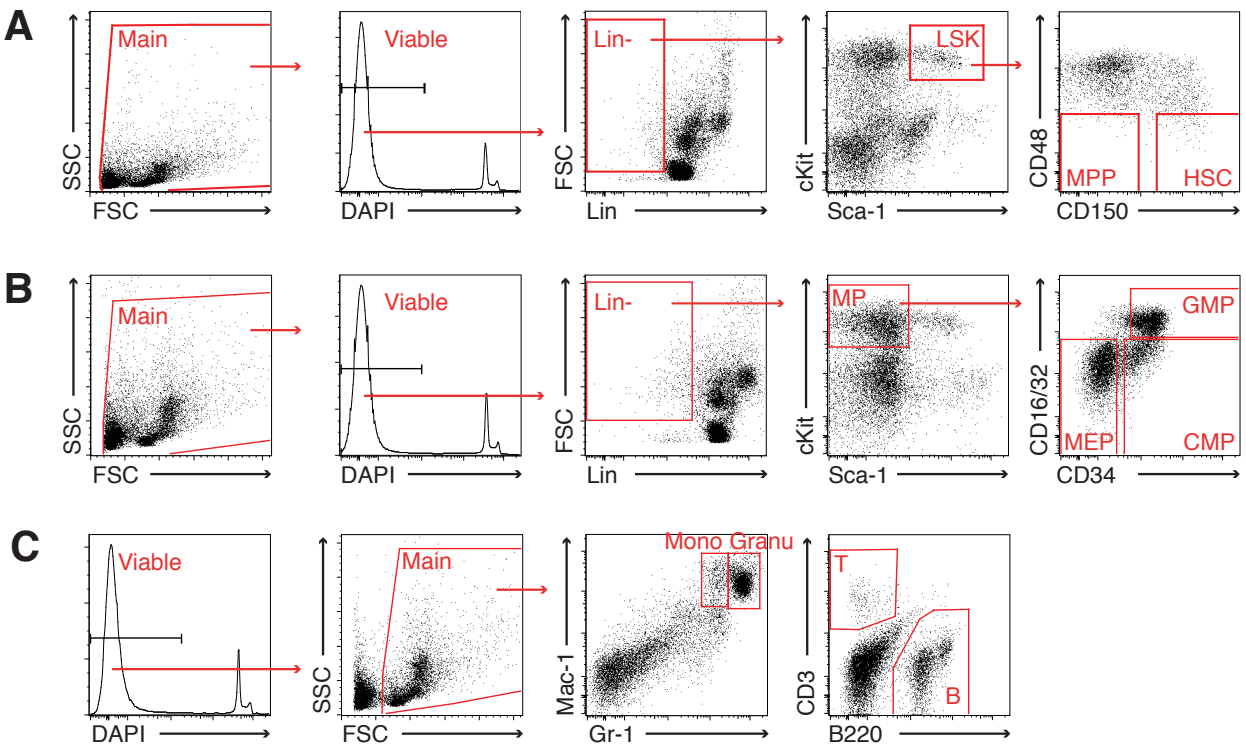
*$p<0.05$ (mutant versus control)

Supplemental Table 3. Summary of recipient mice transplanted with splenocytes from primary diseased $Nras^{G12D/+}$;$Tet2^{+/-}$ mice

Donor cells (#/recipient)	Recipients (#)	Diseased animals (%)	Disease diagnosis	WBC (K/ul)	MO (%)	HB (g/dL)	PLT (K/ul)	Spleen (g)	Liver (g)
Splenocytes ($3x10^6$)	3	100	CMML	5.8±2.1	11.5±2.4	6.4±1.7	105±44.7	1.1±0.5	1.2±0.4
			[a]Reference range	8.9 (4.45 -13.96)	4.61 (2.18 -11.02)	14.2 (10.8 -19.2)	1347 (841 -2159)		

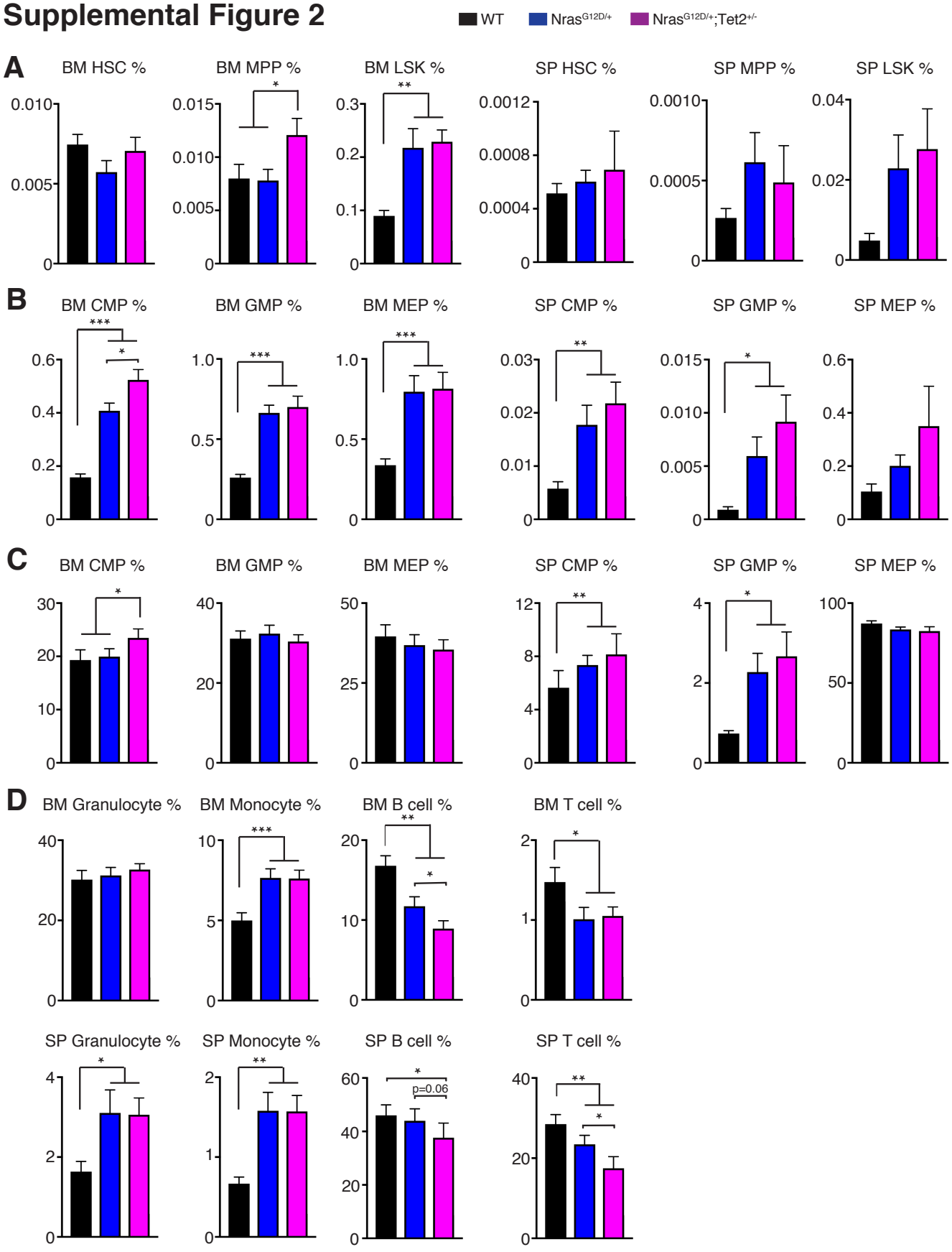
[a]Standard C57BL6 mouse hematology from Charles River Laboratories:
Mean value (standard range) collected from >120 male mice at the age of 8-10 weeks
www.criver.com

Supplemental Figure 1



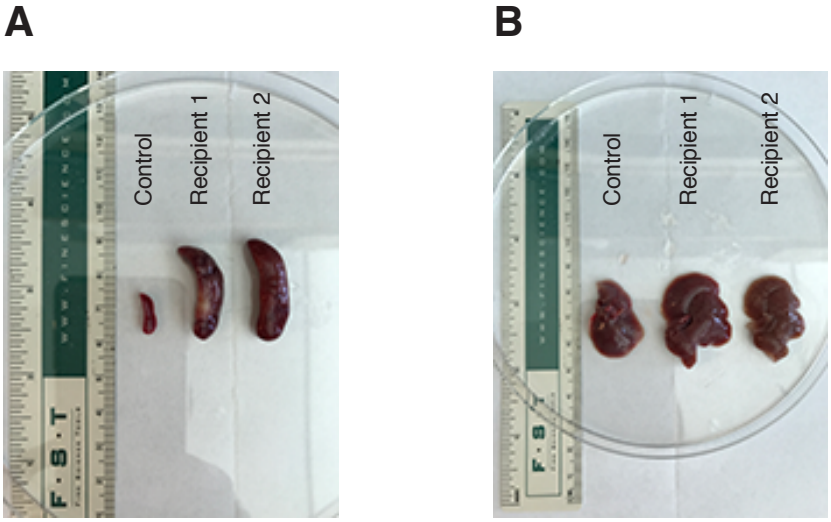
Supplemental figure 1. Representative flow cytometry staining and gating for Lin^{-}Sca-1^{+}c-Kit^{+} cells (LSKs), hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs) (**A**); common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) (**B**); Monocytes, Granulocytes, T cells and B cells (**C**).

Supplemental Figure 2



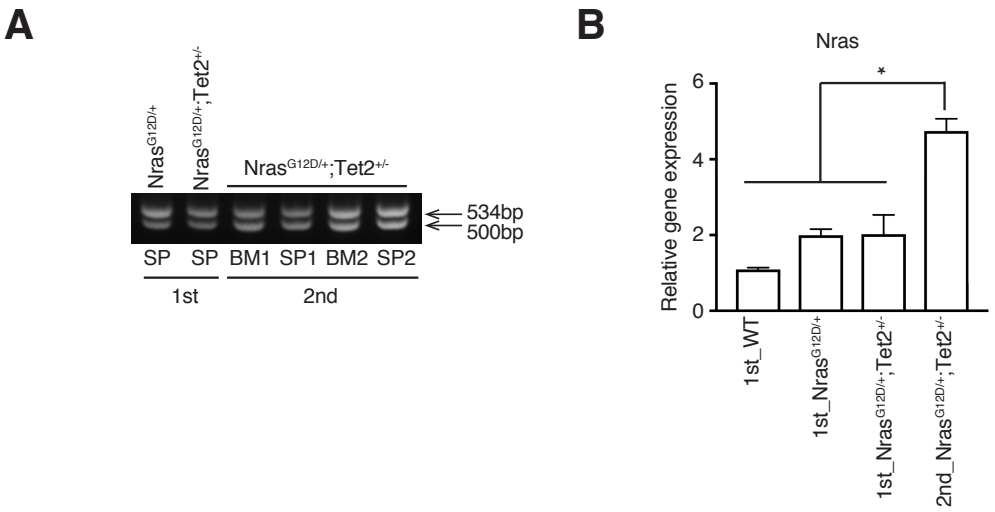
Supplemental figure 2. Frequency of hematopoietic populations in bone marrow and spleen of 6 month-old mice. (A) Frequency of HSCs, MPPs and LSKs relative to total viable cells in the bone marrow and spleen. **(B)** Frequency of CMPs, GMPs and MEPs relative to total viable cells in the bone marrow and spleen. **(C)** Frequency of CMPs, GMPs and MEPs relative to myeloid progenitors (MP, $Lin^{-}Sca1^{-}cKit^{+}$) in the bone marrow and spleen. **(D)** Frequency of mature granulocytes, monocytes, B cells and T cells relative to total viable cells in the bone marrow and spleen. Data represent mean ± SEM. Two-tailed Student's t-tests were used to assess statistical significance. *p≤0.05, **p≤0.01, ***p≤0.001.

Supplemental Figure 3



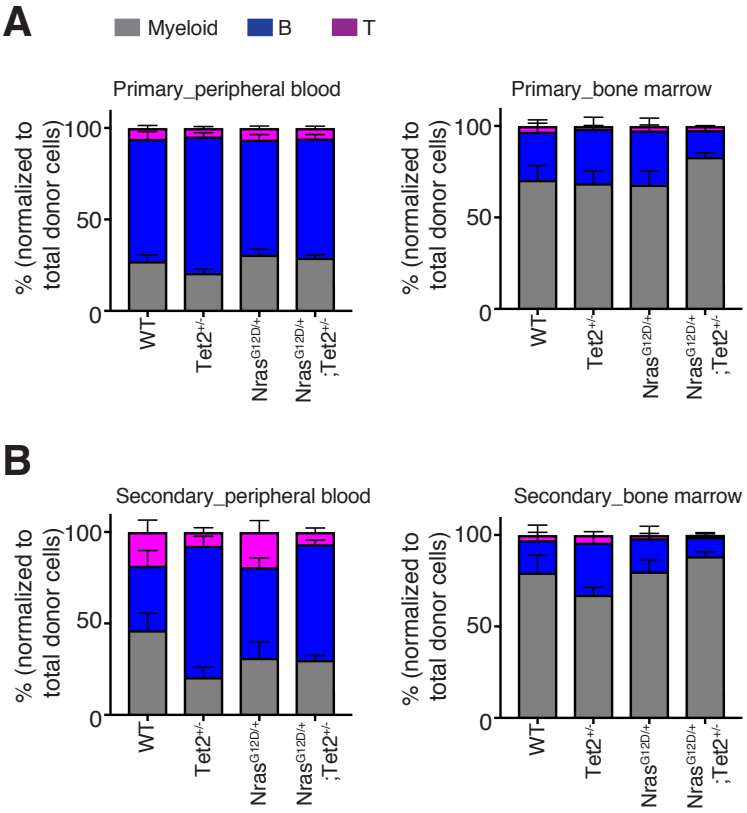
Supplemental figure 3. Representative pictures of spleen **(A)** and liver **(B)** from diseased secondary recipients transplanted with spleen cells from sick primary CMML mice (*Nras*$^{G12D/+}$;*Tet2*$^{+/-}$).

Supplemental Figure 4



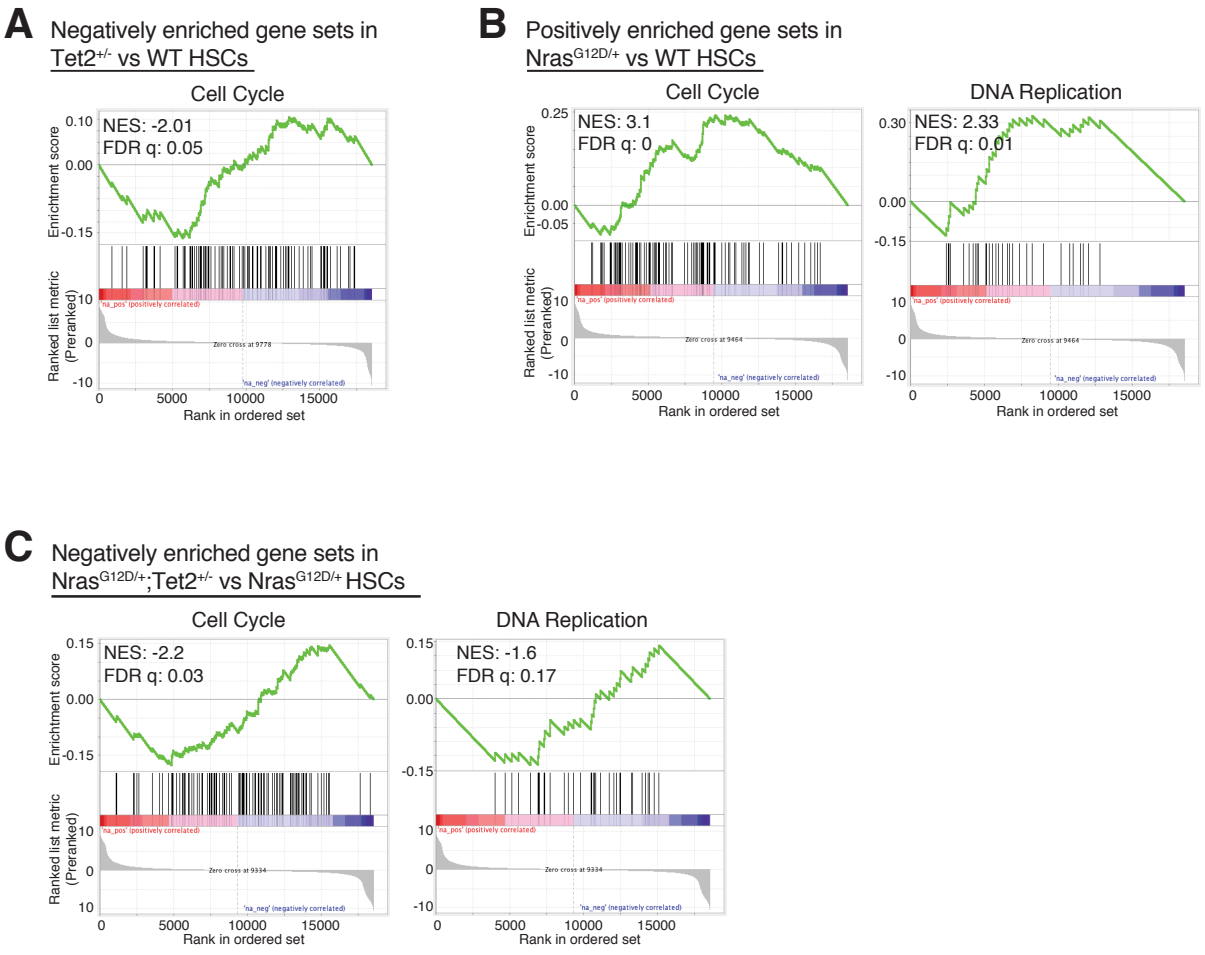
Supplemental figure 4. Dosage analysis of wild type and mutant *Nras* in CMML mice. (A) Semi-quantitative PCR to determine the dose of wild type and recombined mutant *LSL-Nras^{G12D}* alleles (WT allele: 500bp; mutant allele: 534bp) in bone marrow and spleen from secondary CMML recipients. Analysis of spleen DNA from primary mice with CMML (*Nras$^{G12D/+}$* and *Nras$^{G12D/+}$;Tet2$^{+/-}$*) was also shown. **(B)** Quantitative real-time RT-PCR of *Nras* mRNAs in spleen from primary and secondary CMML mice (primary WT is from the 600 day-old cohort). Data represent mean ± SEM (n=3). Two-tailed Student's t-tests were used to assess statistical significance. *p≤0.05, **p≤0.01, ***p≤0.001.

Supplemental Figure 5



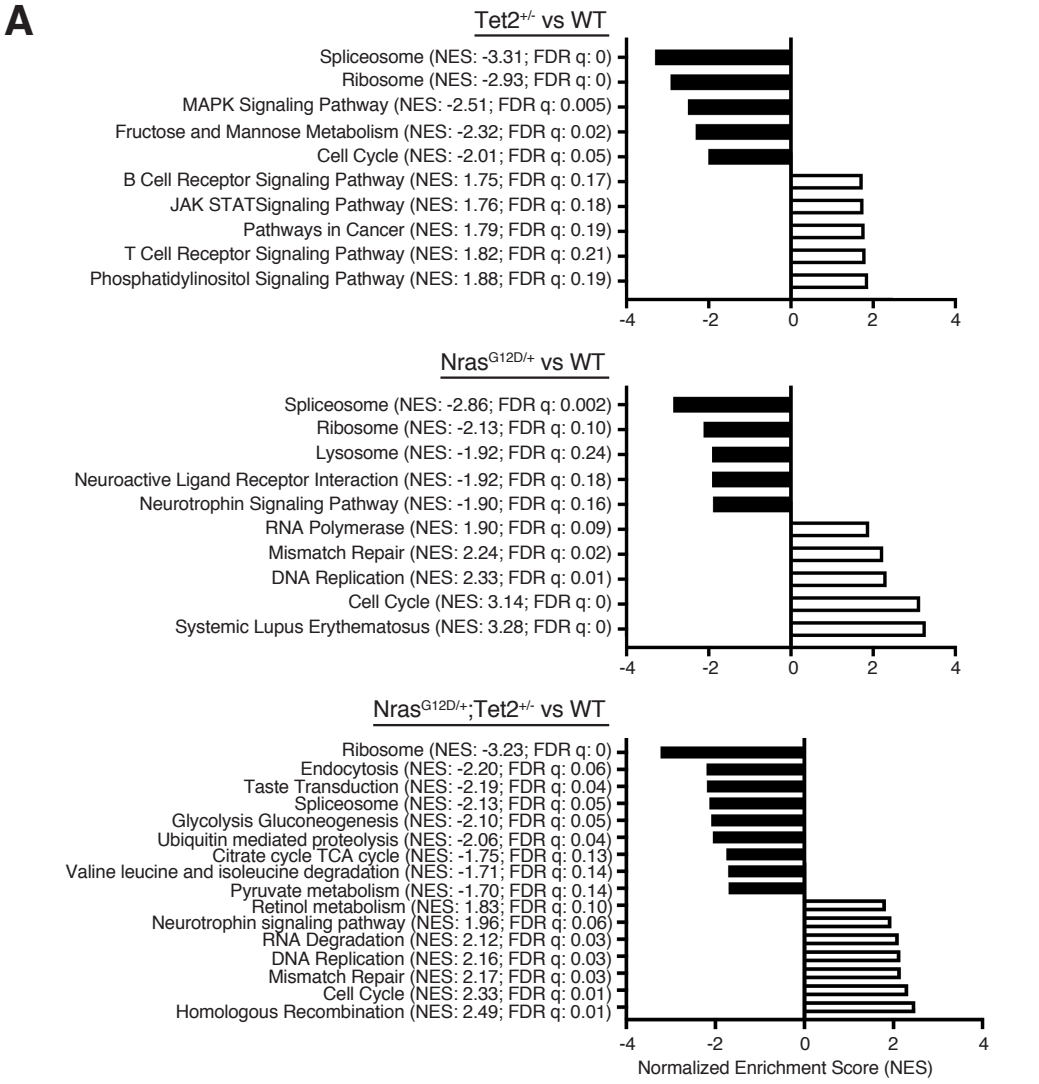
Supplemental figure 5. Relative frequency of myeloid, B and T cells within total donor cells (after removal of red blood cells) in peripheral blood and bone marrow from primary **(A)** and secondary **(B)** transplant recipients. Data represent mean ± SEM. Two-tailed Student's t-tests were used to assess statistical significance. *p≤0.05, **p≤0.01, ***p≤0.001.

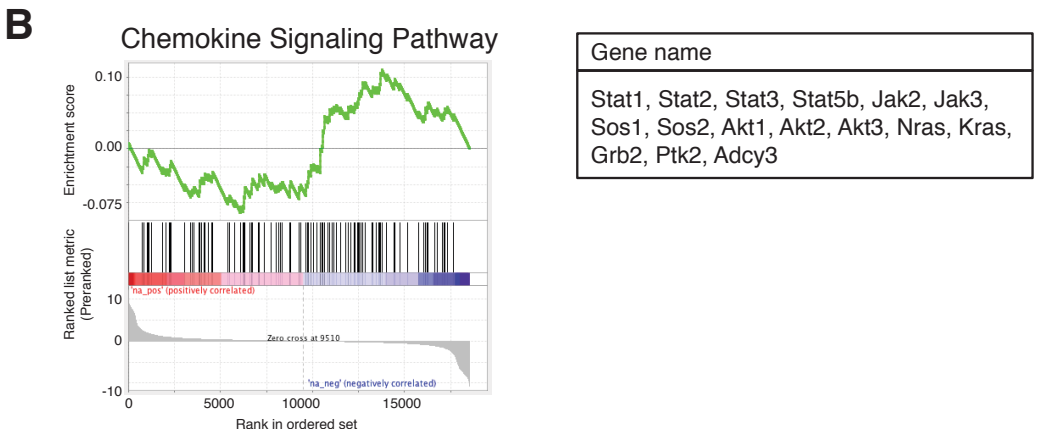
Supplemental Figure 6



Supplemental figure 6. Tet2 haploinsufficiency downregulates cell proliferation-related pathways in HSCs. Enrichment plots from GSEA showing regulation of cell proliferation-related gene sets in *Tet2*$^{+/-}$ vs *WT* HSCs **(A)**; *Nras*$^{G12D/+}$ vs *WT* HSCs **(B)** and *Nras*$^{G12D/+}$*;Tet2*$^{+/-}$ vs *Nras*$^{G12D/+}$ HSCs **(C)**. NES, normalized enrichment score. n=3 per genotype. FDR q<0.25, p<0.05.

Supplemental Figure 7





Supplemental figure 7. Gene set enrichment analysis (GSEA) based on RNA-seq analysis. (A) Top-ranked gene sets positively or negatively enriched in mutant versus control HSCs. FDR q<0.25, p<0.05. n=3 each genotype. **(B)** Representative pathway upregulated in *Nras$^{G12D/+}$;Tet2$^{+/-}$* vs *WT* HSCs, with enriched genes involved in JAK/STAT signaling regulation.