Supplemental Data

Myb drives of B-cell neoplasms and myeloid malignancies in vivo

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Supplemental Methods

Mouse Experiments

R26-Myb mice were generated according to previously described optimized *Rosa26* targeting strategy.[1] To induce hematopoietic or B-cell specific expression, *R26-Myb* mice were crossed to *Vav-iCre* (JAX 008610)[2] mice. A cohort of 21 Myb^{Vav} mice (consisting of 9 males and 12 females) and 14 littermate control mice was established using asynchronously aged mice that originated from two trio breedings. Not all mice were monitored until 80 w and were removed prematurely from the cohort (Figure 1B), including Cre- littermates that were used as controls for flow cytometry analysis of moribund *R26-Myb* mice. This might have resulted in an unconscious bias in the selection of animals to be removed. We considered mice with hematological malignancies to contain at least one of the following criteria: splenomegaly, lymphadenopathy (of mandibular, axillary, mesenteric or inguinal lymph nodes), and liver or bone marrow infiltration. Blood analysis was performed using the Hemavet 950 (Drew scientific group). All experiments on mice were conducted according to institutional, national, and European animal regulations. All *in vivo* experiments were approved by the ethical committee for animal experimentation of the Faculty of Medicine and Health Sciences of Ghent University. Genotyping primers are listed in supplemental Table 1.

Targeting vector assembly

A RMCE-compatible targeting vector, *pRMCE-DV3-Myb*, was constructed by a stepwise multisite Gateway LR reaction, similar as described before[1]. First, three Entry vectors (*pENTR-L4R1 floxed stop*, *pENTR221-Myb*, pEntry 3' IRES-eGFP/Luc[3]) were combined overnight followed by a second overnight reaction together with the *pRMCE-DV3* vector[1] (available at genecorner; LMBP 8189). Several colonies were obtained after transformation of 5 µl of the LR reaction mixture into DH5a bacteria that were subsequently plated onto ampicillin-containing bacterial plates. To create the *pENTR211-Myb* vectors, the ORF of *Myb* (BC011513) was amplified from the *pCMV-SPORT6-Myb* vector (Transomics, Mammalian Gene Collection) with AttB-containing primers listed in Supplemental Table 1, followed by a Gateway BP reaction with the

AttB-flanked PCR fragment and pDONR221 vector (Thermofisher). Five out of five *pRMCE-DV3-Myb* clones were confirmed by restriction enzyme digests and sequencing analysis.

mESC culture, RMCE targeting and validation.

A targeting vector that contained a floxed stop cassette followed by the *Myb* gene and a *EGFP/luciferase* reporter, which was subsequently targeted in mESCs using recombinase-mediated cassette exchange (RMCE). G4 ROSALUC mESCs[4] were cultured on gelatinized recipients containing mouse embryonic fibroblasts (MEFs, TgN (DR4)1 Jae strain) treated with mitomycin C (Sigma-Aldrich, St. Louis, MO), as was previously described[5,6]. For the trap-coupled RMCE experiments, 50% confluent G4 ROSALUC mESCs [4] were cotransfected with the *pRMCE-DV3-Myb* vector and a FlpE-expressing plasmid (pCAGGS-FlpE-IRES-puromycin-pA)[7] in a 1:1 ratio using Lipofectamine 2000 reagent (Thermofisher). G418 selection (200 µg/ml) was started 48 h after transfection. After 7 to 10 days, individual G418-resistant RMCE-targeted *R26-Myb* mESC colonies were observed and were further expanded. Eight colonies were picked and validated by PCR using primers ROSA26 F and Ins R (listed in supplemental Table 1). Correct integration was confirmed by PCR for seven out of eight expanded *R26-Myb* mESC clones (87,5% efficiency, supplemental Figure 1B) and confirmed that mESC targeting via RMCE is very efficient[4-6]. *In vitro* Cre-mediated excision of the floxed stop cassette was performed for two independent *R26-Myb* mESC clones (clone 2 and 3) by electroporation with 5 µg pCAG-NLS-Cre-IRES-Puro-pA. After 24 h, cells were subjected to puromycin selection (1.25 µg/ml; Sigma) for 4 days. For each mESC line, 12 puromycin-resistant colonies were picked, expanded and tested for Luciferase positivity. Based on Luciferase activity, the efficiency of in vitro Cre excision was 92% (11/12) for clone 2 and 75% (9/12) for clone 3.

Diploid embryo aggregation and mice

The generation of chimeras by diploid embryo aggregation was reported previously .[1,5] Briefly, zona-pellucida free E2.5 Swiss embryos were aggregated with clumps (7

to 10 cells) of targeted *RMCE-DV3-Myb* mESCs using depression wells. Aggregates were cultured overnight in microdrops of KSOM with amino acids under mineral oil at 37°C in 95% air and 5% CO_2. The next day, blastocysts were transferred into the uteri of 2.5-dpc pseudopregnant B6CBAF1 females previously mated with vasectomized males. Two correctly ROSA26-targeted mESC clones, clone 2 and 3, were subsequently aggregated with diploid E2.5 Swiss embryos and gave rise to 4 and 9 high-grade chimeric animals, respectively. Chimeras were identified at birth by the presence of black eyes and later by agouti coat pigmentation. After germline transmission, the *R26-Myb* mouse line (derived from clone 2) was established and was backcrossed to three times to C57BL/6 before being crossed to *Vav-iCre* mice.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% [vol/ vol] NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with an additional 1% Triton X-100 plus phosphatase and protease inhibitors. 60 µg protein lysate (in Laemmli buffer) was separated by SDS-PAGE on 10% polyacrylamide gels and blotted onto nitrocellulose transfer membranes (PerkinElmer) that were incubated with antibodies for specific protein detection: anti-Myb (clone D2R4Y, Cell Signaling Technology) and vinculin (clone V9131, Sigma-Aldrich) was used as a loading control with mouse HRP-coupled anti-actin mAb (sc-47778; Santa Cruz Biotechnology). Incubation with primary antibodies was followed by incubation with HRP-coupled secondary antibodies and detection using enhanced chemiluminescence (PerkinElmer Life Sciences). Developed blots were quantified with ImageJ software.

Luciferase assay

To measure luciferase activity, cells were lyzed in Galacto-Star Lysis buffer (Tropix), incubated with ONE-Glo substrate (Promega), and measured on a GloMax 96 Microplate Luminometer (Promega).

qRT-PCR

Total RNA was isolated using RNeasy Plus Mini Kit (QIAGEN). cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche) with oligo(dT) primer starting from equal amounts of RNA as measured by a NanoDrop spectrophotometer (Thermo Scientific). qRT-PCR was performed using the SensiFast SYBR No-Rox Kit (Bioline) and monitored on a LightCycler 480 system (Roche). Gene expression was standardized against reference genes and analyzed using qbase+ (Biogazelle). All primers are listed in supplemental Table 2.

Flow cytometry

For flow cytometry, 5 × 10^6 cells were stained at 4°C in the dark with antibodies (supplemental Table 3). Cells were sorted on FACSAria II (BD) and FACSAria Fusion (BD). Data were acquired on a cell analyzer (LSRFortessa; BD) and analyzed using FlowJo software (Tree Star).

Histology and immunohistochemistry

Tumors were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. Tissue sections were deparaffinized and rehydrated. For histology, slides were stained with hematoxylin and eosin (H&E). For immunohistochemistry, antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer (pH 6.0) in an electric pressure cooker, after which the slides were permeabilized with 0.05% Tween 20 in PBS. Blocking of endogenous peroxidase occurred in 3% H_2O_2 in methanol. Sections were then treated with 1% goat serum/1% BSA in PBS, followed by incubation with primary anti-KI67 (Cell Signaling) or primary anti-Myb (Abcam; clone EP769Y) overnight at 4°C. Biotin-conjugated secondary antibodies (Dako) were detected by the avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA), and developed with diaminobenzidine (Dako).

Transplantation and bioluminescence measurements.

Immunocompromised nonobese diabetic/severe combined immunodeficient γ (NSG) mice were injected at 6-10 weeks of age in the tail vein with 150 µL phosphate-buffered

saline containing $5x10^6$ Myb^{Vav} tumor cells. At regular time points, the bioluminescence was measured using the IVIS Lumina II imaging system (PerkinElmer). Before imaging, the mice were injected intraperitoneally (IP) with 200 µL of a 15 mg/mL *Firefly* d-luciferin potassium salt solution and anesthetized by inhalation of 5% isoflurane. The NSG mice were imaged 10 minutes after luciferin injection. The total bioluminescence signal in each mouse was calculated via the region of interest tool (total counts) in the Living Image software (PerkinElmer).

***Ex vivo* MYBMIM or TG3 treatment**

Frozen leukemia cells (150.000 cells/well; viability after thawing was 50-60%) were cultured in RPMI 1640 with 10% FCS and supplemented with increasing concentrations of either MYBMIM or TG3 and cell viability was checked after 48h through ATP measurement by means of a CellTiter-Glo viability assay (Promega).

Statistical analysis

GraphPad Prism 7.0 (La Jolla, CA) was used for statistical analyses. The Log-rank (Mantel-Cox) test was used to compare mouse groups. An unpaired t test was used to analyze differences between genetic or phenotypic subgroups. p-values less than 0.05 were considered statistically significant. All error bars represent the standard error of the mean (SEM).

References

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3. Haenebalcke L, Goossens S, Dierickx P, et al. The ROSA26-iPSC mouse: a conditional, inducible, and exchangeable resource for studying cellular (De)differentiation. *Cell Rep*. 2013;3(2):335-341.
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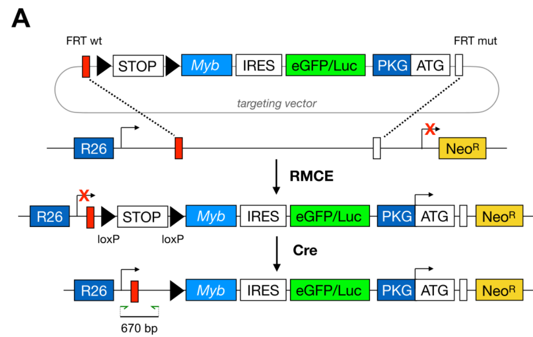
Supplemental Figures

A



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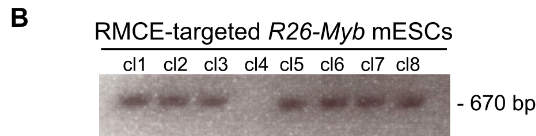

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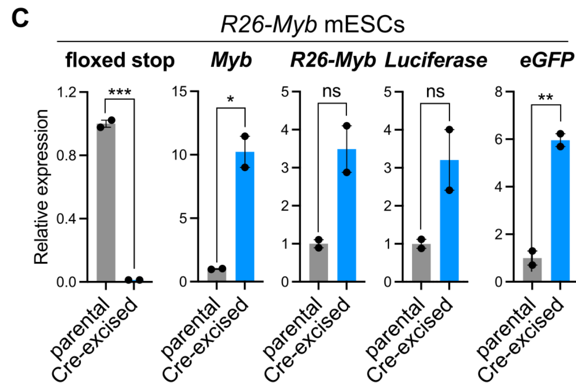

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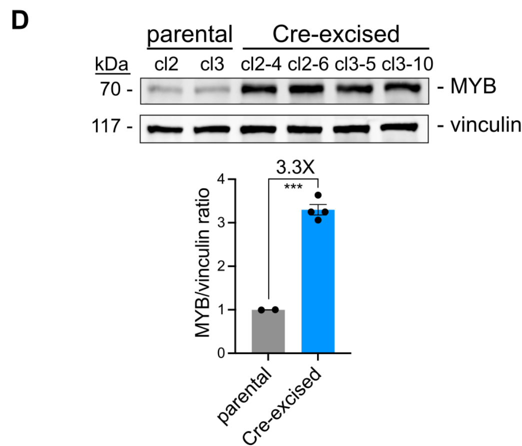

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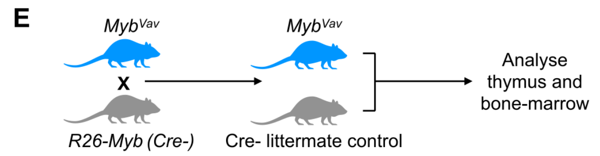

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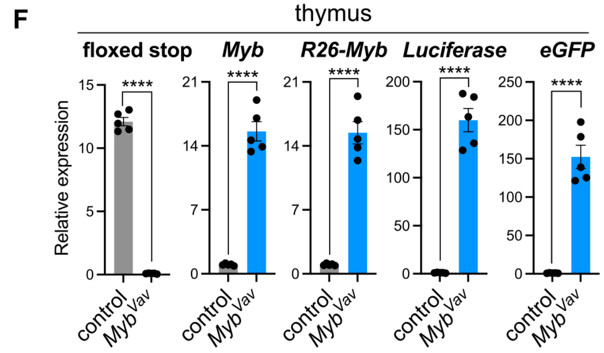

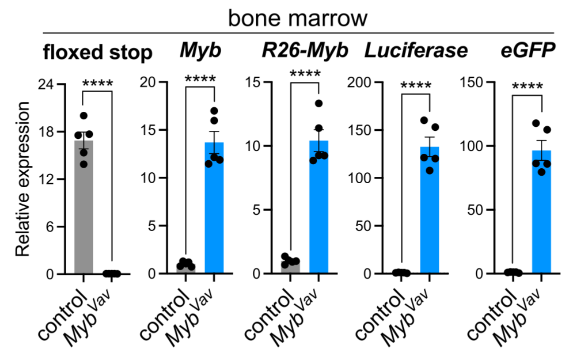

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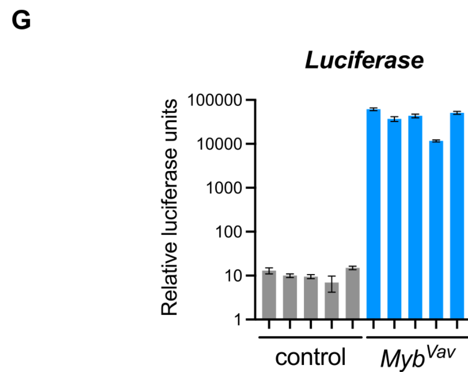
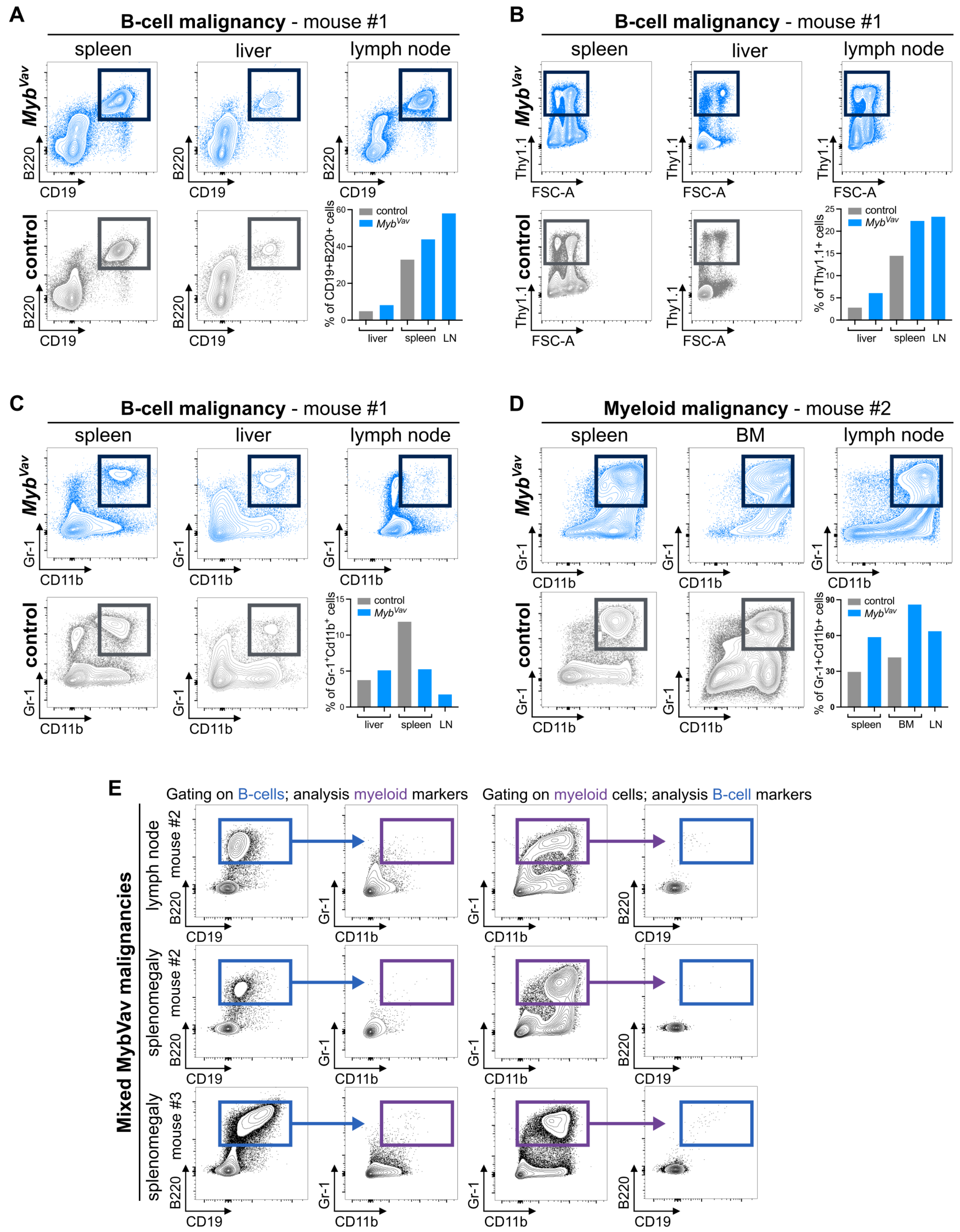


Supplemental Figure 1. Targeting and validation of *R26-Myb* mESCs and mice. (A) Schematic representation of targeting of a conditional Myb overexpression construct to the *Rosa26* locus via recombination-mediated cassette exchange (RMCE) in mouse embryonic stem cells. Upon *Cre*-mediated recombination, the floxed stop cassette is removed and a bicistronic transgene transcript, encoding for Myb and the *eGFP/Luciferase* reporter, is expressed from the *Rosa26* promoter. Green arrows represent a forward primer in the endogenous *Rosa26* locus and a reverse primer in the transgene cassette. The resulting PCR fragment is 670 bp. R26: *Rosa26*. IRES: independent ribosomal entry site. eGFP: enhanced green fluorescent protein. PKG: phosphoglycerate kinase 1. Neo^R: neomycin resistance gene. (B) PCR-based validation of targeted *RMCE-DV3-Myb* mouse embryonic stem cells (mESCs) using primers that are shown in panel A. (C) qRT-PCR analysis for the floxed stop cassette, total and exogenous R26-driven *Myb,* and reporter genes eGFP and Luciferase on two independent parental and two Cre-excised *R26-Myb* mESC clones. For all targets, the average of parental samples was set to one. P-values: *** 0.0005; * 0.0173; ** 0.0064. Error bars represent SEM. (D) Western blot analysis for Myb and vinculin (loading control) on parental and in vitro Cre-excised *R26-Myb* mESCs. Myb bands were quantified, normalized against the loading control and plotted in a graph (bottom). There was a statistically significant increase of Myb levels (3.3-fold increase; P-value: *** 0.0002) in parental (n=2) compared to Cre-excised (n=4) mESCs. Error bars represent SEM. (E) Breeding scheme to obtain homozyogous *Myb^vav^* and Cre-negative littermate control mice that were used for the experiments in F. (F) qRT-PCR analysis for the floxed stop cassette, total and exogenous R26-driven *Myb,* and reporter genes eGFP and Luciferase on cells from thymus and bone marrow of five 10-week-old homozyogous Myb^{vav} mice and five Cre-negative age-matched littermate control mice. Each dot represents a different mouse. For all targets, except for floxed stop, the average of Cre-negative control samples was set to one. P-values: **** <0.0001. Error bars represent SEM. (G) Graph depicting luciferase activity in splenocytes of Myb^{vav} and Cre-negative age-matched littermate control mice. Error bars represent the standard deviation of two technical replicates.


A
B-cell malignancy - mouse #1
spleen
liver
lymph node
Myb^Vav
control
B220
CD19
% of CD19+B220+ cells
control
Myb^Vav
liver
spleen
LN
B
B-cell malignancy - mouse #1
Thy1.1
FSC-A
% of Thy1.1+ cells
C
B-cell malignancy - mouse #1
Gr-1
CD11b
% of Gr-1+Cd11b+ cells
D
Myeloid malignancy - mouse #2
spleen
BM
lymph node
% of Gr-1+Cd11b+ cells
E
Gating on B-cells; analysis myeloid markers
Gating on myeloid cells; analysis B-cell markers
Mixed MybVav malignancies
lymph node
mouse #2
splenomegaly
mouse #2
splenomegaly
mouse #3

Supplemental Figure 2. Flow cytometry of B-cell, myeloid and mixed *Myb^Vav^* malignancies. (A-D) Flow cytometry analysis of B-cell and myeloid neoplasms present in Myb^{Vav} mice, and of their age-matched, Cre-negative healthy littermate control tissues. Single live cells were analyzed for B-cell markers B220 and CD19 (A), T-cell marker Thy1.1 (B), and myeloid markers Gr-1 and Cd11b (C,D). FSC-A: forward scatter area. Insets: Graphs depicting the percentage of T-cells, B-cells or myeloid cells that were pregated for single live cells. (E) Flow cytometry analysis of Myb^{Vav} mice with mixed B-cell and myeloid neoplasms.

Supplemental Tables

Supplemental Table 1. List of cloning and genotyping primers**.**

Primer name	Primer sequence	PCR product
Cloning Primers		
AttB1-Myb-F	*5'GGGGACAAGTTTGTACAAAAAAGCAGGCT*TA **ACC**ATGGCCCGGAGACCCCGACACAGCA-3'	932 bp
AttB2-Myb-R	*5'GGGGACCACTTTGTACAAGAAAGCTGGGT*A***T*** ***CA***CATGACCAGAGTTCGAGCTGAG-3'	
Genotyping primers		
ROSA26 F	5'-CGAGCGGATAACAATTTCACA-3'	tg: 570 bp
Ins R	5'-CCAAGCTTTTTCCCCGTATC-3'	
ROSA26 5' F	AAAGTCGCTCTGAGTTGTTAT	wt: 500 bp
ROSA26 3' mut R	GCGAAGAGTTTGTCCTCAACC	tg: 250 bp
ROSA26 3' wt R	GGAGCGGGAGAAATGGATATG	
Tlox F	5'-ATCATGTCTGGATCCCCATC-3'	tg: 700 bp
Myb R	5'-TAGCATTATCAGTCCGTCCG-3'	
iCre F	5'-AGATGCCAGGACATCAGGAACCTG-3'	tg: 250 bp
iCre R	5'-ATCAGCCACACCAGACACAGAGATC-3'	

Supplemental Table 2. List of qPCR primers**.**

qRT-PCR primers	
Primer name	**Primer sequence**
Floxed Stop F	5’-CACCTTCTACTCCTCCCCTA-3’
Floxed Stop R	5’-TACTTCCATTTGTCACGTCC-3’
Myb F1	5'-TGTACAAAAAAGCAGGCTTAACCA-3'
Myb R1	5'-CACACATCTCAATGTCTTCATCATCT-3'
Myb F2	5'-ATACGAAGTTATTAGGTCCC-3'
Myb R2	5'-TCATCTTCATCGCTACTGTA-3'
eGFP F	5’-CGACAACCACTACCTGAGCAC-3’
eGFP R	5’-CTTGTACAGCTCGTCCATGC-3’
Luc F	5’-CGCTGGAGAGCAACTGCATA-3'
Luc R	5’-CCAGGAACCAGGGCGTATCT-3'
Hprt1 F	5’-GGATTTGAATCACGTTTGTGT-3’
Hprt1 Rv	5’-TGGCAACATCAACAGGACTC-3’
Gapdh F	5’-CCCCAATGTGTCCGTCGTG-3’
Gapdh R	5’-GCCTGCTTCACCACCTTCT-3’
G6pdh F	5’-ATGCAGAACCACCTCCT-3’
G6pdh R	5’-TTCAACACTTTGACCTTCTCA-3’
Rpl13a F	5’-CCTGCTGCTCTCAAGGTTGTT-3’
Rpl13a R	5’-TGGTTGTCACTGCCTGGTACTT-3’
Hmbs F	5’-GAAACTCTGCTTCGCTGCATT-3’
Hmbs R	5’-TGCCCATCTTTCATCACTGTATG-3’
Tbp F	5’-TCTACCGTGAATCTTGGCTGTAAA-3’
Tbp R	5’-TTCTCATGATGACTGCAGCAAA-3’
Actb F	5’-GCTTCTAGGCGGACTGTTACTGA-3’
Actb R	5’-GCCATGCCAATGTTGTCTCTTAT-3’
Eef1a1 F	5’-TCGCCTTGGACGTTCTTTT-3’
Eef1a1 R	5’-GTGGACTTGCCGGAATCTAC-3’
Oaz1 F	5’-ATTGCTGTTTAAGATGGTCAGG-3’
Oaz1 R	5’-GGGGAGGTGACACTATTTTTCC-3’
Matr3 F	5'-TGGACCAAGAGGAAATCTGG-3'
Matr3 R	5'-TGAACAACTCGGCTGGTTTC-3'
B2m F	5'-CGGCCTGTATGCTATCCAGAA-3'
B2m R	5'-GGCGGGTGGAACTGTGTTA-3'
Ubc F	5'-AAAGCCCCTCAATCTCTGGAC-3'
Ubc R	5'-TGCATCGTCTCTCTCACGGA-3'

Supplemental Table 3. List of flow cytometry antibodies**.**

Marker	clone	fluofore	dilution	company
T-cell panel				
Thy1.2/CD90.2	53-2.1	BV500	1/250	BD Biosciences
CD4	RM4-5	AF700	1/250	eBioscience
CD8	53-6.7	PE-Cy7	1/250	eBioscience
fixable viability dye		eFluor450	1/2000	eBioscience
Myeloid and B-cell				
CD45	30-F11	AF700	1/200	Life technologies
fixable viability dye		eFluor506	1/400	eBioscience
B220	RA3-6B2	BV605	1/200	BD Biosciences
CD19	1D3	APC-Cy7	1/200	BD Biosciences
CD11b/Mac1	M1/70	PE	1/200	BD Biosciences
Gr-1/Ly-6G		PE-Cy7	1/100	