Amplification of mixed lineage leukemia gene perturbs hematopoiesis and cooperates with partial tandem duplication to induce acute myeloid leukemia

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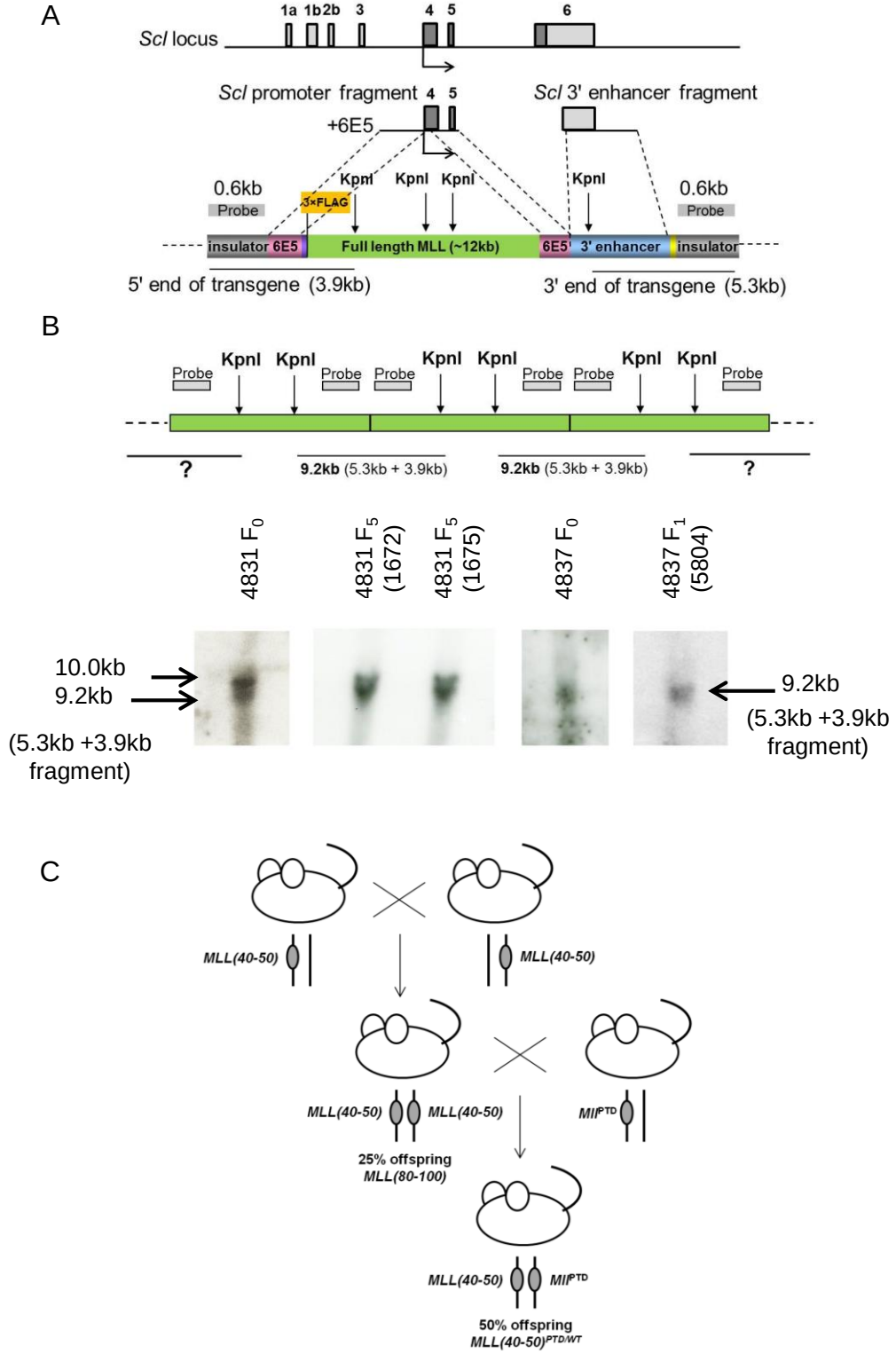
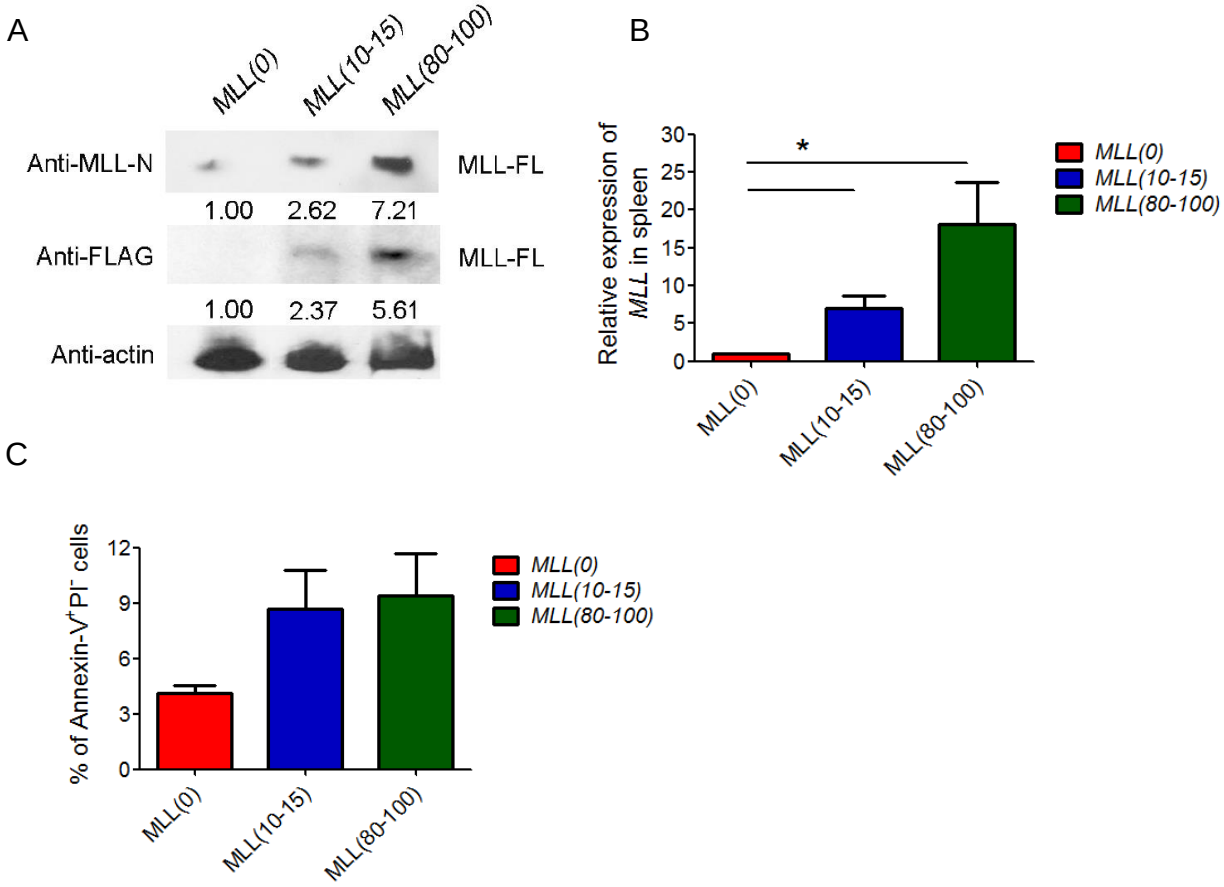
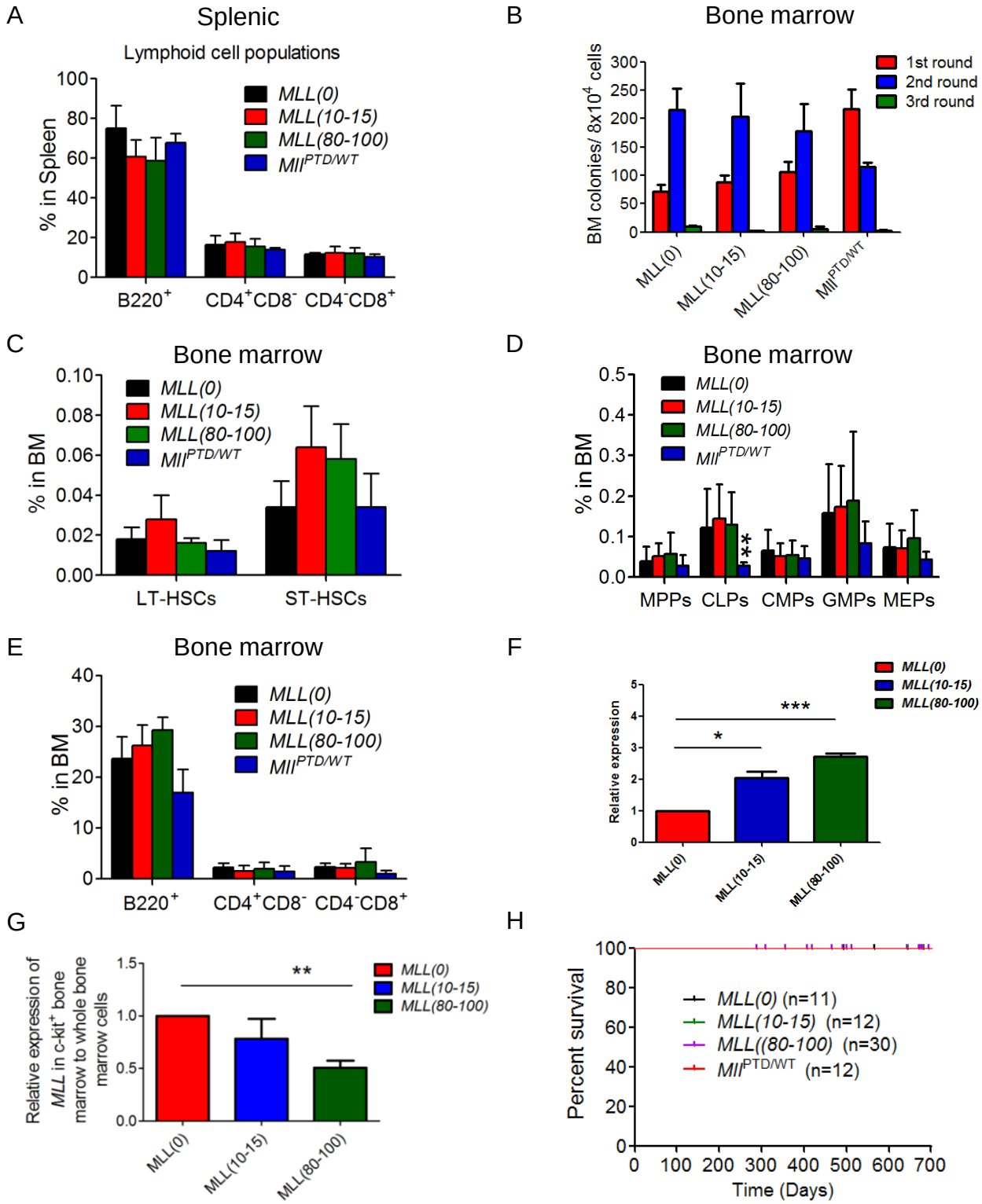
Supplementary Figures



Supplementary Figure S1. Generation of *MLL(n)* transgenic mice and double mutant *MLL(40-50)*$^{PTD/WT}$ mice. (A) Schematic representation of the construct used for generation of transgenic mice. Locations of *Kpn*I restriction site in the *MLL*/6E5 construct used in Southern blotting were also indicated. (B) The insulator sequence was used as the probe for hybridization of transgenes. Founder 4831 and its F5 offspring 1672 and 1675 produced a 9.2kb band, which corresponds to the transgene concatemer, and a 10.0kb band, which corresponds to one of the bands released from either the 5' or 3' end of an integration site. Founder 4837 and its F1 offspring 5804 produced a 9.2kb band, which confirmed transgene concatemerization and its integrity. (C) Schematic diagram showing the breeding strategy used to produce *MLL(80-100)* and *MLL(40-50)*$^{PTD/WT}$ mice.

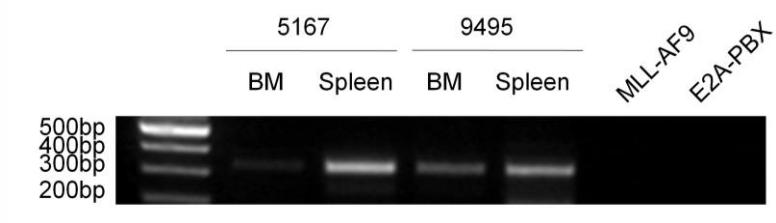


Supplementary Figure S2. Expression of *MLL* transgene in transgenic mice. (A) Western blotting of total and transgenic *MLL* protein using whole-cell lysates prepared from spleens of transgenic mice. The 430kDa full-length (*MLL*-FL) of *MLL* protein were detected by anti-*MLL* N-terminus and anti-FLAG antibodies. β-actin was used as a loading control. Normalized *MLL* expression levels relative to β-actin levels were quantified by ImageJ (NIH). (B) Relative *MLL* expression level in the spleen of *MLL(10-15)* and *MLL(80-100)* mice compared to *MLL(0)* controls at 3 months old. (C) % of apoptotic cells (Annexin-V^+PI^-) cells in the spleen of *MLL(n)* mice at 3 months old. Results in panel B and C were obtained from 5 and 4 independent experiments respectively. Bar graph shows mean+S.E.M. (ns = not significant, *P<0.05 and **P<0.01, paired t-test).

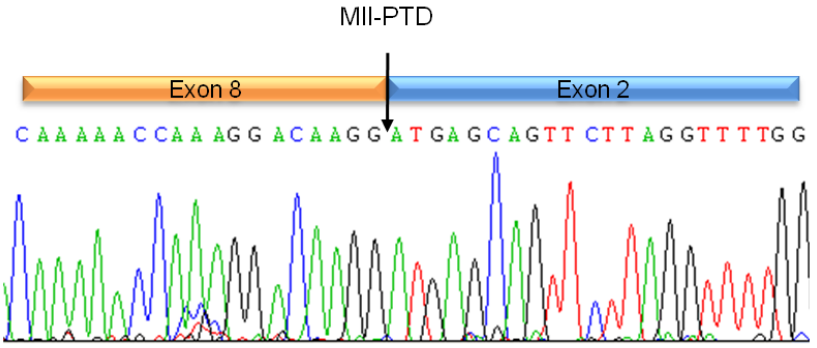


Supplementary Figure S3. Effect of *MLL* amplification on hematopoiesis in transgenic mice. (A) Relative proportions of B- (B220$^+$) and T- (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$) cell populations in spleen. (B) Bone marrow cells from different mice (8×10^4 cells) were seeded into methylcellulose for replating assays. Enumeration of colonies and replating of cells to the next round were performed after six days of culture. Results were obtained from 5 independent experiments and the bar graph show mean+S.E.M. Relative proportions of (C) HSCs and (D) progenitor cell populations in bone marrow. Long-term hematopoietic stem cells, LT-HSCs; short-term hematopoietic stem cells, ST-HSCs; multipotent progenitors, MPPs; common lymphoid progenitors, CLPs; common myeloid progenitors, CMPs; granulocyte megakaryocyte progenitors, GMPs; megakaryocyte-erythroid progenitors, MEPs. Relative proportions of (E) B- (B220$^+$) and T- (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$) cell populations in bone marrow. (F) Relative *MLL* expression level in c-kit$^+$ bone marrow hematopoietic stem and progenitor cells of *MLL(n)* mice at 3 months old. (G) Relative *MLL* expression level in c-kit$^+$ bone marrow hematopoietic stem and progenitor cells compared to relative *MLL* expression level in whole bone marrow cells of *MLL(n)* mice at 3 months old. (H) Survival curves of *MLL(0)*, *MLL(10-15)*, *MLL(80-100)* and *Mll*$^{PTD/WT}$ mice. None of *MLL(0)* (n=11), *MLL(10-15)* (n=12), *MLL(80-100)* (n=30) and *Mll*$^{PTD/WT}$ (n=12) mice developed AML in two years. Results in panel A to F were obtained from 5 independent experiments and bar graphs show mean+S.E.M (ns = not significant, *P<0.05, paired t-test).

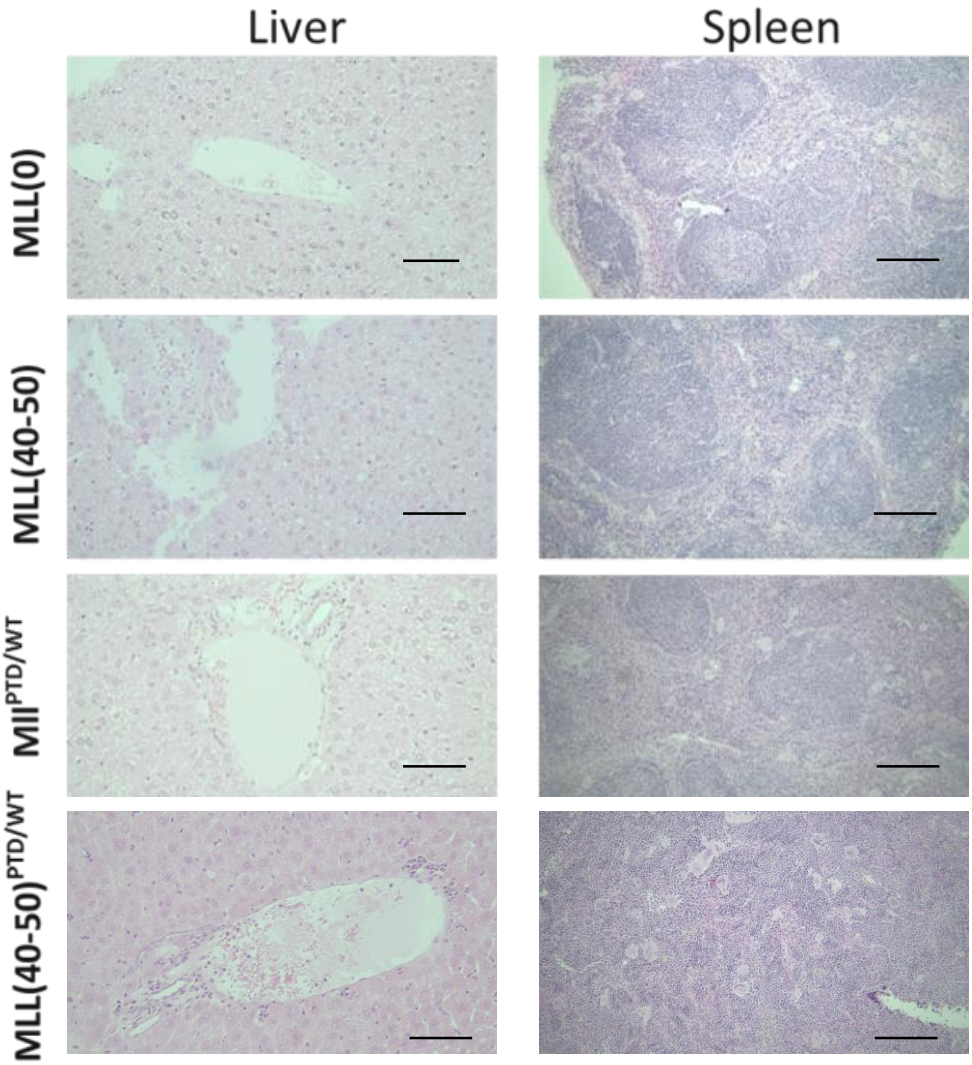
A



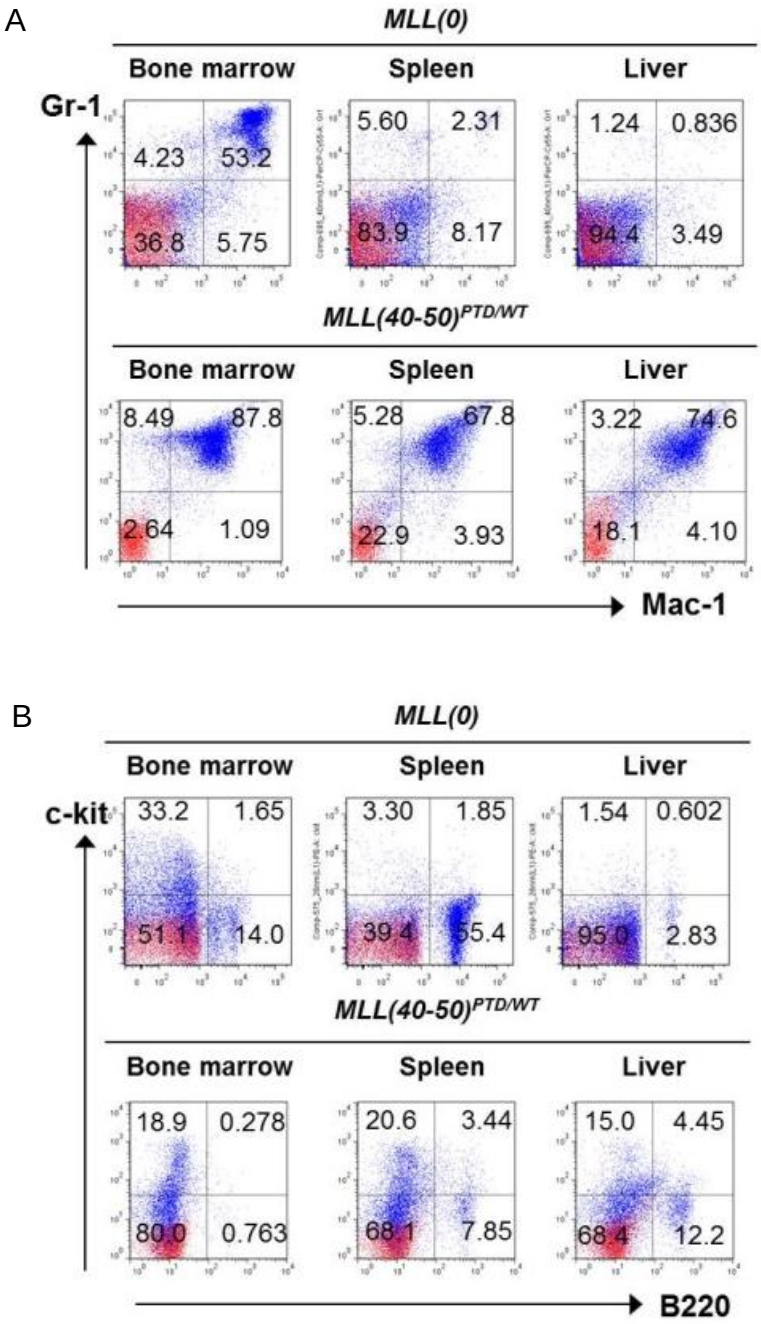
B



Supplementary Figure S4. Demonstration of MLL-PTD expression. (A) The *Mll*-PTD transcript was amplified in *MLL(40-50)*$^{PTD/WT}$ bone marrow and splenic cells to give a 335bp signal, whereas no signal was observed in *MLL-AF9* and *E2A-PBX* cell lines. (B) Sequencing of RT-PCR products verified the presence of *Mll*-PTD exon 6-2 junction.

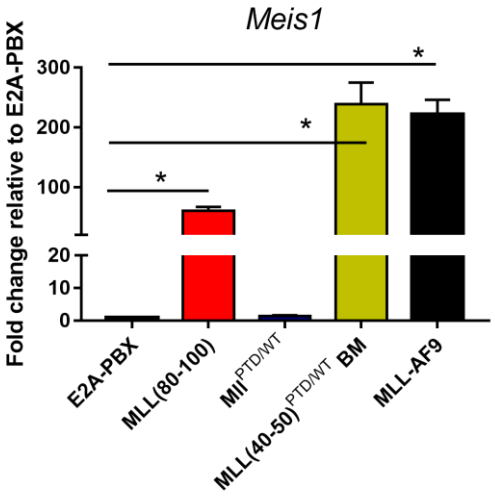


Supplementary Figure S5. Infiltration of leukemic blasts into the liver and spleen of leukemic mice. Infiltration of leukemic blasts into the tissues of liver and spleen of morbid *MLL(40-50)$^{PTD/WT}$* mice resulting in alteration in their normal architecture. Scale bars represent 12.5 (liver) or 25 (spleen) µm.



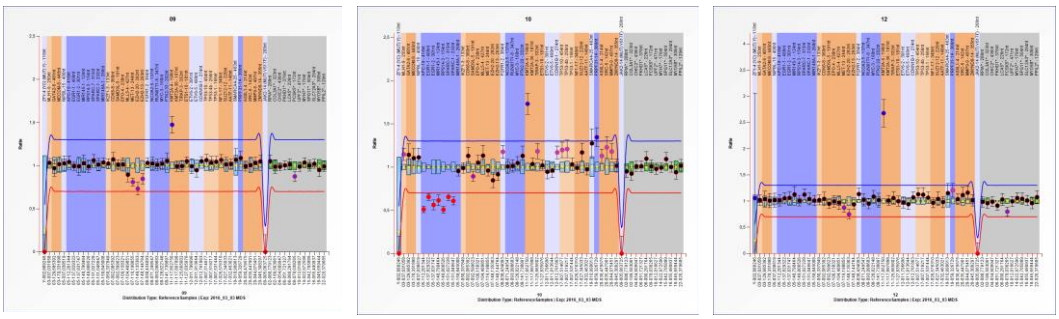
Supplementary Figure S6. Expansion of myeloid blasts in the bone marrow, spleen and liver of leukemic mice. (A) Representative flow cytometry plots showing expansion of myeloid blasts in different compartments of leukemic *MLL(40-50)$^{PTD/WT}$* mice compared to *MLL(0)* controls. Red dots, unstained cells; blue dots, cells stained with indicated antibodies. (B) Representative flow cytometry plots showing reduction in B220^{+} lymphoid cells in bone marrow and spleens of *MLL(40-50)$^{PTD/WT}$* mice compared to *MLL(0)* controls. Red dots, unstained cells; blue dots, cells stained with indicated antibodies.

A



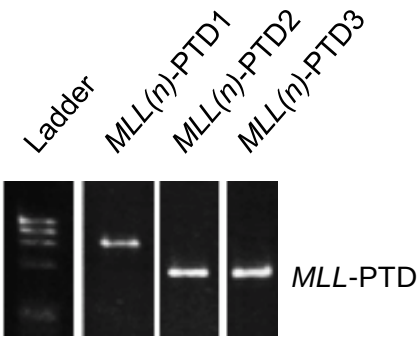
B

(i)



MLL(n)-PTD*1* *MLL(n)*-PTD2 *MLL(n)*-PTD3

(ii)



Supplementary Figure S7. RNA expression of Meis1 in *MLL(40-50)*$^{PTD/WT}$ mice and *MLL*-PTD in *MLL(n)*-PTD patient samples. (A) Relative expression levels of *Meis1* in *MLL(n)*, *Mll*$^{PTD/WT}$, leukemic *MLL(40-50)*$^{PTD/WT}$ bone marrow and *MLL-AF9* leukemic cells compared to *E2A-PBX* leukemic cells by q-RT-PCR. (B) (i) Multiplex Ligation-dependent Probe Amplification (MLPA) and (ii) PCR for the 3 human bone marrow *MLL(n)*-PTD samples showing *MLL* amplification and partial tandem duplication in each of the samples.

Supplementary Table S1 - Clinical features of *MLL(40-50)$^{PTD/WT}$* mice with AML

	MLL(40-50)$^{PTD/WT}$ **(n=3)**	***MLL(40-50)****	***MII$^{PTD/WT}$****	***MLL(0)****
Disease Latency (days; mean)	395	N/A	N/A	N/A
Bone marrow	Pale in color	Red	Red	Red
Spleen size (cm)	2.5±0.2	1.3±0.2	1.4±0.3	1.5±0.2
WBC count (×10^7):				
Bone marrow (two femurs)	8.73±1.57	3.03±1.82	3.38±1.88	2.81±1.02
Spleen	22±3.96	5.86±3.24	11.84±3.63	9.53±3.03
Thymus	8.67±3.05	7.17±4.31	8.73±5.38	5.47±2.75

*No animal comes down with disease and data were collected from 4 mice
N/A: not applicable

Supplementary Table S2 – Primers used in this paper

Primer	Nucleotide sequence (5' to 3')	Application
MLL ex.13F	TCT CGG ACT ACC AGC CAT TT	PCR genotyping of *MLL(n)* mice
MLL ex.17R	AGT TGG TGA GTC TGG TTC TCC	
MLL ex.20F	GGG TTG CTG TCT CAC ATC CT	
MLL ex.23R	TAT GGG CAA TGG TCC TGT TT	
3' enhancer F	CAG GTG GGT CAT TCT GTA GG	
3' insulator R	CCA ACT CGA ATT CAT TGC AG	
PTD DNA forward	GAG CCT TGG CCC GAA TGA AAC TGT	PCR genotyping of *Mll*$^{PTD/WT}$ mice
PTD DNA reverse	CCG GCG AAC GTG GCG AGA AA	
Scl intron6 F	CCC CCT ACC CGG ACA CA	Transgene copy number estimation by Taqman q-PCR
Scl intron6 R	TGG GTA ACC CTT TAC TTA GAT TCA TGA	
Scl intron6 probe	CCC CCT GCC CAT GTA AAC CTG TTA CCT	
PTD exon 6 Forward	CAG GCC AGA GCA AAG CAA GCA	RT-PCR of *Mll*-PTD cDNA
PTD exon 3 Reverse	CCC CAG GGA AGG TAG GAG GTC	
Mouse *Gapdh* F	Mouse GAPD (GAPDH) endogenous control (VIC/MGB Probe, Primer Limited) (Applied Biosystems)	Endogenous Control gene assay in Taqman q-PCR
Mouse *Gapdh* R		
Mouse *Gapdh* probe		
Human *MLL* F	ATA AAG TCC AGG AAG CTC GAT CA	Transgene expression Analysis by Taqman q-PCR
Human *MLL* R	CAG CAT CCT CAA ACC GTT AAC A	
Human *MLL* probe	ATG CCC GCC TAA AGC AGC TCT CAT TT	
Mouse *Hoxa9* F	CCG AAC ACC CCG ACT TCA	*Hoxa9* expression analysis by Taqman q-PCR
Mouse *Hoxa9* R	TTC CAC GAG GCA CCA AAC A	
Mouse *Hoxa9* probe	TGC GCT TCC AGT CCA AGG CGG	
Mouse *Meis1* F	CCTCGGTCAATGACG CTTTAA	*Meis1* expression analysis by Taqman q-PCR
Mouse *Meis1* R	GGTACAAGTAGCTAATTCACA	
Mouse *Meis1* probe	ACACCCCCTCTTCCCTCTCTTAGCA CTGA	
Human *HOXA9* F	GCCGGCCTTATGGCATTAA	*HOXA9* expression analysis by Taqman q-PCR
Human *HOXA9* R	AGGGACAAAGTGTGAGTGTCAA	
Human *HOXA9* probe	ACCGCTGTCGGCCAG	
Hoxa9-F	CTG TCT CCG CTT CCA TTC	SYBR Green q-PCR after ChIP
Hoxa9-R	TCA GCC CTG ATT CCA ATT TC	

Supplementary Table S3 - Cell surface markers used to identify different hematopoietic stem and progenitor cell populations

Cell populations	Cell surface markers	References
LT-HSCs	$Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{-}Flt3^{-}$	Yang et al., 2005
ST-HSCs	$Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{+}Flt3^{-}$	
MPPs	$Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{+}Flt3^{+}$	
CMPs	$Lin^{-}IL\text{-}7R^{-}c\text{-}kit^{+}Sca\text{-}1^{-}CD34^{+}FcγR^{lo}$	Akashi et al., 2000
MEPs	$Lin^{-}IL\text{-}7R^{-}c\text{-}kit^{+}Sca\text{-}1^{-}CD34^{-}FcγR^{lo}$	
GMPs	$Lin^{-}IL\text{-}7R^{-}c\text{-}kit^{+}Sca\text{-}1^{-}CD34^{+}FcγR^{hi}$	
CLPs	$Lin^{-}IL\text{-}7R^{+}c\text{-}kit^{lo}Sca\text{-}1^{lo}$	Kondo et al., 1997

Supplementary Table S4 - Antibodies used for staining of different cell lineages

	Staining for HSCs and progenitors in BM	**Dilution**
1	Sca-1-Pacific Blue (D7)	1:50
2	c-kit-PE-Cy7 (2B8)	1:100
3	CD34-FITC (RAM34)	1:25
4	CD16/32-APC (2.4G2)	1:75
5	IL-7R_-Biotin (A7R34)/ Streptavidin-Alexa Fluor 700	1:100/ 1:200
6	FLT3-PE (A2F10)	1:50
7	Unconjugated rat antibodies: Mac-1 (M1/70), Gr-1 (RB6-8C5), CD3_ (145-2C11), Ter119 (Ter-119), CD4 (RM4-5), B220 (RA3-6B2) and CD8 (53-6.7)/ Goat anti-rat-PE-Cy5	1:40 each/ 1:50

	General staining for all lineages	**Dilution**
1	Gr-1-PE-Cy5.5 (RB6-8C5)	1:200
2	Mac-1-PE-Cy7 (M1/70)	1:200
3	c-kit-PE (2B8)	1:200
4	B220-Pacific Blue (RA3-6B2)	1:200
5	CD4-Alex Flour 700 (RM4-5)	1:200
6	CD8-APC-Cy7 (53-6.7)	1:200

Supplementary methods

Mice

Transgenic mice with *MLL* amplification were generated by microinjection of eggs harvested from super-ovulated C57BL/6J × CBA females with the *MLL/6E5* construct was performed. The *MLL/6E5* construct was generated by subcloning the pFLAG-CMV1-tagged human *MLL* cDNA into a vector containing the murine *Scl* exon 4 promoter (*+6E5*) and 3' enhancer as previously described [1]. The chicken β-globin HS4 core sequence (the insulator) released from the pNI-CD plasmid (gift from Suming Huang, University of Florida, USA) was subcloned at the 5' and 3' end of the transgenic construct to prevent transgene silencing resulting from the spread of heterochromatic region [2].

To obtain a pure C57BL/6J background, founder 4837 was subsequentially backcrossed five times although most of the data were generated using the F_2. As the transgene transmission in all cases was consistent with Mendelian inheritance, it was concluded that all transgene copies in founder 4837 were located on a single chromosome. Since all the litters produced in subsequent matings consistently have 10-15 copies of *MLL*, founder 4837's offspring were concluded to contain 10-15 copies of *MLL*. The 40-50 transgene copies in founder 4831 were also found located on one chromosome because of the observed Mendelian inheritance pattern. To obtain transgenic mice with a higher copy number of *MLL*, F_1 mice from founder 4831 were first backcrossed five times to a pure C57BL/6J background before being bred among themselves to produce mice with 80-100 copies of the transgene (Supplementary Figure S1C). Most of the data were generated using the F_1 *MLL(80-100)* mice. $Mll^{PTD/WT}$ knock-in mice with *Mll* exons 2-6 inserted into intron 1 in a pure C57BL/6J background were obtained from Michael Caligiuri

(Ohio State University, USA) [3]. Primers for PCR genotyping of *MLL(n)* transgenic and *Mll*$^{PTD/WT}$ knock-in mice were summarized in Supplementary Table 2.

MLL(40-50)$^{PTD/WT}$ mice were generated by mating *MLL(80-100)* with *Mll*$^{PTD/WT}$ mice (Supplementary Figure S1C). Since transmission of both mutations followed Mendelian inheritance, *MLL(40-50)*$^{PTD/WT}$ and *MLL(40-50)* mice were produced in a 1:1 ratio. Twelve *MLL(40-50)*$^{PTD/WT}$, fourteen *MLL(40-50),* and twelve *Mll*$^{PTD/WT}$ mice were kept for disease observation over two years.

Estimation of *MLL* transgene copy numbers in transgenic mice

Estimation of *MLL* transgene copy numbers in transgenic mice was achieved by quantification of *Scl* enhancer copy number*s* using q-PCR.

[delta]Ct,sample - [delta]Ct,reference = [delta][delta]Ct

2–[delta][delta]Ct = relative quantity (RQ)

- [delta]Ct,sample is the Ct value of *Scl* enhancer in a transgenic mouse sample normalized to the endogenous housekeeping gene
- [delta]Ct, reference is the Ct value of *Scl* enhancer in a wild-type mouse normalized to the endogenous housekeeping gene.

In the case of a transgenic mouse has an average Ct 22.73 for the *Gapdh* and an average Ct 24.51 for the *Scl* enhancer while the wildtype mouse was found to have an average Ct 22.88 for the *Gapdh* and an average Ct 29.28 for *Scl* enhancer.

The RQ of *Scl* enhancer in the transgenic mouse is therefore

[delta]Ct,sample = 24.51 - 22.73 = 1.78

[delta]Ct,reference = 29.28 - 22.88 = 6.40

[delta][delta]Ct = [delta]Ct,sample - [delta]Ct,reference

= 1.78 - 6.40

= -4.62

RQ = 2–[delta][delta]Ct

= 2–(-4.62)

= **24.60**

The RQ of *Scl* in the wild-type mouse is set to 1, as

[delta][delta]Ct = [delta]Ct,sample - [delta]Ct,reference = 0

RQ = 2–[delta][delta]Ct

= 2–(0)

= **1**

Since the wild-type mouse always has two copies of endogenous *Scl* in the genome and the RQ value is set to 1, the copy number of *Scl* enhancer in the transgenic mouse can thus be estimated using its RQ

1 / 2 = 24.60 / total *Scl* copy number

Total *Scl* copy number in the transgenic mouse = 49.2

As the copy numbers of *Scl* enhancer is equal to the copy numbers of *MLL* transgenes in transgenic mice,

To minus the two copies of endogenous *Mll*:

49.2 - 2 = 47.2

Therefore, in this case the transgenic mouse has approximately 47 copies of *MLL* transgene.

Same calculations were applied to all the mice to obtain their transgene copy numbers. The RQ values from three independent experiments for each mouse were averaged for calculation.

In this study, transgenic mice are defined based on their transgenic copy numbers: *MLL(0)* refers to the non-transgenic littermates, *MLL(10-15)* refers to founder 4837's offspring containing 10-15 copies of *MLL*, *MLL(40-50)* refers to founder 4831's offspring containing 40-50 copies of *MLL* and *MLL(80-100)* refers to founder 4831's offspring containing 80-100 copies of *MLL*.

Southern blot

Southern blotting was performed according to standard protocols [4]. To study transgene integration site, 10μg of genomic DNA extracted from tail snips was first digested with *Kpn*I at 37°C overnight. The digested DNA was then resolved by agarose gel electrophoresis (0.7%) at 50V until the desired size range was obtained. The DNA was then transferred from the agarose gel to the GeneScreen Plus® Hybridization Transfer membrane (PerkinElmer). After transfer, crosslinking of DNA onto the membrane was achieved using a Stratalinker UV crosslinker (Stratagene). Probe labeling was performed in a 50μl reaction volume using Ready-To-Go™ DNA labeling Beads (-dCTP) (GE Healthcare) according to manufacturer's instructions. Unincorporated nucleotides were removed from the labeled probe using illustra MicroSpin™ G-50 Columns (GE Healthcare) according to manufacturer's instructions. Hybridization of the membrane with the labeled probe was carried out using Amersham Rapid-hyb™ Buffer (GE Healthcare). The buffer was first mixed with denatured salmon testis DNA (Sigma Aldrich) and added to a hybridization bottle containing the membrane. The membrane was incubated at 65°C for 1 hour with rotation prior to addition of the labeled probe and then incubated for at least 3 hours. After hybridization, the membrane was washed three times at 65°C for 10 minutes with rotation using wash solution (2× SSC, 1% SDS). The membrane was exposed, between two intensifying screens, to the X-ray film (X-OMAT™, Kodak) at -80°C.

Preparation of single-cell suspensions from mouse tissues

Mice were culled in a CO_2 chamber according to Schedule 1 of the Animal (Scientific Procedures) Act 1986 set by the Home Office. After dissection, the bone marrow, the spleen and the thymus of each mouse were harvested into PBS. The bone marrow cells were flushed out, undergone an erythrocyte lysis treatment at room temperature for 10 minutes [Red cell lysis buffer: 10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM EDTA (pH 8.0)] and passed through a 40μm cell-strainer (BD Biosciences) as previously described [5]. For the spleen and thymus, they were homogenized by passing through a 40μM cell strainer using the plunger from a 5ml syringe. The splenocytes then underwent the same erythrocyte lysis treatment.

Taqman quantitative PCR

To estimate the transgene copy number in *MLL*/6E5 transgenic mice by Taqman quantitative PCR, mouse *Gapdh* was used as an endogenous control while endogenous *Scl* was used as a standard for calculation of the transgene copy number. We have designed primers to amplify the 3' *Scl* enhancer sequence within the construct for estimation of transgene copy number in transgenic mice (Supplementary table 2). Since a wild-type mouse always carries two copies of *Scl*, transgene copy number can therefore be calculated from the fold increase in *Scl* signal in transgenic mice. In the reaction for the endogenous control, each reaction mix contained 1μl diluted genomic DNA biopsy material, 1×TaqMan Fast Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and 1μl mouse *Gapdh* endogenous control master mix (VIC/MGB Probe, Primer Limited) (Applied Biosystems). In the reaction for the transgene, each reaction mix contained 1μl diluted genomic DNA, 1× TaqMan Fast Universal PCR Master Mix, No AmpErase UNG, 0.3μM of forward and reverse primer, and 0.1μM of the double-dye oligo

that are specific for the *Scl* 3' enhancer fragment. The copy number of *Scl* calculated in transgenic mice is equal to the copy number of *MLL*.

To study gene expression by TaqMan quantitative RT-PCR, total RNAs from BM cells and splenocytes with or without culture were first extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNAs by the SuperScript™ II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. The reaction was then performed in the same way as transgene copy number estimation. Primer Express 3.0 (Applied Biosystems) was used to design all the primers and probes and they are summarized in Table 2. All real-time PCR reactions were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with an initial DNA denaturation step of 95°C for 20 seconds followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Data were analyzed using the sequence detection system (Applied Biosystems) and reported as relative quantity (RQ) values, which represent the fold differences when normalized to the endogenous control on the same 96-well plate.

Western blot

Whole cell lysates for western blotting were prepared using a high salt lysis buffer [0.3M NaCl, 20mM HEPES pH 7.3, 25% Glycerol, 1.5mM MgCl2, 0.2mM EDTA, 0.2mM PMSF, protease inhibitor cocktails (Sigma Aldrich), 0.2mM dTT and 0.1% Triton X]. Lysates were undergone SDS-PAGE and then transferred to the Hybond™ ECL™ membrane (GE Healthcare) according to standard protocols. Anti-MLL (N4.4, Upstate) and anti-FLAG (M2, Sigma Aldrich) antibodies were used to detect the level of MLL expression (1:300 and 1:500 dilution respectively). Anti-beta-actin-HRP (Abcam) was used as the loading control (1:20,000 dilution).

***In vitro* replating assay**

To assess the self-renewal potential of hematopoietic stem and progenitor cells, cells of interest (8×10^4 bone marrow cells and 20×10^4 splenocytes) were incubated in MethoCult M3231 methylcellulose (StemCell Technologies) supplemented with 10ng/ml IL-3, 10ng/ml IL-6, 10ng/ml GM-CSF, 20ng/ml SCF, 100IU/ml penicillin and 100μg/ml streptomycin. After incubation for 6 days, enumeration of colonies was performed under the microscope before replating to the next round. The procedures of colony counting and replating were performed as previously described [6].

Colony forming cell assay

Myeloid CFC assays were performed to quantify multi-potential and lineage-restricted progenitors in the hematopoietic system. 8×10^4 bone marrow cells or 20×10^4 splenocytes were seeded into 1ml MethoCult™ GF M3434 methylcellulose (StemCell Technologies) pre-supplemented with IL-3, IL-6, SCF and Epo. Colonies in each well were enumerated and classified based on the morphology according to manufacturer's description after 14 days in culture.

Flow cytometry

Cell lineages and hematopoietic subsets within the bone marrow, the spleen and the thymus were assessed by flow cytometry. For a general study of different cell lineages, a cocktail of antibodies specific for Gr-1, Mac-1, c-kit, B220, CD4 and CD8 was used. Staining of different hematopoietic stem, progenitor, B- and T- cell populations were performed as previously described [7-12]. Flow cytometric analysis was performed using BD LSRII (BD Biosciences) as previously described [5]. Before staining the cells with each antibody cocktail, optimization of fluorescence compensation for each color was performed to allow subsequent valid multicolor analyses. Cell surface markers used to identify each hematopoietic cell population are

summarized in Supplementary Table 3. Antibodies used in each staining are summarized in Supplementary Table 4. Data analysis was carried out using FlowJo version 7.5.

May-Grünwald-Giemsa stain

Mouse bone marrow and splenic cells were stained by May-Grünwald stain (Sigma Aldrich) and Giemsa stain (VWR) according to manufacturer's instructions. Cardiac puncture was performed to obtain peripheral blood samples from the moribund mice to prepare blood smears. The smears were air-dried for 10 minutes before underwent the same staining procedure.

Histology

The excised tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 to 5μm, and stained with hematoxylin and eosin (H&E) for histological analysis.

Cell lines and WDR5-0103 treatment

Primary mouse bone marrow and splenic cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), 20% WEHI conditioned medium, 100IU/ml penicillin and 100μg/ml streptomycin (R20/20 medium). The mouse *MLL-AF9* and *E2A-PBX* cell lines (Eric So and Michael Cheung, Kings College London, UK), generated by retroviral transduction of wild-type mouse c-kit$^+$ bone marrow cells with human *MLL-AF9* and *E2A-PBX* constructs, were also cultured in R20/20 medium. Enumeration of viable cell numbers was performed on a regular basis by trypan blue exclusion using a hemocytometer. All cultures were maintained at a concentration of 1.5-2 × 10^5 cells/ml. Mouse bone marrow cells and splenocytes (1 × 10^5 cells/ml) treated with 40μM WDR5-0103 (Item No. 13945, Cayman) for 5 days were counted by trypan blue, and then subjected to May-Grünwald-Giemsa stain and Taqman quantitative PCR to examine cell differentiation and RNA levels of MLL targets.

Chromatin immunoprecipitation

The ChIP assay kit was used according to the manufacturer's protocol (Upstate Biotech) with some modifications. Briefly, the cells were fixed with 1% formaldehyde for 15 minutes at room temperature and then quenched with 0.125M glycine for 5 minutes. To generate DNA fragments of 0.2-1kb, 1×10^6 cells were sonicated by Bioruptor (Diagenode, Liege, Belgium) on maximum power for 15minutes with an on-off interval of 30 seconds. Chromatin fragments were incubated with 3µg of each antibody overnight and collected in protein-A agarose (UpstateBiotech). Anti-H3 (K9, K14) acetylation (Upstate Biotech), anti-H3K4 trimethylation (Active Motif), anti-H3K9 dimethylation (Abcam) and anti-H3K79 dimethylation (Abcam) antibodies were used. Cross-linked products were reversed by heating overnight at 65°C and then treated with proteinase K at 45°C for 1 hour. The eluted DNA was purified using the QIAquick PCR purification kit (Qiagen) and resuspended in elution buffer according to manufacturer's instructions. Quantification of histone acetylation and methylation at the *Hoxa9* promoter was achieved by SYBR green q-PCR (Bioline). Primers were designed to amplify the promoter sequence of *Hoxa9* (Supplementary table 2). Each reaction (20µl) used 2µl of the DNA template, 0.2µM of each primer and 10µl of SYBR Green PCR Master Mix (Bioline) and was normalized to the input DNA. All real-time PCR reactions were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) with an initial DNA denaturation step of 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The specificity of PCR products was analyzed by addition of a melting curve cycle, which consisted of 1 cycle each of 95°C for 15 seconds, 60°C for 60 seconds and 95°C for 15 seconds.

Human Material

Human material was collected according to institutional ethical procedures. Bone marrow mononuclear cells were separated with Ficoll gradient centrifugation according to manufacturer's instructions. Viable cells were preserved in liquid nitrogen (after decreasing temperature 1ºC/minute to -70ºC) in 10% (v/v) DMSO; 50% RPMI 1640 plus L-glutamine 2 mM and 100 U/mL penicillin / 100 µg/mL streptomycin; and 40% fetal calf serum (all from Gibco-Thermo Fisher Scientific, Waltham, MA USA).

MLPA

DNA was isolated with protein salting-out method[13]. Concentration and purity was assessed with nanoDrop (Thermo Scientific). Multiplex ligation-dependent probe amplification (MLPA) was performed with SALSA MLPA probemix P414-A1 MDS (MRC-Holland, Amsterdam, the Netherlands.) according to manufacturer recommendations and analyzed with Coffalyser.net (MRC-Holland, Amsterdam, The Netherlands).

RT-PCR

RNA was extracted with Tri reagent (Sigma) and retro-transcribed with Super Script IV (Invitrogen-Thermo Fisher Scientific) using Protector RNase inhibitor (ROCHE) and Primer Random (ROCHE). Amplification was performed with AmpliTaq Gold (Thermo Fisher Scientific) 1.25 U in a 50µL mix containing Forward and Reverse primers and 2mM MgCL2. PCR Annealing temperature was 57ºC. PCR products were run in a 2.5% (W/V) agarose gel with DNA molecular weight marker IX (ROCHE).

Statistics

All statistical tests were performed in GraphPad Prism 5 software. Correlations between categorical values were performed using unpaired Student's t tests. Comparisons of survival curves were performed using log-rank (Mantel-Cox) tests. p value less than 0.05 was considered

statistically significant. The lower the p-value the more significant it is. This was indicated by the number of asterisks in each figure (*=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$).

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