Setd2 regulates quiescence and differentiation of adult hematopoietic stem cells by restricting RNA polymerase II elongation

Yile Zhou,[1,2] Xiaomei Yan,[2] Xiaomin Feng,[2] Jiachen Bu,[2,3] Yunzhu Dong,[2] Peipei Lin,[2] Yoshihiro Hayashi,[2] Rui Huang,[2] Andre Olsson,[4] Paul R. Andreassen,[2] H. Leighton Grimes,[4] Qian-fei Wang,[3] Tao Cheng,[5] Zhijian Xiao,[5] Jie Jin[1,*] and Gang Huang[2,*]

[1]Department of Hematology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [2]Division of Pathology and Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, OH, USA; [3]Laboratory of Genome Variations and Precision Bio-Medicine, Beijing Institute of Genomics, Chinese Academy of Sciences, China; [4]Division of Immunobiology and Center for Systems Immunology, Cincinnati Children's Hospital Medical Center, OH, USA; and [5]State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital and Center for Stem Cell Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China

**JJ and GH contributed equally to this study as joint senior authors*

 doi:10.3324/haematol.2018.187708

Received: Juanary 3, 2018.

Accepted: April 6, 2018.

Pre-published: April 12, 2018.

Correspondence: gang.huang@cchmc.org or jiej0503@zju.edu.cn

Supplementary materials

Supplementary methods:

Flow cytometry

Single cell suspensions were prepared from BM, spleen or peripheral blood, followed by red blood cell lysis. Cell surface markers were stained with standard protocol. We used the following antibodies to stain surface markers: anti-Sca-1 (BD Biosciences 553108), anti-CD150 (Biolegend 115914), anti-CD117 (Biolegend 105825), anti-CD105 (Biolegend 120411), anti-CD41 (Biolegend 133913), anti-CD16/32 (Biolegend 101323), anti-CD48 (Biolegend 103404), streptavidin (Biolegend 405247), anti-CD45.1 (BD Biosciences 553776), and CD45.2 (BD Biosciences 558702). We used the following antibody cocktail to exclude lineage+ cells: anti-CD11b (BD Biosciences 553309), anti-Gr1 (BD Biosciences 553125), anti-B220 (BD Biosciences553086), anti-CD19 BD Biosciences 553784), anti-CD3 (BD Biosciences 553060), anti-CD4 (BD Biosciences 553045), anti-CD8 (BD Biosciences 553029), and anti-Ter119 (BD Biosciences 553672). Dead cells were excluded with 7-Amino-Actinomycin D (BD Pharmingen 559925) or Zombie Aqua (Biolegend 423101). Ki67 analysis was performed with anti-Ki67 (Biolegend 652409) and BD Fxiation/Permeabilization Kit (BD Biosciences 554714). Annexin V apoptosis staining was performed using the Annexin V Apoptosis Detection Kit (BD Biosciences 559763). Analysis was performed on FACSCanto or LSR Fortessa and sorting was performed on a FACSAria II (BD Biosciences). Flow cytometry results were analyzed with FlowJo (TreeStar).

BrdU assay

1.5 mg BrdU was intraperitoneally injected into each mouse at 3 days before sacrifice. Cells were stained with cell surface antibodies as described above and then processed with BrdU staining using the FITC-BrdU Flow kit (BD Biosciences 559619) following the manufacturer's instructions.

Single-cell differentiation assay

Single SLAM-HSCs were sorted by flow cytometry and cultured in Stemspan medium (Stemcell 09650) containing murine SCF and murine TPO for the first 48h. Then culture the cells in differentiation medium (IMDM+10%FBS+SCF+TPO+Il-3+G-CSF+EPO) for another 8-12 days. Clones derived from SLAM-HSCs were harvested, and examined for the presence of myeloid lineages (neutrophil, erythroid, macrophage and megakaryocyte).

Histology

Thymus, spleen, and femur or sternum were fixed in formalin and embedded in paraffin. Organs were sectioned and stained with hematoxylin and eosin (H&E) or reticulin. Images were captured using Motic BA310 biological lab microscope with Motic Image Plus 2.0 software.

Colony formation assays

For CFU assays, indicated numbers of bone cells were plated in triplicate into 1ml MethoCult GF M3434 (Stem Cell Technologies) and colonies were scored every 7 days. For CFU-E assays, indicated numbers of bone cells were plated in triplicate into 1ml MethoCult SF M3436 (Stem Cell Technologies) and colonies were scored 48 hours later.

Bone marrow transplantation assays

Six- to eight-week-old B6-SJL (CD45.1+) mice were lethally irradiated (7.50+4.25 Gy). Four

hours after irradiation, mice were transplanted with donor BM cells via tail vein injection. For competitive transplantation, we mixed equal numbers of competitor B6-SJL BM and tester CD45.2 BM cells. For non-competitive transplantation, we injected 2×10^6 tester CD45.2 BM cells. Donor contribution was determined by flow cytometry at different time points and calculated by CD45.2+/(CD45.1+ + CD45.2+) × 100% within the indicated cell type.

Immunoblotting

The LSK populations in the BM were sorted by flow cytometry. The LSK fractions were restained for western blot. Cell extract was prepared by lysing cells in sodium dodecyl sulfate (SDS) sample buffer containing 10 mM NaF, 10mM β-Glycerophosphate, 1mM phenylmethylsulfonyl fluoride, 0.2mM Na3VO4, 2.5mM dithiothreitol, 5% 2-mercaptoethanol, 1mM 4-Amidinophenylmethanesulfonyl Fluoride Hydrochloride, and proteinase inhibitors followed sonication. Samples were boiled at 95 °C for 5 minutes and loaded to SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The following antibodies were used: anti-Setd2 (Abcam, ab31358), anti-β-Actin (Santa Cruz , sc-1616R), anti-H3K36me3 (Abcam, ab9050), anti-H3K36me1 (Abcam, ab9048), anti-H3K36me2 (Millipore, 07-369-I), anti-H3K4me3 (Abcam, ab12209), anti-H3K27me3 (Active Motif, 39157), anti-H3K27ac (Active Motif, 39134), anti-H3K79me2 (Abcam, ab3594), anti-H3 (Abcam, ab1791), anti-RNA polymerase II Ser2 (Biolegend, 920202), anti-RNA polymerase II Ser5 (Biolegend, 920302), anti-Myc (Abcam, ab32072), anti-NSD1 (Millipore, 04-1565), anti-NSD2 (Bethyl, A303-094A), anti-NSD3 (Lifespan Biosciences, LS-C334150-50), anti-Gata1 (CST, 3535s), anti-Gata3 (CST, 5852s), anti-Klf1 (Sigma, AV31493). Horseradish peroxidase-conjugated

antibody to rabbit (NA934V, GE Healthcare) was used as the secondary antibody. Super Signal West Dura Chemiluminescent Substrate (Pierce) was used for ECL detection.

Quantitative PCR (qPCR)

RNA was extracted using RNeasy Mini Kit (QIAGEN 74104) and cDNA was generated using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Relative gene expression was measured using TaqMan universal PCR master mix (ThermoFisher 4304437) or SYBR Green PCR master mix (appliedbiosystems 1711564). Reactions were carried out on a StepOnePlus Real-time PCR System (Applied Biosytems). Relative expression was calculated using the ΔΔCT method. The following primers were used: Setd2 (Life Technologies, Mm 01250225_m1), Ash1l (Life Technologies, Mm01212704-g1), Nsd1 (Life Technologies, Mm00464682_m1), Nsd2 (Life Technologies, Mm01211104_m1), Nsd3 (Life Technologies, Mm01218484_m1), β-Actin (Life Technologies, Mm 00607939_s1). All the primers used in SYBR reactions are listed below.

RNA-seq and data analysis

LSKs were isolated from *Setd2*$^{\Delta/\Delta}$ and littermate control mice by FACS sorting. Total RNA was purified using a RNeasy microKit column system (Qiagen). Total RNAs were sent to CCHMC DNA Core for sequencing, including library construction. Libraries were sequenced on a HiSeq 2500 platform (Illumina Inc.) using the single-end 75 bp sequencing strategy.

Gene Set Enrichment Analyses (GSEA) was performed using javaGSEA software (http://software.broadinstitute.org/gsea/index.jsp). Our RNA-seq datasets have been deposited in the Gene Expression Omnibus database under GSE112550.

ChIP qPCR

4-6M c-kit^{+} BM cells were isolated from *Setd2*$^{\Delta/\Delta}$ and littermate control mice by autoMACS Pro and cross-linked with 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M to stop cross-linking. Then, the cross-linked chromatin was fragmented to a size range of 0.2–0.9 kb with 5-7U micrococcal nuclease for 15minutes at 37°C. ChIP was performed using proper amount ChIP-grade antibodies to bound magnetic beads. Following immunoprecipitation, the bound nucleoprotein complexes were extensively washed 4 times with wash buffer II and once with wash buffer III. The ChIP enriched DNA was eluted and purified by Qiagen PCR purification kit (QIAGEN 28104). This purified DNA was further processed for qPCR. The following antibodies were used: Setd2 (Epigentek, A-3720), H3K36me1 (Abcam, ab9048), H3K36me2 (Millipore, 07-369-I), H3K36me3 (Abcam, ab9050), Pol II (Ser2) (BioLegend, 920202), Pol II (Ser5) (BioLegend 920302). The primers used were listed below.

Statistics

Data were analyzed by Prism 6.0 (GraphPad Software), P values < 0.05 were considered to be significant. Continuous variables were analyzed by using Student t test or one-way ANOVA.

Study approval

All animal studies were performed according to an approved Institutional Animal Care and Use Committee protocol and federal regulations.

qPCR primers		
Myc	F	CTGGATTTCCTTTGGGCGTT
	R	TGGTGAAGTTCACGTTGAGGG
Mycn	F	CCTCACTCCTAATCCGGTCAT
	R	GTGCTGTAGTTTTTCGTTCACTG
Myb	F	AGACCCCGACACAGCATCTA
	R	TAGTTTTCCCCAAGTGACGCT
CDK6	F	TCTCACAGAGTAGTGCATCGT
	R	CGAGGTAAGGGCCATCTGAAAA
Bcl2	F	GCTACCGTCGTGACTTCGC
	R	CCCCACCGAACTCAAAGAAGG
Hmgb2	F	CGGGGCAAAATGTCCTCGTA
	R	ATGGTCTTCCATCTCTCGGAG
Brd2	F	AATGGCTTCTGTACCAGCTTTAC
	R	CTGGCTTTTTGGGATTGGACA
Brd3	F	GGGCGAAAGACTAACCAACTG
	R	GAAAGGCCAGGCAAACTGATG
Brd4	F	CCTCCCAAATGTCTACAACGC
	R	TGAGCAGATATTGCAGTTGGTT
Erg	F	ACCTCACCCCTCAGTCCAAA
	R	TGGTCGGTCCCAGGATCTG
Lmo2	F	ATGTCCTCGGCCATCGAAAG
	R	CGGTCCCCTATGTTCTGCTG
Scl	F	CTCGGCCATTCGTACATGGAA
	R	GGATACCTCTGCACCGTAGC
Gata1	F	GCCCAAGAAGCGAATGATTG
	R	GTGGTCGTTTGACAGTTAGTGCAT
Gata3	F	CTCGGCCATTCGTACATGGAA
	R	GGATACCTCTGCACCGTAGC
Klf1	F	CAGCTGAGACTGTCTT ACCC
	R	AATCCTGCGTCTCCTCAGAC

Myc ChIP-qPCR primers		
-1935	F	CTTCGCAACGCTGTGGTCTC
	R	GTTGTGGCTCTCGGATTTGT
-1344	F	TGCACAGCGTAGTATTCAGG
	R	CCTTCGTATGTGTGTGTTAAGC
-1030	F	GGCATATTCTCGCGTCTAGC
	R	CATACACCTCCACACAGTTCCA
-958	F	CCACAGGGGCAAAGAGGATT
	R	GAGGAGTCTCTGCCGGTCTA
-596	F	TTATCCCTAAGTGGCTCTCCAAG
	R	GTGTGTGGAGTGATAGAGGGTG
-345	F	TATTGTGTGGAGCGAGGCAG
	R	CGGCTGAACTGTGTTCTTGC
-93	F	CCGAGTTCCCAAAGCAGAGG
	R	GGAATATAAAGGGCGGGTGG
+220	F	AGTAATTCCAGCGAGAGACAGAG
	R	GATGTTGGGTCAGTCGCAGG
+1285	F	GACTTGGGGGAAACCAGAGG
	R	TTCCTAGCAATTCAGGGGCG
+1485	F	GGTTTAGAGTGTAGAAGGGAGGTG
	R	CGATTGCTGACTTGGAGGAGAG
+1776	F	CGCCTTTCTCCTTCAGGTGG
	R	CATCGGCCTTGGCTTCAGAG
+2575	F	CAGGACTGTATGTGGAGCGG
	R	CTCAGGCTGGTGCTGTCTTT
+2895	F	AGTGCTGCATGAGGAGACAC
	R	AACCTCTCGCTTCCTACCCT
+3667	F	TTTGCAGCTATCCCTCACGG
	R	CACACCCAAAGCAAAGCACA
+4044	F	GGTGTCTGTGGAGAAGAGGC
	R	TTGTGCTGGTGAGTGGAGAC
+4631	F	ACAGAACTGATGCGCTGGAA
	R	AGCTTACAGTCCCAAAGCCC
+1.3Mb	F	TGTTCCTGCCACTCATGCTT
	R	GCAAGTGCAGCAAAGGTCAT
BENC-A	F	CTGCCAGGCTCAAGAGTCAAG
	R	AATACAAATGGGAAAGGGCACA
BENC-C	F	CACAAAGGTATGCACCGAGG
	R	GTGCTCTCAATACCAGGCTCA
BENC-D	F	GGAGTCCAGTCAGTAGTTTGG
	R	TGCTGTTTTGCACCAACGTATTTTA
BENC-E	F	TCCTCAAATGCTAGGGCACAA
	R	TCATACCTGGGCTTACTGCAAA
BENC-G	F	AAGAAGTGGGGCCTATGGAG
	R	GCTACATTGGGTGGGAAAGTG

Table S1. *Setd2$^{f/w}$/Tie2-Cre* × *Setd2$^{f/f}$*

litter	*Setd2$^{f/f}$/Tie2-Cre*	Mendel ratio	p value
1	0/6 (0%)	25%	0.0478
2	0/7 (0%)	25%	
3	0/8 (0%)	25%	
total	0/21 (0%)	25%	

Table S2. Cell classification of BM and spleen

Tissue	Lineage	*Setd2$^{f/f}$* (n=4)	*Setd2$^{\Delta/\Delta}$* (n=4)
BM cytospin	Erythroid (%)	20±2	42±4
	Myeloid (%)	65.5±1	55±4
	Lymphoid (%)	12.5±1	3±0.5
	Megakaryocyte (absolute number per slide)	11~18	32~38
Spleen histology	Megakaryocyte (absolute number per slide)	5~48	241~321

Table S3. GO analysis of differentially expressed genes: lineage differentiation related

Category	Name	p-value	Hit in Query List
GO: Biological Process GO:0030218	erythrocyte differentiation	1.89E-08	**GATA1, GATA3**, STAT5A, CITED2, SOX6, PTBP3, ANK1, CASP3, RHAG, TGFBR3, **KLF1**, HBA1
GO: Biological Process GO:0030099	myeloid cell differentiation	5.16E-07	PDE1B, **GATA1, GATA3**, STAT5A, CITED2, GPC3, CA2, SOX6, PTBP3, ANK1, CASP3, IFI16, RHAG, TFRC, TGFBR3, **KLF1**, HBA1, SCIN, PRDM16
GO: Biological Process GO:0002521	leukocyte differentiation	1.53E-04	PDE1B, HLA-DQA1, **GATA1, GATA3**, CD46, STAT5A, CITED2, JAG2, FZD7, CCR2, GPC3, CA2, CHD7, IFI16, LGALS3, TFRC, CD74, PRDM16
GO: Biological Process GO:0045582	positive regulation of T cell differentiation	8.48E-04	HLA-DQA1, **GATA3**, CD46, STAT5A, CCR2, CD74
GO: Biological Process GO:0045621	positive regulation of lymphocyte differentiation	1.73E-03	HLA-DQA1, **GATA3**, CD46, STAT5A, CCR2, CD74
GO: Biological Process GO:0060374	mast cell differentiation	2.28E-03	**GATA3**, STAT5A

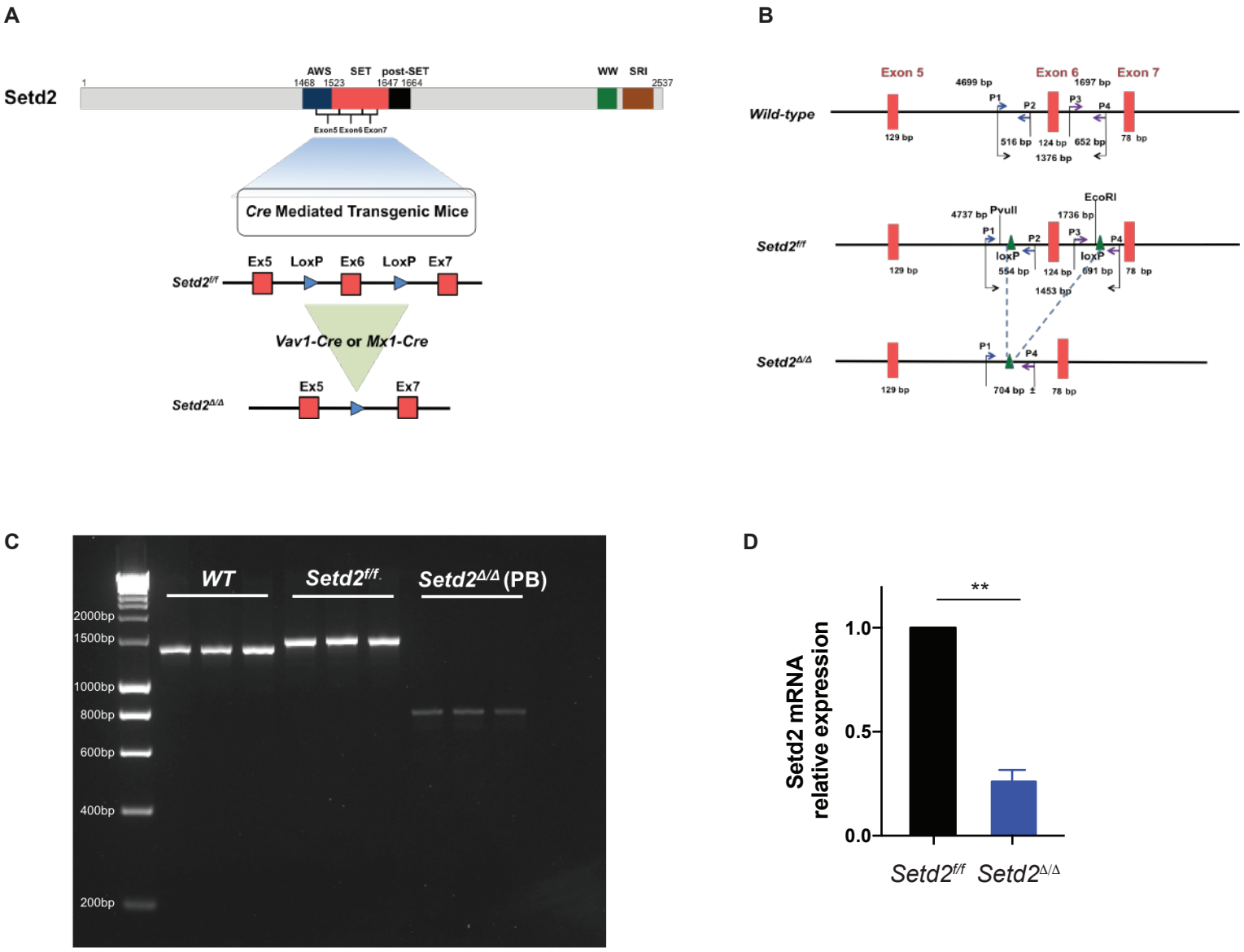

Figure S1. (A) Diagram of wild-type *Setd2* and *Cre* mediated transgenic mice. Homology motifs of SETD2 are shown as colored bars with the following indicated: AWS, associated with SET; SET, Su(var)3-9/Enhancer of zeste/Trithorax; post-SET, Cysteine-rich motif following a subset of SET domains; WW, domain with 2 conserved Trp(W) residues; SRI, Set2 Rpb1 interacting. A frame-shift deletion is designed by flanking exon 6 with LoxP sites. Thus Cre recombinase-mediated excision trigger nonsense-mediated decay of the mutant transcript and produce a null allele. (B). Diagram of wild-type SETD2 and Cre mediated transgenic mice. (C). The genotyping using tail tissue and peripheral blood by genomic PCR. (D).Relative *Setd2* gene expression levels were determined by qrt-PCR using c-Kit+ BM cells.

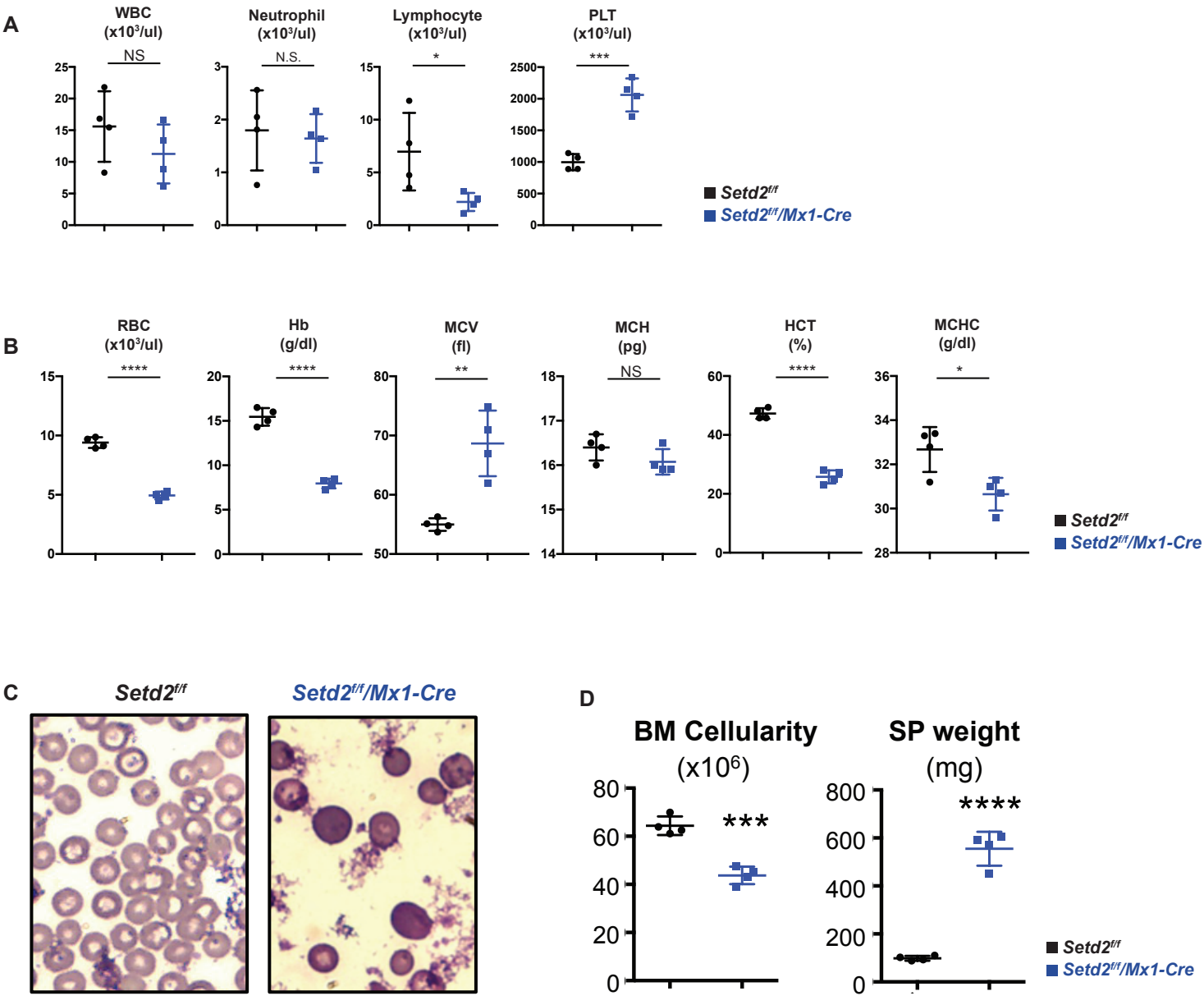

Figure S2. (A). Complete blood count of *Setd2$^{f/f}$/Mx1-Cre* mice, showing reduced white blood cells, lymphocytes, neutrophils, and platelet. (*n*=4 each genotype; mean ± SEM) (B). Complete blood count of *Setd2$^{f/f}$/Mx1-Cre* mice, showing reduced red blood cells, hemoglobin content, red blood cell specific volume (HCT), mean corpuscular hemoglobin concentration (MCHC), but increased mean corpuscular volume of red cells (MCV) and mean corpuscular hemoglobin (MCH).(*n=4* each genotype; mean ± SEM) (C). Representative photos of Wright's stained peripheral blood smear of Setd2f/f and Setd2f/f/Mx1-Cre mice. (D). BM cellularity and spleen weight of *Setd2$^{f/f}$* and *Setd2$^{f/f}$/Mx1-Cre* mice. (*n=4* each genotype; mean ± SEM)

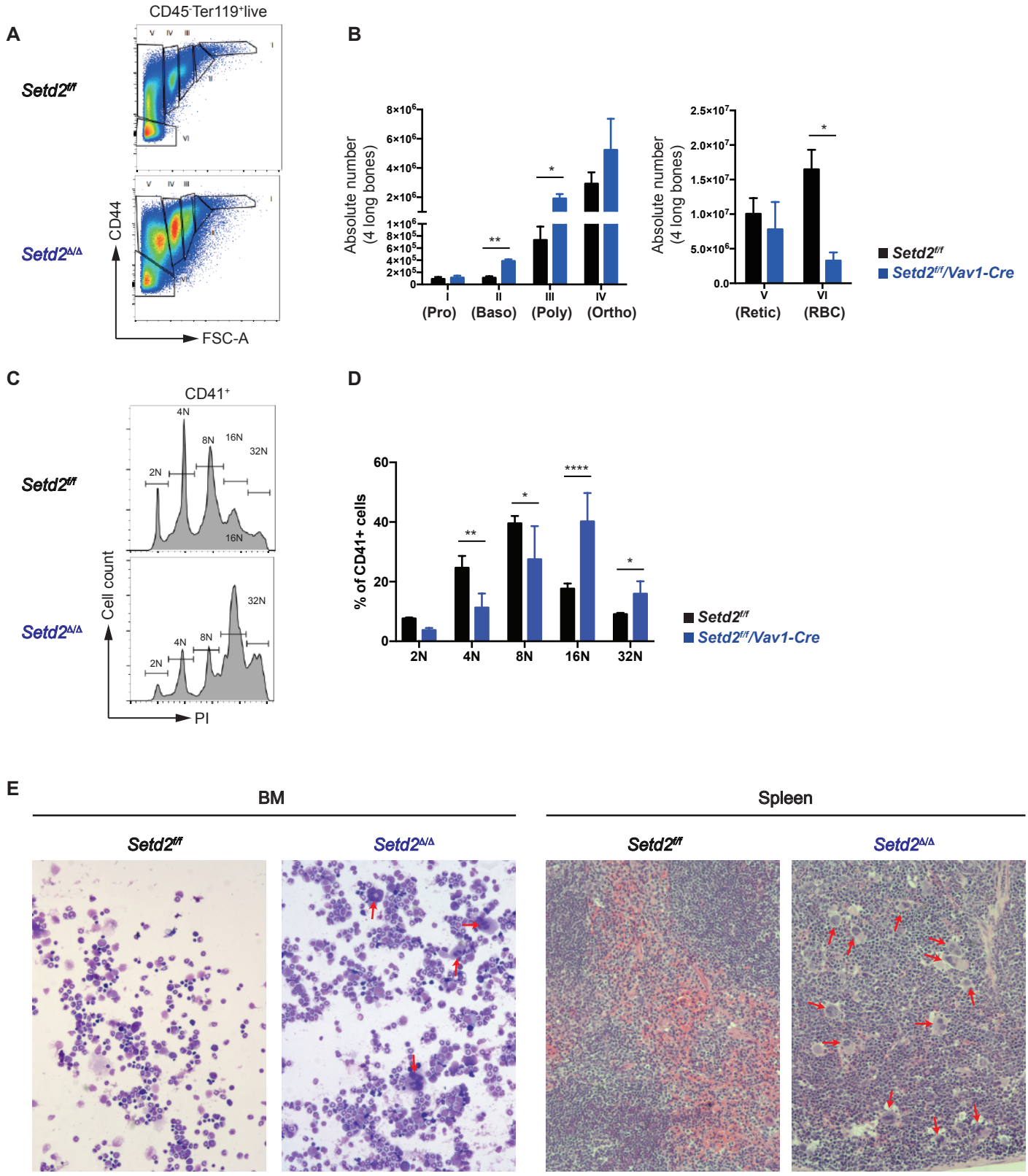

Figure S3. (A).Flow cytometry analysis of erythroid differentiation of *Setd2*$^{f/f}$ and *Setd2*$^{f/f}$*/Vav1-Cre* mice BM cells. (B). Absolute number of proerythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts (IV), reticulocytes (V), and mature RBCs (VI). (*n*=4 each genotype; mean ± SEM) (C). Flow cytometry analysis of ploidy distribution (PI staining) of CD41$^+$ bone marrow cells. (D). Percent of ploidy distribution in *Setd2*$^{f/f}$ and *Setd2*$^{f/f}$*/Vav1-Cre* mice CD41$^+$ BM cells. (*n*=3 each genotype; mean ± SEM) (E). Representative photos of Wright's stained BM cytospins and HE stained spleens of *Setd2*$^{f/f}$ *and Setd2*$^{f/f}$*/Vav1-Cre* mice: megakaryocytes were indicated by the red arrows.

Figure.S4 Zhou. *et al.*

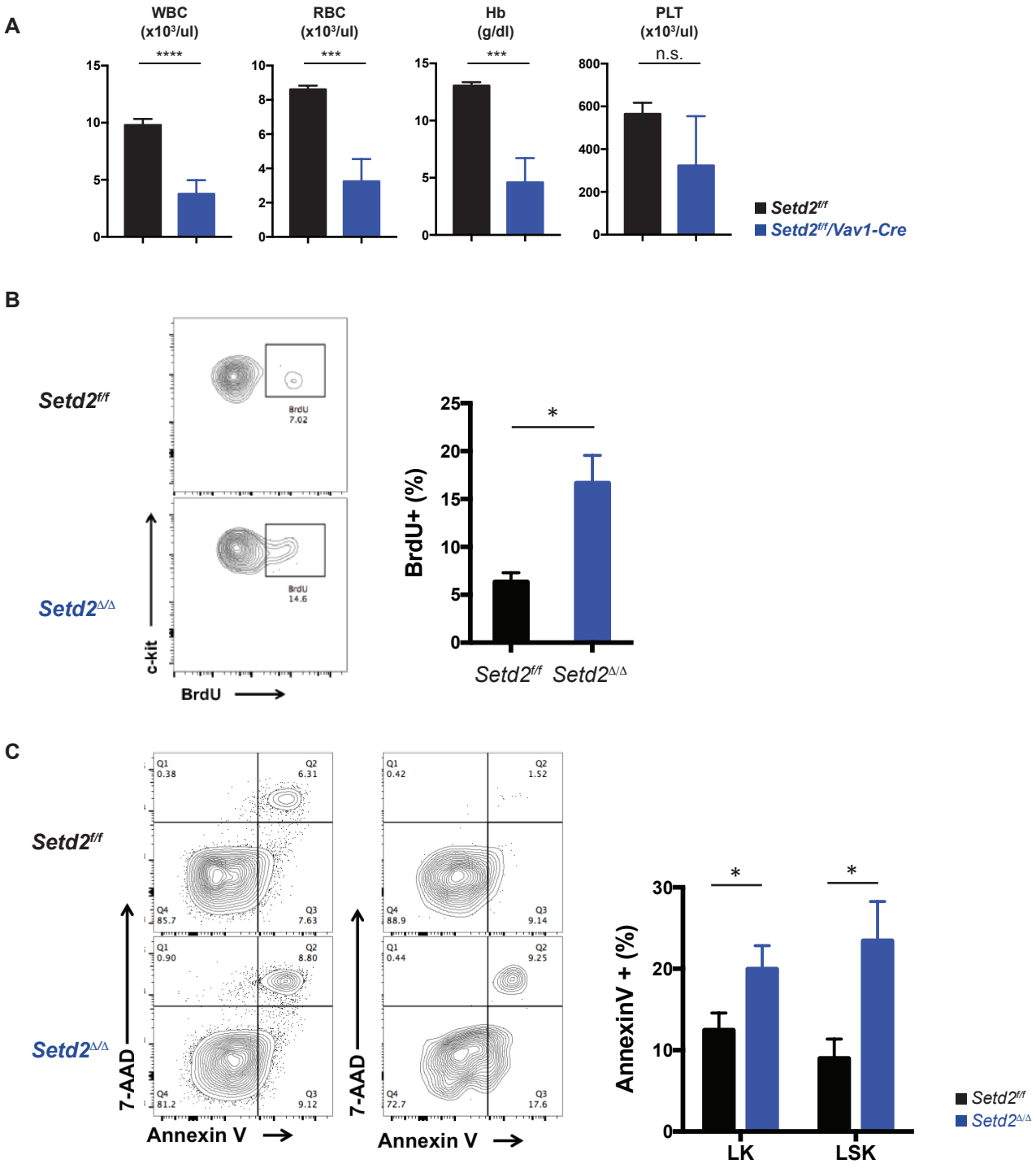

Figure S4. (A). Complete blood counts of peripheral blood were analyzed 4 weeks after BMT.(*n*=6 each genotype; mean ± SEM) (B). Flow cytometry analysis of *Setd2*$^{f/f}$ and *Setd2*$^{f/f}$*/Vav1-Cre* BM cells with BrdU in SLAM-HSC population.(*n*=3 each genotype; mean ± SEM) (C). Flow cytometry analysis of Setd2f/f and Setd2f/f/Vav1-Cre BM cells with Annexin V and 7-AAD in LK and LSK populations. (*n*=4 each genotype; mean ± SEM)

Figure.S5 Zhou. *et al.*

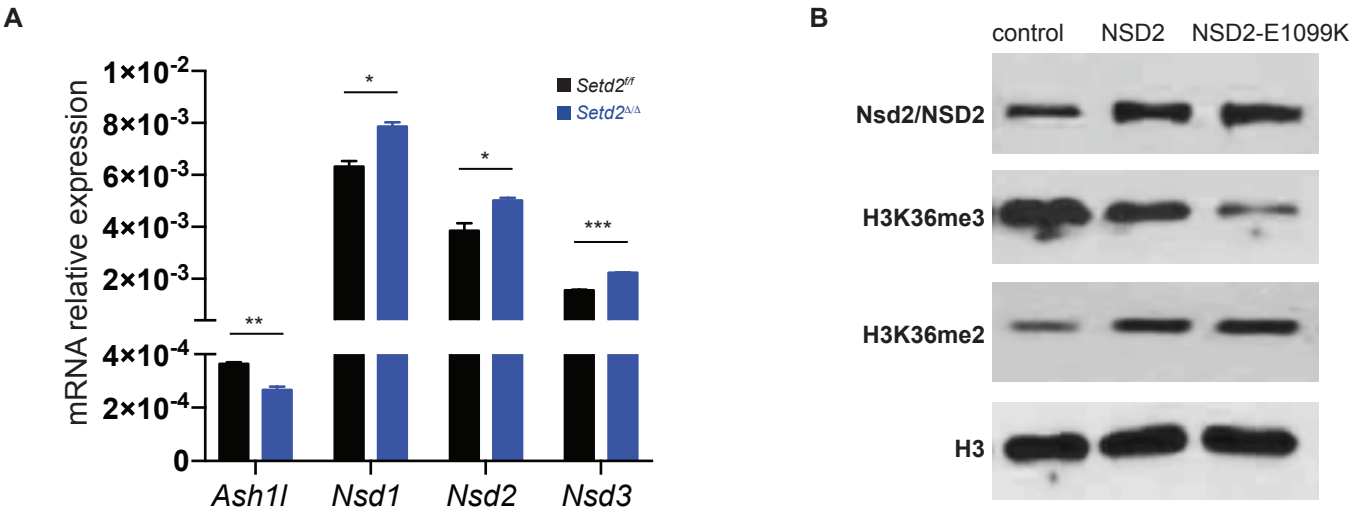

Figure S5. (A). Rrelative gene expression levels of Ash1l and Nsd1/2/3 were determined by qrt-PCR using *Setd2*$^{f/f}$ and *Setd2*$^{f/f}$*/Vav1-Cre* BM LSK cells. (*n*=2; mean ± SEM) (B). Mouse leukemia cell line transduced with MSCV-control, MSCV-NSD2-WT, and MSCV-NSD2-E1099K. Then cells were collected to determine the protein levels of Nsd2/NSD2, H3K36me2, H3K36me3, and H3.

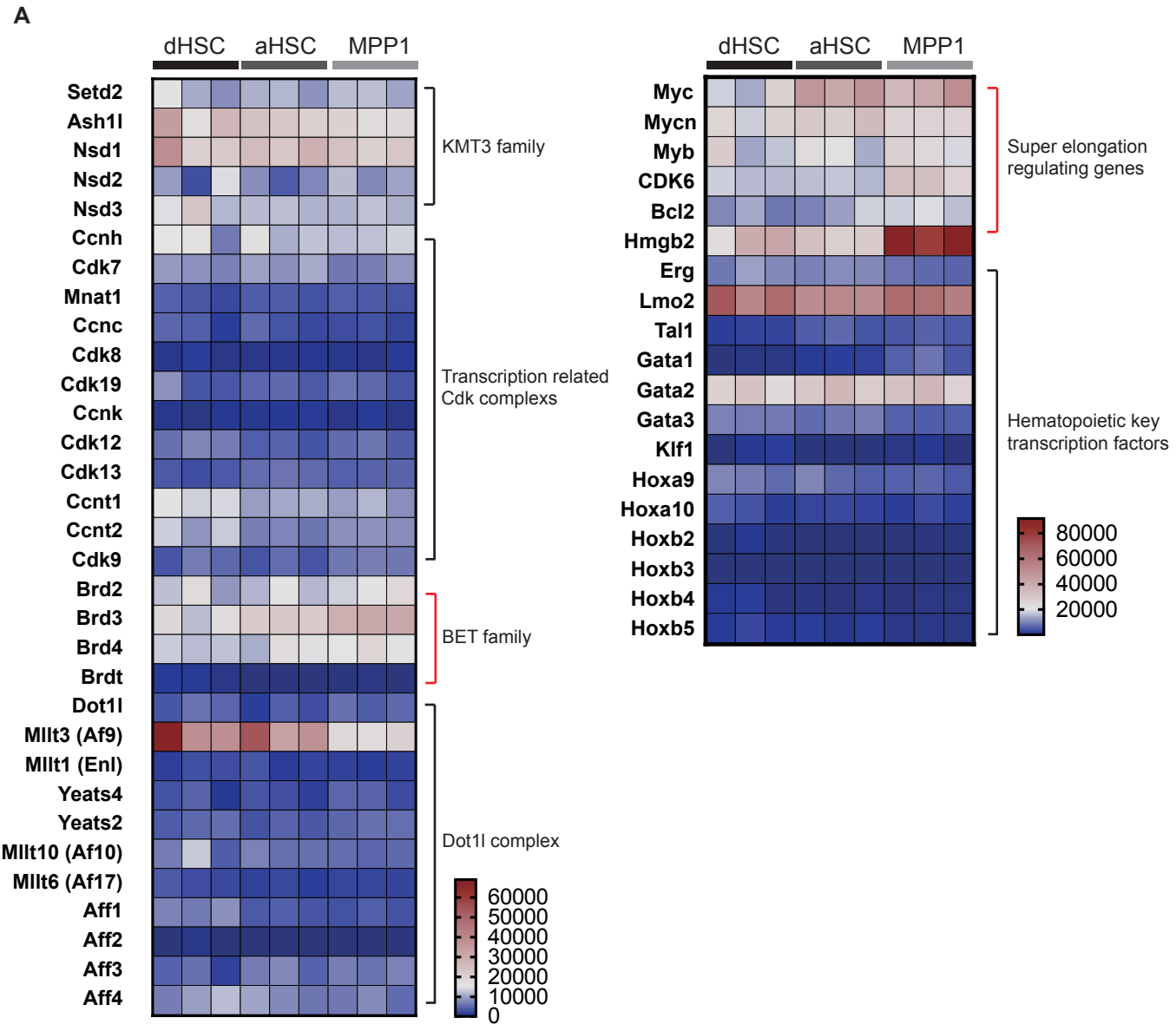

Figure S6. Heatmap illustration of gene expression profiles of selected genes in dormant HSC (dHSC), active HSC (aHSC), and multiple progenitors 1 (MPP1), using published RNA-seq-based transcriptional profiling (Cabzas-Wallscheid N, Buettner F, Sommerkamp P, Klimmeck D, Ladel L, Thalheimer FB, et al. Vitamin A-Retinoic Acid Signaling Regulates Hematopoietic Stem Cell Dormancy. Cell. 2017 May 18;169(5):807-23 e19.).

A GSE19429
Leukemia 2010 Apr; 24(4):756-64

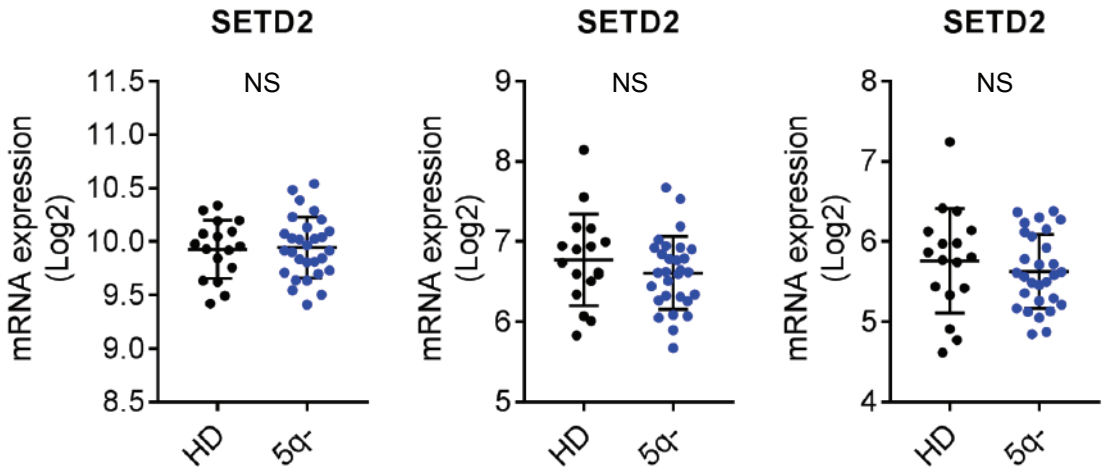

Figure S7. (A). The SETD2 expression levels were compared between healthy donors (HD) and MDS del(5q-) patients using published data (Leukemia 2010 Apr; 24(4):756-64).