# ASXL2 regulates hematopoiesis in mice and its deficiency promotes myeloid expansion

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## ABSTRACT

Chromosomal translocation t(8;21)(q22;q22) which leads to the generation of oncogenic RUNX1-RUNX1T1 (AML1-ETO) fusion is observed in approximately 10% of acute myelogenous leukemia (AML). To identify somatic mutations that co-operate with t(8;21)-driven leukemia, we performed whole and targeted exome sequencing of an Asian cohort at diagnosis and relapse. We identified high frequency of truncating alterations in *ASXL2* along with recurrent mutations of *KIT, TET2, MGA, FLT3,* and *DHX15* in this subtype of AML. To investigate in depth the role of ASXL2 in normal hematopoiesis, we utilized a mouse model of ASXL2 deficiency. Loss of ASXL2 caused progressive hematopoietic defects characterized by myeloid hyperplasia, splenomegaly, extramedullary hematopoiesis, and poor reconstitution ability in transplantation models. Parallel analyses of young and >1-year old Asxl2-deficient mice revealed age-dependent perturbations affecting, not only myeloid and erythroid differentiation, but also maturation of lymphoid cells. Overall, these findings establish a critical role for ASXL2 in maintaining steady state hematopoiesis, and provide insights into how its loss primes the expansion of myeloid cells.

## Introduction

Chromosomal translocation t(8;21) (q22;q22) is a frequent cytogenetic abnormality observed in approximately 10% of acute myelogenous leukemia (AML). This rearrangement involves *RUNX1* gene on chromosome 21 and *RUNX1T1* gene on chromosome 8 and results in generation of oncogenic RUNX1-RUNX1T1 fusion protein (also known as AML1-ETO).[1] However, expression of RUNX1-RUNX1T1 alone, either as a transgene or using viral transduction in hematopoietic cells, is insufficient to cause leukemia in mice.[2-4] Loss of sex chromosome and other chromosomal aberrations, as well as recurrent somatic mutations of *KIT, FLT3, NRAS* and *KRAS,* are associated with t(8;21) AML.[2,4] Recent high-throughput sequencing

approach has identified additional mutagenic events involving *ASXL2, ASXL1* and *DHX15* genes in t(8;21) AML.[5-10] Several secondary genetic events that co-operate with RUNX1-RUNX1T1 fusion in inducing leukemia have been demonstrated using mouse models.[2-4,11]

ASXL1, ASXL2 and ASXL3 are human homologs of Drosophila Asx (Additional sex combs) and function as epigenetic regulators through recruitment of Polycomb group repressor complexes (PRC) and Trithorax group activator complexes.[12,13] ASXL proteins can interact with BAP1, NCOA1, EZH2, WTIP and nuclear receptors, which suggests diverse functions of ASXL family members in epigenetic and transcriptional regulation.[12,13] Somatic mutations of *ASXL1* occur in a range of hematologic disorders,[14-20] and its deletion in mouse hematopoietic cells results in multilineage cytopenias and dysplasia, which are associated with global reduction of H3K27 trimethylation.[21] In contrast, somatic mutations of *ASXL2* are observed exclusively in t(8;21) AML,[5,8] while mutations of *ASXL3* are not reported in hematologic malignancies.[13] Silencing of *ASXL2* is partially embryonic lethal depending on the genetic background, and leads to congenital heart defects. Surviving homozygous *Asxl2* mutant mice are smaller than wild-type littermates, demonstrate skeletal homeotic transformations consistent with disruption of Polycomb/Trithorax complex functions, develop cardiac dysfunction, and exhibit decreased bone mineral density.[22-26] More recently, its role in normal hematopoietic development and leukemic development has been investigated.[27,28]

In the present study, we used exome sequencing to identify somatic mutations co-operating with RUNX1-RUNX1T1 rearrangement in AML. Frequent truncating mutations of *ASXL2* were identified in our t(8;21) AML cohort. Using an Asxl2-deficient mouse model, we aimed to clarify the function of ASXL2 in hematopoiesis. We demonstrate that ASXL2 is crucial to maintain hematopoietic stem cell (HSC) number and function. Loss of ASXL2 leads to myeloproliferation and extramedullary hematopoiesis, as well as to progressive defects in multilineage differentiation. These results establish ASXL2 as a key component of the epigenetic machinery involved in maintaining hematopoietic development.

## Methods

### Acute myelogenous leukemia samples

t(8;21) AML was diagnosed by karyotypic analysis and/or reverse transcriptase PCR assay for the detection of fusion transcript of RUNX1-RUNX1T1. Samples were collected with informed consent at diagnosis, during complete remission, and at time of frank relapse in accordance with the Declaration of Helsinki. Bone marrow (BM) mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation (1.077 g/mL; Amersham Pharmacia, Sweden). The study was approved by the institutional review boards of the respective institutes.

### Exome sequencing and somatic variant discovery

DNA was sheared using a Covaris instrument and assessed on a 2100 Bioanalyzer. Library preparation and exome sequencing were performed as described previously.[29] For whole exome sequencing, DNA were captured using SureSelect Human All Exon 50Mb kit (Agilent), according to the manufacturer's instructions. For targeted capture library, Agilent's SureSelect XT2 Target Enrichment System for Illumina Multiplexed Sequencing was used. RNA baits were designed to capture exons of 530 genes (*Online Supplementary Table S1*) and libraries were sequenced on HiSeq 2000 (Illumina).

100 bp paired-end reads were aligned to human reference genome (reference build: hg19) using bwa-mem aligner with default parameters. PCR duplicate reads were marked with samblaster.[30] Resulting BAM files were further processed according to GTAK best practices including INDEL re-alignment and base quality recalibration (*https://software.broadinstitute.org/gatk/best-practices/bp_3step.php?case=GermShortWGS*). Somatic variants were detected using Varscan2 somatic command.[31] Raw variants were processed with processSomatic command with the *P*-value set to 0.05 to obtain high confident variants. Variants were further processed using fpFilter per script to remove potential false positives (*https://github.com/ckandoth/variant-filter*). Resulting variants were annotated using Variant Effect Predictor and filtered against germline variants in both dbSNP and ExAC, while keeping deleterious and clinically significant variants.[32] For samples without germline controls, variants were called using MuTect using a panel of normal derived from in-house cohort of AML remission samples, as described previously.[29,33] Oncoplots were drawn using maftools Bioconductor package.[34] All the somatic mutations for whole exome sequencing and about 90% mutations reported for targeted exome sequencing were validated using PCR amplification and Sanger sequencing.

### Mice

Mice with *Asxl2* gene-trap allele [referred to as knockout (KO) allele in this study] have been described previously.[24] *Asxl2* heterozygous KO mice were maintained on C57BL/6J (B6) and 129Sv (129) genetic backgrounds. *Asxl2* homozygous null mice [and corresponding wild-type (WT) controls] used in all experiments were B6 x 129 F1 obtained by crossing *Asxl2* heterozygous B6 and 129 mice. Mouse colonies were housed and maintained at the animal facility of Comparative Medicine Centre, National University of Singapore (NUS). All mice experiments were approved by Institutional Animal Care and Use Committee, NUS, Singapore.

### Flow cytometry

Cells were incubated with fluorochrome-conjugated antibodies for 30 min on ice, washed and resuspended in SYTOX Blue Dead Cell Stain (ThermoFisher Scientific) before acquisition on FACS LSR II flow cytometer (BD Biosciences). Sorting of cells was performed on FACSAria cell sorter (BD Biosciences) and data were analyzed using FACSDIVA software (BD Biosciences). See *Online Supplementary Table S2* for the list of antibodies used for flow cytometric staining.

### Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 software.

### Accession numbers

The accession numbers for the sequencing data reported in this study are SRP122878 (exome sequencing) and GSE106798 (RNA sequencing).

## Results

### Mutational profile of RUNX1-RUNX1T1 AML

We performed whole-exome sequencing of 10 paired samples of newly-diagnosed and relapsed AML with RUNX1-RUNX1T1 rearrangement from an Asian cohort,

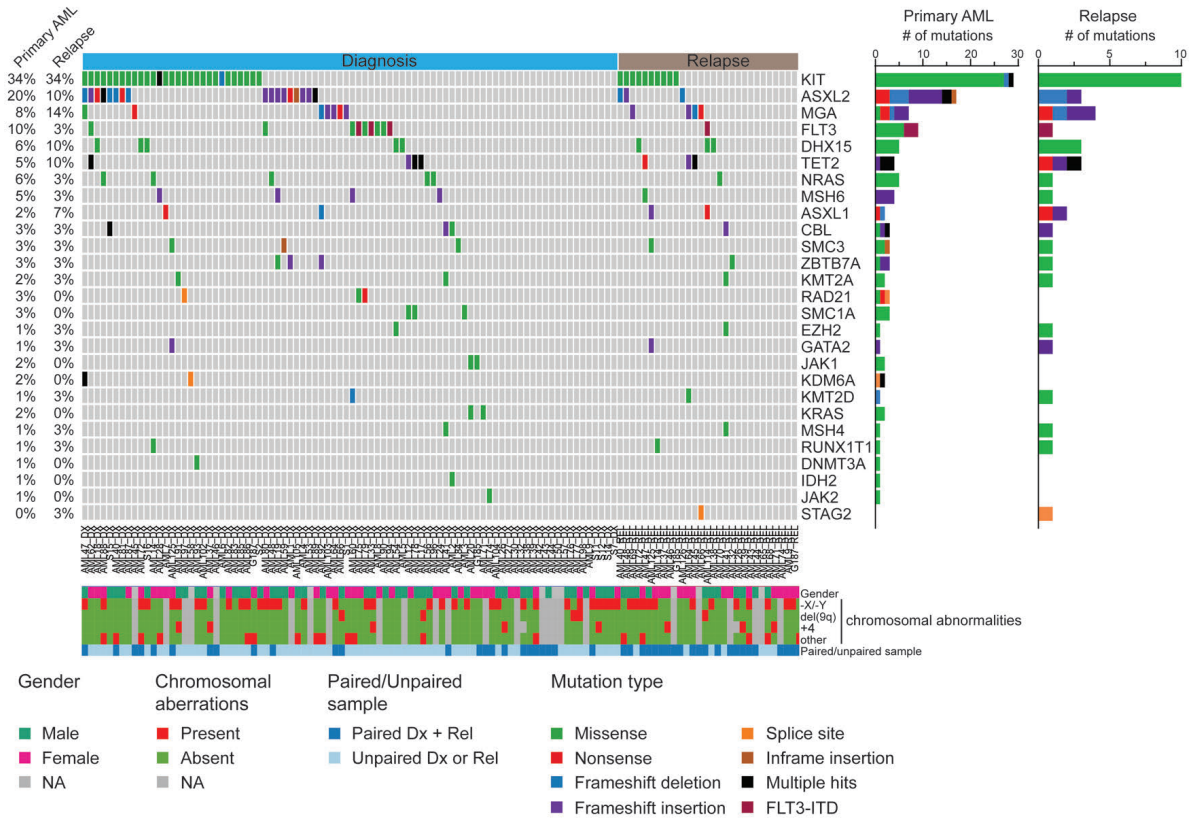


**Figure 1. Mutational landscape of t(8;21) acute myelogenous leukemia (AML).** Matrix displays individual somatic mutations detected in diagnosis and relapse AML with RUNX1-RUNX1T1 fusion. Mutational frequencies are illustrated on the left and bar graphs on the right depict absolute number and type of mutations. The annotation bars at the bottom display patient information including the cytogenetic aberrations detected in karyotype analysis. NA: information not available.

along with matched germline (complete remission) DNA (*Online Supplementary Table S3*). We achieved a mean depth of 100x (range 64-271x); an average 68% of nucleotides were covered by at least 20 reads (*Online Supplementary Figure S1*). We identified 55 somatic mutations (in 52 genes) in newly-diagnosed cases and 76 mutations (in 69 genes) at relapse, which included recurrent mutations in *KIT, TET2, DHX15* and *MGA* (*Online Supplementary Table S4*). We assessed the stability of mutations in diagnosis and relapse samples analyzed using whole exome sequencing. Overall, the disease evolution followed the pattern reported previously for AML,[35] as the founding clone or a subclone at diagnosis survived the therapy, gained additional mutations, and expanded at relapse (*Online Supplementary Figure S2*). Interestingly, all four mutations of *TET2* (in 3 cases) were acquired at relapse (*Online Supplementary Table S4*).

Further to uncovering the repertoire of co-operating mutations in t(8;21) AML, we analyzed mutational status of 530 genes (*Online Supplementary Table S1*) in 76 newly-diagnosed and 19 relapse t(8;21) AML cases using targeted-exome sequencing (*Online Supplementary Table S3*). In this cohort, the mean sequencing coverage across targeted bases was 96x (range 76-319x), with 70% of bases covered greater than 20x (*Online Supplementary Figure S1*).

Overall, we observed that the mutations of *KIT* were most frequent at both diagnosis and relapse in our cohort, along with recurring alterations in *ASXL2, MGA, DHX15, TET2* and *FLT3* genes (Figure 1, *Online Supplementary Table S5* and *Online Supplementary Figure S3*). Somatic mutations of *ASXL2* were detected in 20% of newly-diagnosed (17 of 86) and 10% of relapsed (3 of 29) cases while *ASXL1* was mutated at a much lower frequency (2% of newly-diagnosed cases and 7% of relapsed cases) (Figure 1, *Online Supplementary Table S5* and *Online Supplementary Figure S3*). Mutations of *DHX15*, a RNA helicase, occurred exclusively at Arg222 residue in 7 cases. *MGA* and *TET2* genes predominantly harbored nonsense and frameshift mutations spread throughout the transcript (Figure 1, *Online Supplementary Figure S3 and Online Supplementary Table S5*).

*ASXL2* was the second most frequently mutated gene, and interestingly, all alterations were truncating mutations located in exons 11 and 12 (Figure 1 and *Online Supplementary Figure S3*). Unlike alterations of *ASXL1*, which are recurrent in several hematologic diseases, recent reports[5,8,9] have highlighted the incidence of *ASXL2* mutations specifically in the t(8;21) subtype of AML. In this study, we aimed to investigate the consequences of deficiency of ASXL2 on hematopoietic development using a mouse model.

## Impaired hematopoiesis in *Asxl2*-deficient mice

Our RT-PCR analysis showed that *Asxl2* is expressed in a broad range of murine hematopoietic cell types (*Online Supplementary Figure S4A*). To investigate the physiological role of ASXL2 in steady-state hematopoiesis, we used gene-trap mice (referred to as *Asxl2* KO mice) described previous-

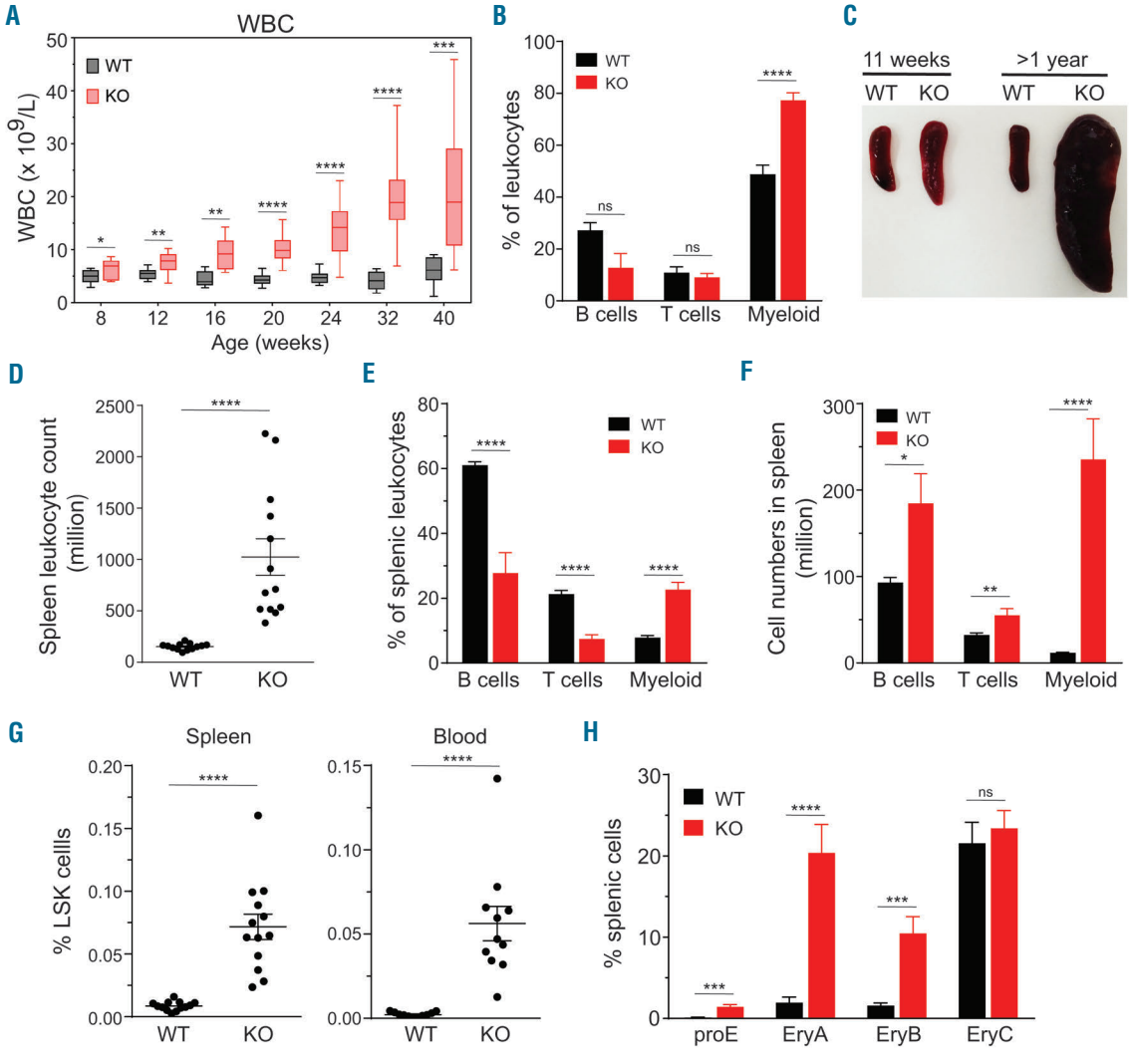


**Figure 2 Myeloproliferation and extramedullary hematopoiesis in *Asxl2*-deficient mice.** (A) White blood cell counts in peripheral blood of 8-10 week old wild-type (WT) and *Asxl2*-deficient mice. Whiskers extend from the minimum to the maximum values. (B) Proportion of B cells (CD19$^+$), T cells (CD3$^+$), and myeloid cells (CD11b$^+$) in the peripheral blood of >1-year old WT and *Asxl2* knockout (KO) mice determined using flow cytometry (B and T cells, n=3; myeloid cells, n=11). (C) Representative photograph of spleens isolated from young (11-week old) and old (>1-year old) WT and *Asxl2* KO mice. (D) Total leukocyte counts in spleens from >1-year old mice. (E and F) Frequencies (E) and absolute numbers (F) of lymphoid and myeloid cells in spleens of old WT and KO mice. Flow-cytometric analysis on splenocytes was similar to (B) (n=12-13). (G) Frequency of LSK cells in spleen and peripheral blood of old WT and KO mice. (H) Proportion of erythroid progenitors detected in flow cytometric analysis (staining with CD71 and TER119 antibodies) of spleens from old WT and KO mice (n=10). Bars represent mean±Standard Error of Mean. *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001, ns: not significant.

ly.[24] As reported,[24] *Asxl2* KO mice had a significantly shorter life span compared with the WT mice (*Online Supplementary Figure S4B*). We observed a significant reduction in *Asxl2* transcript and protein levels in the BM, spleen and thymus of *Asxl2* KO mice compared to WT mice (*Online Supplementary Figure S5A-F*), whereas the expression of *Asxl1* was not affected (*Online Supplementary Figure S5G-I*). We cultured the BM cells from WT and KO mice for two weeks in myeloid differentiation-promoting conditions and observed an increase in the proportion of CD11b$^+$ cells with a concomitant decrease in Lin$^-$Kit$^+$ cells in the cultures from *Asxl2*-deficient BM, suggesting altered myeloid differentiation (*Online Supplementary Figure S6A* and *B*). Moreover, in re-plating assays, we observed a sustained ability of *Asxl2* KO BM cells to generate myeloid colonies compared with WT cells (*Online Supplementary Figure S6C*).

To gain initial insights into the effect of ASXL2 deficiency on hematopoiesis *in vivo*, we periodically analyzed the peripheral blood (PB) from *Asxl2* KO and WT mice beginning at eight weeks of age. We observed an age-dependent increase in white leukocyte count in the PB of *Asxl2*-deficient mice, while the numbers were unchanged in the WT mice (Figure 2A). This was associated with reduced red blood cell (RBC) counts and hemoglobin in peripheral blood of *Asxl2* KO mice with increasing age (*Online Supplementary Figure S7*). This observation suggested progressive defects in the hematopoietic compartment in *Asxl2* KO mice, and therefore we investigated systematically hematopoietic development in >1-year old mice. Flow cytometric analysis of blood leukocytes from these mice showed a higher proportion of myeloid (CD11b$^+$) and a reduced proportion of lymphoid (CD19$^+$ and CD3$^+$) cells in KO mice (Figure 2B and *Online Supplementary Figure S8A*). Old *Asxl2* KO mice exhibited extensive splenomegaly (Figure 2C and D) which was associated with marked myeloproliferation evident by increased fre-

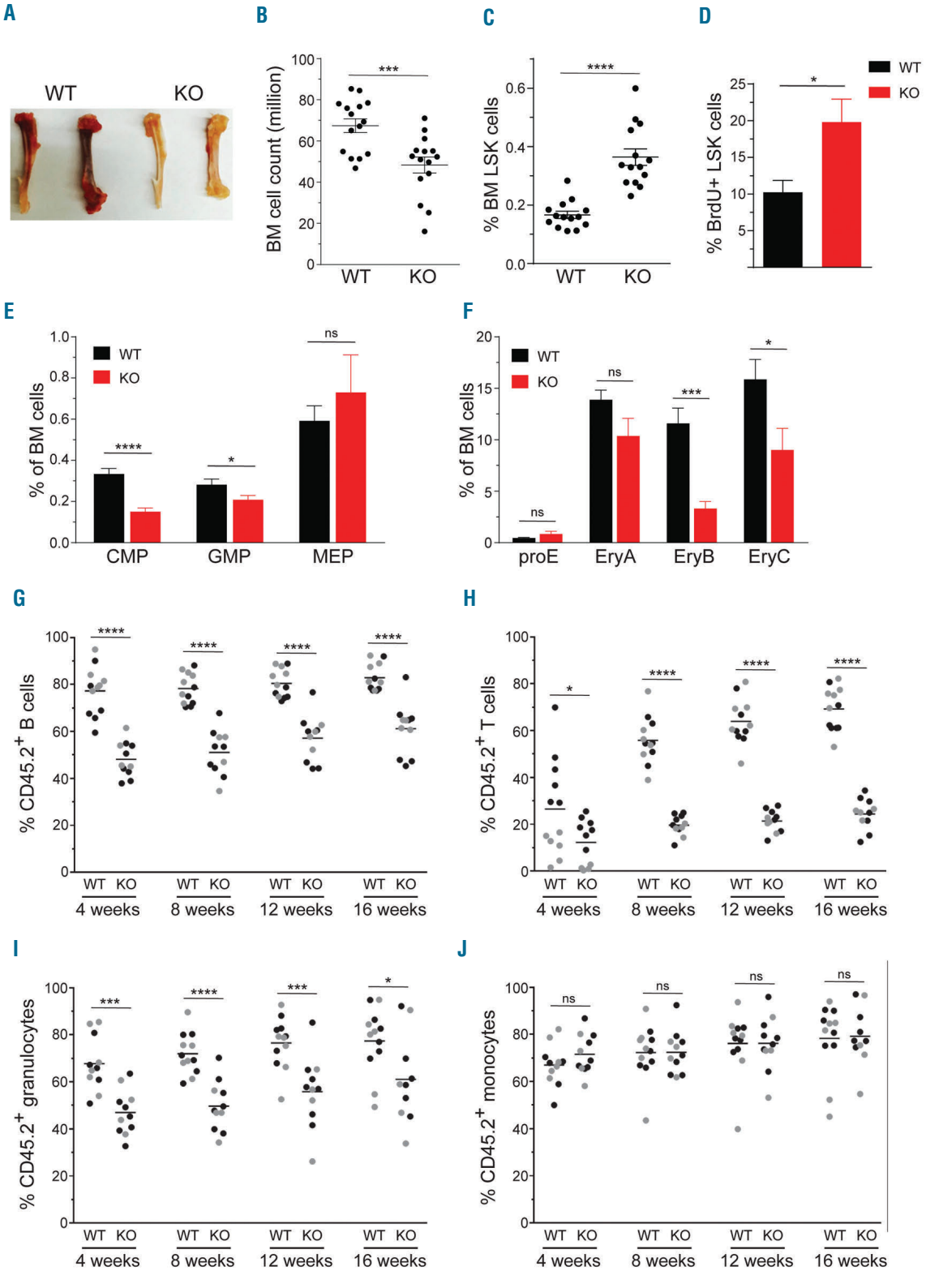


**Figure 3. ASXL2 deficiency perturbs frequency and function of hematopoietic stem cells (HSCs.)** (A) Representative photograph of tibias and femurs, respectively, from >1-year old wild-type (WT) and *Asxl2* knockout (KO) mice. (B) Bone marrow (BM) cellularity (two femurs and two tibias) in old WT and *Asxl2* KO mice. (C) Proportion of LSK cells in the BM of old mice (n=14). (D) Frequency of BrdU$^+$ LSK cells in the BM of 10-20-weeks old WT and *Asxl2* deficient mice (n=5). (E) Proportions of CMP, GMP and MEP in the BM of >1-year old WT and KO mice (n=12). (F) Frequency of erythroid precursors (proE: CD71$^+$TER119$^{lo}$, EryA: CD71$^+$TER119$^+$FSC$^{hi}$, EryB: CD71$^+$TER119$^+$FSC$^{lo}$ and EryC: CD71$^-$TER119$^+$FSC$^{lo}$) in BM of >1-year old WT and *Asxl2* KO mice (n=8). (G-J) Competitive repopulation assay: 4000 (black circles) or 2000 (gray circles) LSK cells were injected in recipient mice and proportion of donor-derived cells (CD45.2$^+$) in peripheral blood was determined at 4, 8, 12 and 16 weeks post transplantation. Ability of WT and *Asxl2* KO LSK cells to reconstitute B cells (CD19$^+$) (G), T cells (CD3$^+$) (H), granulocytes (CD11b$^+$Gr1$^+$), (I) and monocytes (CD11b$^+$Gr1$^-$F4/80$^+$) (J) was analyzed using flow cytometry. Bars represent mean±Standard Error of Mean. *$P$<0.05, ***$P$<0.001, ****$P$<0.0001, ns: not significant.

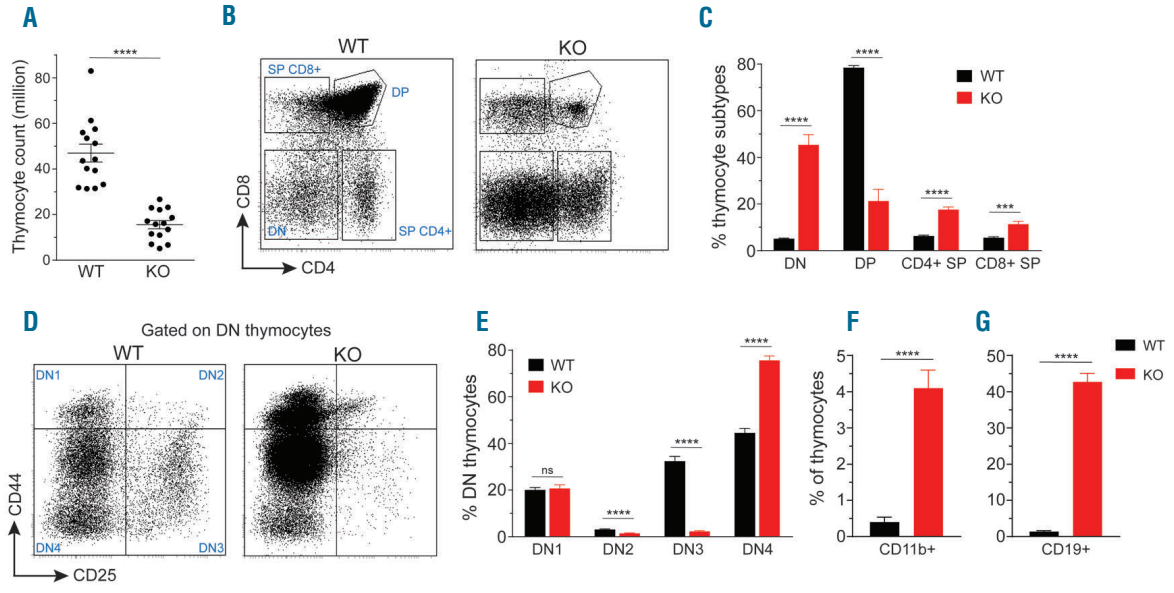


**Figure 4. ASXL2 is essential for normal thymocyte maturation.** (A) Thymocyte count in >1-year old wild-type (WT) and *Asxl2* knockout (KO) mice. (B) Representative FACS plots depict the proportion of DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), $CD4^+$ single positive (SP) and $CD8^+$ SP cells in the thymus of old WT and *Asxl2* KO mice. (C) Frequencies of DP, DN, $CD4^+$ SP and $CD8^+$ SP populations in old WT (n=17) and *Asxl2* KO (n=18) mice. (D) FACS plots show representative flow cytometric staining for surface expression of CD44 and CD25 within the DN population in the thymus of old mice. (E) Proportions of DN1 ($CD44^+CD25^-$ DN), DN2 ($CD44^+CD25^+$ DN), DN3 ($CD44^-CD25^+$ DN) and DN4 ($CD44^-CD25^-$ DN) sub-populations within the DN compartment of thymus of old WT and KO mice. (F and G) Frequencies of myeloid cells ($CD11b^+$) (F) and B cells ($CD19^+$) (G) in the thymus of >1-year old WT and KO mice (n=5-7). Data are represented as mean±Standard Error of Mean. ***$P$<0.001, ****$P$<0.0001.

quency of $CD11b^+$ cells and myeloperoxidase-positive cells (Figure 2E and *Online Supplementary Figure S8B* and *C*). The absolute numbers of $CD11b^+$ myeloid cells were significantly increased (20-fold) while the number of lymphoid cells were 2-fold higher in the spleens of KO mice compared with the WT mice (Figure 2F). Histological examination of spleens demonstrated loss of normal architecture in KO mice compared with the WT mice (*data not shown*). Moreover, extramedullary hematopoiesis in the KO spleens was evident by an elevated proportion and numbers of total $Lin^-Kit^+$ and $Lin^-Kit^+Sca1^+$ (LSK) cells (Figure 2G and *Online Supplementary Figure S9A-D*) as well as significantly higher frequency of erythroid progenitors (populations proE, EryA and EryB) (Figure 2H and *Online Supplementary Figure S9E*). We also detected markedly increased frequency of stem/progenitor cells in the peripheral blood of old KO mice (Figure 2G and *Online Supplementary Figure S9F*).

Parallel analyses of spleens from young KO mice (8-14 weeks old) revealed modestly increased spleen size with a trend towards elevated proportion and number of LSK cells (*Online Supplementary Figure S10A-C*). A propensity for increased frequencies of myeloid cells ($CD11b^+$) and erythroid progenitors was also noted in the spleens of young KO mice (*Online Supplementary Figure S10D* and *E*). This indicates an onset of extramedullary hematopoiesis in young mice, which manifests by marked myeloid and erythroid cell expansion as the mice grow older.

## ASXL2 deficiency results in defective differentiation and function of hematopoietic stem cells

ASXL2 deficiency resulted in paler bones and decreased marrow cellularity in old mice (Figure 3A and B). Notably, BM of old *Asxl2* KO mice had higher frequency and absolute number of LSK cells compared with the WT mice (Figure 3C and *Online Supplementary Figure S11A* and *B*). The proportion of LT-HSCs ($CD34^-Flt3^-LSK$), ST-HSCs ($CD34^+Flt3^-LSK$) and MPPs ($CD34^+Flt3^+LSK$) within the LSK compartment was largely unaltered (*Online Supplementary Figure S11C*), albeit an overall increase in the absolute number of these subpopulations occurred in the old KO mice (*Online Supplementary Figure S11D* and *E*). We also observed reduced BM cellularity in young KO mice; and although the proportion of LSK cells was not significantly altered, the frequency and absolute numbers of LT-HSCs were significantly higher, suggesting early defects in maintaining HSC frequency (*Online Supplementary Figure S11F-J*). *In vivo* BrdU incorporation assay uncovered a significantly higher frequency of $BrdU^+$ LSK cells in the BM of *Asxl2* KO mice, indicating increased cycling of stem/progenitor cells lacking ASXL2 (Figure 3D). These results illustrate that ASXL2 is required for maintaining the number and self-renewal of HSCs during steady-state hematopoiesis.

Flow cytometric analyses also demonstrated decreased proportion and number of common myeloid precursors (CMP; $Lin^-Kit^+Sca1^-CD34^+FcγRII/III^{lo}$) and granulocyte monocyte precursors (GMP; $Lin^-Kit^+Sca1^-CD34^+FcγRII/III^{hi}$) in >1-year old *Asxl2* KO mice (Figure 3E and *Online Supplementary Figure S12A* and *B*). Furthermore, a marked reduction in the frequencies of erythroid precursors was noted in the old KO mice (Figure 3F and *Online Supplementary Figure S12C*), indicating impaired erythropoiesis. Reduction in erythroid precursors as well as CMP and GMP populations was also apparent in the BM of the young KO mice, signifying an early inception of defects in erythroid and myeloid differentiation because of ASXL2 deficiency (*Online Supplementary Figure S12D-F*).

Further, multi-lineage reconstitution ability of *Asxl2*-deficient HSCs was assessed in a competitive repop-

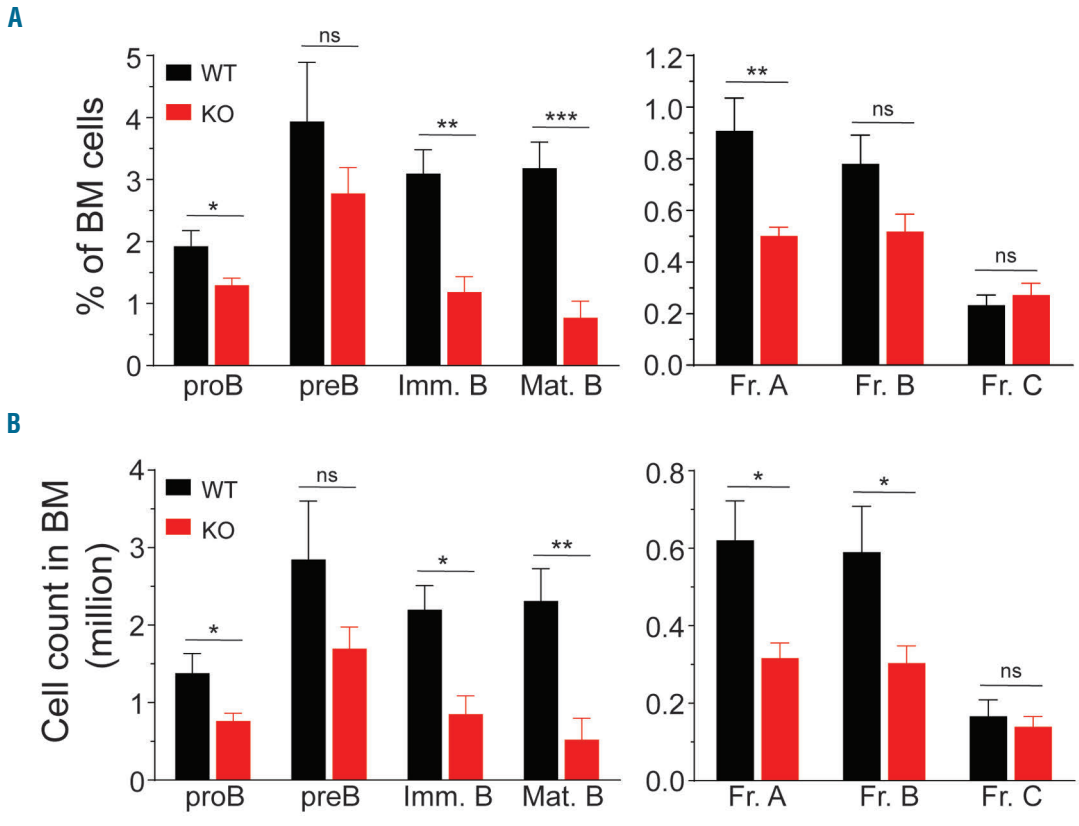


**Figure 5. Impaired B-cell lymphopoiesis in *Asxl2*-deficient mice.** (A and B) Proportion (A) and absolute number (B) of cells at different stages of B-cell development in the bone marrow of >1-year old WT and *Asxl2* KO mice (n=4-6). proB: $CD43^+B220^+$, preB: $CD43^-B220^+IgM^-$, immature B (Imm. B): $CD43^-B220^+IgM^+$, mature B (Mat. B): $CD43^-B220^{hi}IgM^+$, Fraction A (Fr. A): $CD24^-BP1^-$ proB cells, Fraction B (Fr. B): $CD24^+BP1^-$ proB cells, Fraction C (Fr. C): $CD24^+BP1^+$ proB cells. Data are represented as mean±Standard Error of Mean. **P*<0.05, ***P*<0.01, ****P*<0.001, ns: not significant.

ulation assay. ASXL2 deficiency resulted in poor reconstitution of lymphoid lineage (both B and T cells) compared with the WT cells (Figure 3G and H). The reconstitution of granulocytes was also impaired in mice transplanted with *Asxl2*-deficient LSK cells (Figure 3I), while the proportion of donor-derived monocytes was similar to the recipients transplanted with WT cells (Figure 3J). These repopulation assays demonstrated that ASXL2 is essential for differentiation into multiple hematopoietic lineages including the lymphoid lineage, prompting us to investigate the development of lymphoid lineage in the *Asxl2* KO mice.

## Impaired thymopoiesis in mice lacking ASXL2

A marked reduction of thymus size and cellularity was a consistent feature in >1-year old *Asxl2* KO mice compared with the WT mice (Figure 4A). Flow cytometric analyses revealed a striking reduction in $CD4^+CD8^+$ double positive (DP) cells and a concomitant increase in the proportion and number of $CD4^-CD8^-$ double negative (DN) cells in the thymi of *Asxl2* KO mice (Figure 4B and C and *Online Supplementary Figure S13A*). Within the DN compartment, the proportion of DN1 ($CD44^+CD25^-$) subpopulation was unchanged, whereas a significant reduction was detected in the proportion of DN2 ($CD44^+CD25^+$) and DN3 ($CD44^-CD25^+$) subpopulations, indicating a partial block in differentiation of earliest thymocytes (Figure 4D and E). A large number of DN cells in the thymi of KO mice consisted primarily of $CD25^-$ cells, present in the DN1 and DN4 gates (Figure 4D and *Online Supplementary Figure S13B*). Further characterization showed that the vast majority of cells in the DN gate were $CD19^+$ (which contributed to 40% of total thymus-residing hematopoietic cells), and the frequency and number of $CD11b^+$ myeloid cells were also noticeably increased in the thymus of KO mice compared with the WT mice (Figure 4F and G and *Online Supplementary Figure S13C-F*).

Young *Asxl2* KO mice exhibited decreased thymus cellularity, but displayed less pronounced effect on major thymic compartments compared with the >1-year old mice (*Online Supplementary Figure S13G-I*). These findings illustrate that ASXL2 is essential for maintaining normal thymopoiesis in mice and its deficiency leads to progressive impairment in thymocyte maturation, which is manifested by a partial block from the DN to the DP stage, and an accumulation of myeloid and B cells in the thymus of the old mice.

## Defects in B-cell development in *Asxl2* knockout mice

We also examined the consequences of ASXL2 deficiency on B-cell development in the BM by examining the expression of several surface antigens, which define the advancing stages of B-cell maturation. While the young *Asxl2* KO mice showed largely conserved subsets during B-cell lymphopoiesis, a notable decrease in the proportion of mature B cells ($CD43^-IgM^+B220^{hi}$) compared with the WT mice was observed (*Online Supplementary Figure S14*).

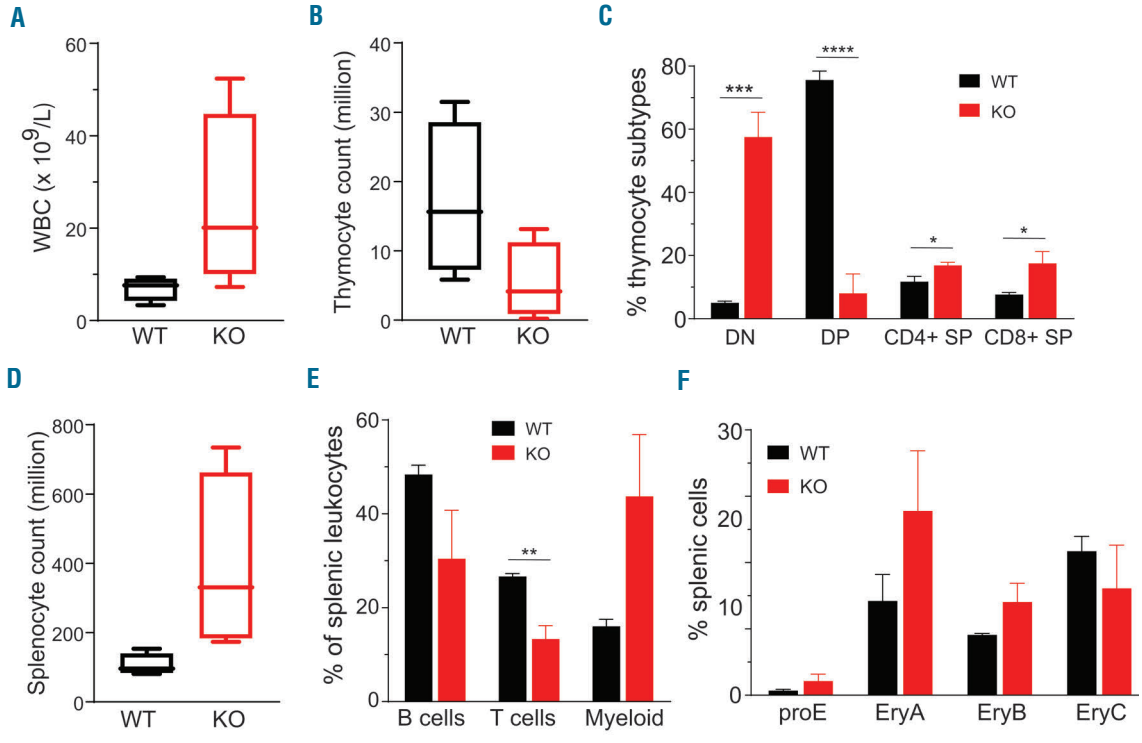


**Figure 6. Cell-intrinsic effects of *Asxl2* deficiency on lymphoid and myeloid lineages.** Lethally irradiated mice transplanted with either wild-type (WT) or *Asxl2* knockout (KO) bone marrow (BM) cells were analyzed after one year. (A) Number of white blood cells (WBCs) in the peripheral blood of recipient mice. (B) Number of thymocytes in recipient mice. (C) Proportion of donor-derived (CD45.2$^+$) DN, DP, CD4$^+$SP and CD8$^+$SP cells in thymi of recipient mice. (D) Spleen cellularity. (E) Percentages of CD45.2$^+$ B, T and myeloid cells in the spleen of mice transplanted with either WT or KO cells. (F) Frequencies of erythroid precursors in spleens of recipient mice. Bars represent mean±Standard Error of Mean. *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001.

However, the BM of old KO mice showed a substantial reduction in the frequencies and absolute number of proB (CD43$^+$B220$^+$), preB (CD43$^-$IgM$^-$B220$^+$), immature B (CD43$^-$IgM$^+$B220$^+$), and mature B cells (Figure 5A and B), indicating an age-dependent paucity in B-cell development. Further fractionation of proB cells using the scheme proposed previously,[36] revealed a lower proportion of CD24$^-$BP1$^-$ (Fraction A) and CD24$^+$BP1$^-$ (Fraction B) proB cells in the old *Asxl2* KO mice (Figure 5A and B), demonstrating a partial arrest of B-cell maturation.

## Cell-intrinsic role of ASXL2 in hematopoiesis

To test the cell-autonomous function of ASXL2 in hematopoietic development, we transplanted lethally irradiated mice with either WT or *Asxl2* KO BM cells and analyzed the hematopoietic compartment one year later. We observed that the recipient mice transplanted with *Asxl2* KO BM cells tended to have higher WBC counts in peripheral blood (Figure 6A), similar to the age-dependent increase in WBC observed in KO mice (Figure 2A). We also noted decreased thymocyte cellularity and accumulation of donor-derived DN cells in the thymi of mice transplanted with *Asxl2* KO BM compared with those transplanted with WT BM (Figure 6B and C). Spleens of *Asxl2* KO BM-recipients were enlarged and exhibited elevated proportion of myeloid cells (Figure 6D and E). Moreover, our analysis also indicated a higher proportion of erythroid precursors in spleens of mice reconstituted with *Asxl2* KO BM cells compared with the WT cells (Figure 6F), similar to the phenotype observed in old *Asxl2* KO mice. Overall, the recipient mice recapitulated hematopoietic features of old *Asxl2* KO mice, suggesting a hematopoietic cell-intrinsic function of ASXL2.

## Identification of altered gene expression in ASXL2-deficient hematopoietic stem cells

To determine the molecular basis of the defects observed in *Asxl2* KO HSCs, we performed global gene expression profiling (RNA-Sequencing) of sorted LSK cells from old and young WT and *Asxl2* KO mice. Comparison of transcriptomic profiles of LSK cells from old WT and *Asxl2* KO identified more than 2500 genes differentially expressed (983 genes up-regulated and 1653 genes down-regulated in the KO cells; FDR<0.1) (*Online Supplementary Table S6*), including those involved in myeloid differentiation such as *Csf1, Gfi1b, Gata2, Hoxa9, Hoxa5, Mpl, Cdk6, Ccr1* and *Ets1* (*Online Supplementary Figure S15A*). *Asxl2*-deficient LSK cells from old mice exhibited more pronounced changes in gene expression compared with the young mice (Figure 7A and B and *Online Supplementary Figure S15B*). However, a significant overlap of genes commonly up-regulated and down-regulated in the KO LSK cells was noted within the two age groups (Figure 7C and *Online Supplementary Table S6*). Interestingly, GSEA analysis revealed that the expression of RUNX1-RUNX1T1 targets[37] inversely correlated in the KO cells (Figure 7D and *Online Supplementary Figure S15C*). Also, genes down-regulated in immature BM progenitors upon silencing of *CBFA2T3* (also called *ETO2*)[38] were also suppressed in the LSK cells from both old and young *Asxl2* KO mice (Figure 7E and *Online Supplementary Figure S15D*). *CBFA2T3* is involved in the translocation t(16;21)(q24;q22) with

*RUNX1* in AML, a disease with phenotypic and transcriptional profile reminiscent of t(8;21) AML.[39,40]

## Discussion

This study, along with recent reports,[5-10] provides a comprehensive landscape of the mutational spectrum of t(8;21) AML, which is distinctive from other subtypes of AML and characterized by high frequency of alterations of *KIT, ASXL2, DHX15* and *MGA* genes; and rare mutations of other commonly altered genes in myeloid leukemia (*DNMT3A, RUNX1, IDH2, NPM1*). Truncating mutations of *ASXL2* occurred in 20% of newly-diagnosed t(8;21) AML cases, and were localized to exons 11 and 12, a pattern highly reminiscent of the mutational profile for *ASXL1* in hematologic malignancies.[41,42] However, unlike *ASXL1*, which is altered in a diverse range of hematologic diseases, mutations of *ASXL2* are specific to the t(8;21) AML subtype. We also noted that the mutational frequency of RAS pathway genes (*NRAS* and *KRAS*) and *ZBTB7A* in our Asian cohort of t(8;21) AML were notably lower

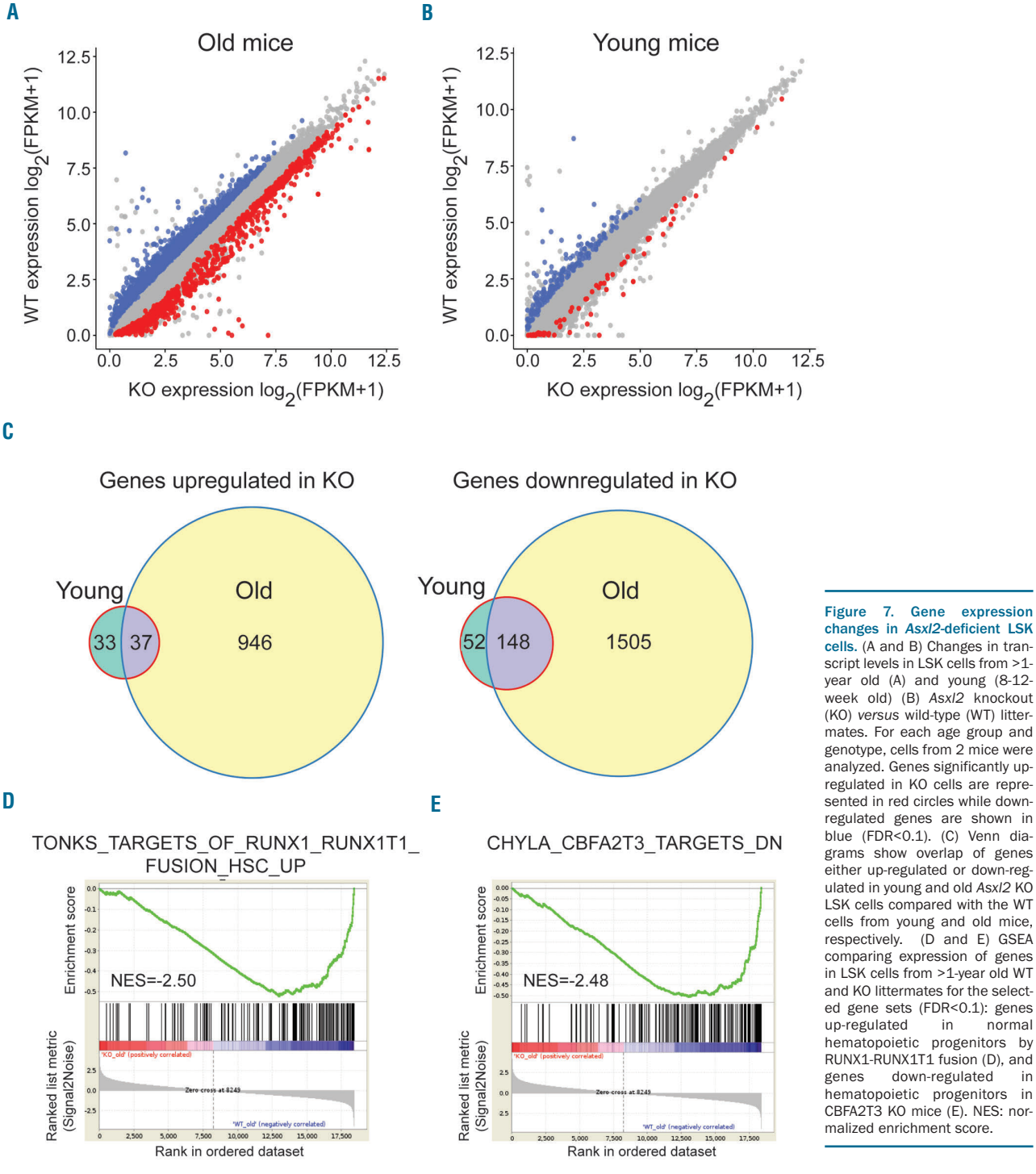


**Figure 7. Gene expression changes in *Asxl2*-deficient LSK cells.** (A and B) Changes in transcript levels in LSK cells from >1-year old (A) and young (8-12-week old) (B) *Asxl2* knockout (KO) *versus* wild-type (WT) littermates. For each age group and genotype, cells from 2 mice were analyzed. Genes significantly up-regulated in KO cells are represented in red circles while down-regulated genes are shown in blue (FDR<0.1). (C) Venn diagrams show overlap of genes either up-regulated or down-regulated in young and old *Asxl2* KO LSK cells compared with the WT cells from young and old mice, respectively. (D and E) GSEA comparing expression of genes in LSK cells from >1-year old WT and KO littermates for the selected gene sets (FDR<0.1): genes up-regulated in normal hematopoietic progenitors by RUNX1-RUNX1T1 fusion (D), and genes down-regulated in hematopoietic progenitors in CBFA2T3 KO mice (E). NES: normalized enrichment score.

than previous reports from Western populations.[5,7-9,40,43] We obtained a mutational rate of 6% for *NRAS* and 2% for *KRAS* compared to 13-26% for *NRAS* and 4-7% for *KRAS* observed in previous studies.[5,7-9,43] Similarly, *ZBTB7A* mutations that are frequent in Caucasian t(8;21) AML (9-23%)[9,40,43] were observed in only 3% of our Asian patient cohort. Although this might reflect a true difference between genetic backgrounds, this requires further confirmation in independent t(8;21) AML cohorts from different genetic backgrounds.

In this study, we focused on functional characterization of *Asxl2* in hematopoietic differentiation using a mouse model of *Asxl2* deficiency. Mice used in this study were C57BL/6 x 129Sv F1 as the animals in either C57BL/6 or 129Sv background die perinatally,[23] which precludes investigation of *Asxl2* knockout on an inbred genetic background. Moreover, this mouse model exhibits constitutive loss of *Asxl2*, which is distinct from the truncating somatic mutations of *ASXL2* observed in t(8;21) AML that may possibly generate a C-terminal truncated protein. Despite these limitations, our study strengthens the findings concerning the key role of ASXL2 in hematopoiesis reported in two recent studies.[27,28] Furthermore, our study reports several additional features of *Asxl2* deficient mice, which had not been described previously. While the significance of ASXL2 in maintaining normal hematopoiesis is evidenced from these studies, we performed a parallel in-depth analysis in young (8-14 weeks old) *versus* old (>1-year old) mice, which helped establish the progressive phenotype associated with *Asxl2* deficiency, including uncovering the crucial function of ASXL2 in development of the lymphoid lineage. Age-dependent defects in hematopoiesis in the knockout mice were characterized by an increased proportion of LSK cells and reduced frequencies of CMP and GMP cells in the BM of >1-year old KO mice. *Asxl2*-deficient mice also exhibited a progressive myeloproliferative phenotype, accompanied by increased peripheral WBC counts, splenomegaly and extramedullary hematopoiesis, indicating perturbed hematopoiesis. In addition, erythroid maturation was impaired in the old mice lacking ASXL2, signifying age-dependent defects in erythroid development. Both previous studies showed that ASXL2 deficiency led to poor reconstitution ability of HSCs in transplantation models, consistent with our findings. We observed that *Asxl2*-deficient LSK cells exhibited poor reconstitution ability of the lymphoid lineage and flow cytometric analyses of >1-year old mice demonstrated defects in both T-cell maturation in the thymus and B-cell development in the BM of the old KO mice. Apart from the intrinsic effect of ASXL2 on lymphoid differentiation, chronic overproduction of myeloid cells and accompanying inflammatory signals in the mice lacking ASXL2 may also negatively affect the lymphoid output. These findings establish ASXL2 as an essential component of hematopoietic development in mice.

Gene expression analysis of sorted LSK cells from WT and *Asxl2* KO BM identified several key regulators of hematopoiesis as downstream targets of ASXL2 in HSCs. Prominently, GSEA analysis revealed that the expression of genes regulated by RUNX1-RUNX1T1 correlated with the expression of ASXL2, indicating ASXL2 is possibly required for transcriptional activity of RUNX1-RUNX1T1. An identical finding was described recently for *Asxl2*-deficient LSK cells in an independent mouse model. Here the authors also detected binding of ASXL2 to the genomic loci occupied by RUNX1 and RUNX1-RUNX1T1, although no direct interaction between ASXL2 and either RUNX1 or RUNX1-RUNX1T1 was observed.[27] Further studies are needed to investigate whether ASXL2 acts as a likely co-regulator of RUNX1-RUNX1T1 during leukemogenesis. GSEA also indicated a correlation between *Asxl2* deficiency and the expression levels of reported targets of BMI1 (a PRC1 member)[44] and SUZ12 (a PRC2 member)[45] (*Online Supplementary Figure S15E* and *F*), consistent with a role for ASXL2 in transcriptional regulation mediated through the PRC complexes.[12]

In summary, the current study identified frequent *ASXL2* mutations in t(8;21) AML and characterized its role in hematopoietic development in mice. This work demonstrates that ASXL2 plays a critical role in multi-lineage differentiation and highlights how its loss leads to progressive hematopoietic defects and promotes myeloid expansion, thereby advancing our understanding of epigenetic machinery that regulates hematopoiesis.

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