

NSUN2-FOSB reciprocity facilitates leukemogenesis in an m⁵C-dependent manner by increasing BCL2L1 expression

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Supplemental methods

Reagents

Cytarabine (Ara-c, TargetMol, Boston, MA, USA), Cladribine (Cla, TargetMol), homoharringtonine (HHT, TargetMol), decitabine (DAC, TargetMol), 5-Azacytidine (AZA, TargetMol), puromycin (TargetMol), MY-1B (TargetMol), and tucidinostat (Tuc, TargetMol) were dissolved in dimethyl sulfoxide (DMSO) and deposited at -86 °C until use.

Western blot

Western blot analysis was performed according to standard procedures. Briefly, cells were washed twice with ice-cold PBS, and total protein lysates were extracted by RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA, USA). After incubation on ice for 30 min, cell lysates were centrifuged at 12,000×g for 15 min. The supernatants were collected, and protein concentration was measured using a BCA assay (Thermo Scientific). Equal amounts of proteins were loaded, separated by 8-12% SDS-PAGE, and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk at room temperature for 2 h and subsequently incubated with primary antibodies at 4°C overnight. After washing five times with 1×TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Signals were detected using chemiluminescent reagents (Thermo Scientific), and images were captured digitally using a ChemiDoc MP (Bio-Rad, Richmond, CA, USA). The following antibodies were used: NSUN2 (1:5000, ab259941, Abcam, Cambridge, MA, USA); FOSB (1:2000, ab11959, Abcam); YBX1 (1:2000, ab76149, Abcam); cleaved poly (ADP-ribose) polymerase (PARP, 1:2000, #5625S, Cell Signaling Technology, Beverly, MA, USA); BCL2L1 (1:2000, #2764, Cell Signaling Technology); BAX (1:2000, ab32503, Abcam), MCL1 (1:2000, #4572, Cell Signaling Technology),

BCL2 (1:5000, #15071, Cell Signaling Technology), β -actin (1:10000, ab6276, Abcam), and GAPDH (1:10000, ab8245, Abcam) were used as endogenous controls.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer's instructions. Normal and leukemic cells were lysed in TRIzol and incubated at room temperature for 15 min. Chloroform was thoroughly mixed with the TRIzol solution. After centrifugation, the aqueous phase was carefully extracted and mixed with an equal volume of isopropanol. Total RNA was precipitated and washed with 75% ethanol. RNA concentration and quality were analyzed by measuring the absorbance at 260 nm using a spectrophotometer (DS-11, DeNovix, Wilmington, DE, USA). RNA was used as a template for cDNA synthesis using the Primescript™ RT Master Mix (RRO36A, Takara Bio, Tokyo, Japan). qRT-PCR was performed using cDNA as a template by TB Green Premix Ex Taq™ (RR820A, Takara Bio) on the ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Human GAPDH and murine β -actin were used as endogenous controls for human and murine samples, respectively. Each sample was run in triplicate, and relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. All primer sequences are listed in Table S2.

Apoptosis assay

Apoptosis was measured using Annexin V/7-AAD staining (BD PharMingen, San Diego, CA, USA). Briefly, leukemic cells plated at 2×10^5 cells/mL in six-well plates were collected, washed with $1 \times$ binding buffer, and treated in 100 μ L of $1 \times$ binding buffer containing 5.0 μ L of Annexin V-APC and 5.0 μ L of 7-AAD for 15 min at room temperature, protected from light. Cells were then resuspended in 400 μ L of $1 \times$ binding buffer, and apoptotic cells were analyzed by flow cytometry (CytoFLEX LX, Beckman-Coulter, Brea, CA, USA) within 30 min after staining. The percentage

of apoptotic cells was calculated as Annexin V⁺/7-AAD⁺ (late apoptosis) + Annexin V⁺/7-AAD⁻ (early apoptosis).

CCK-8 assay

Leukemic cells (100 μ L, 1×10^5 cells/mL) were seeded in 96-well plates. After adding 10 μ L of CCK-8 solution (Dojindo, Kumamoto, Japan), cells were incubated for 2–4 h. Absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

Cell death assay by 7-AAD staining

Leukemic cells (2×10^5 cells/mL) in 12-well plates were collected and washed with $1 \times$ PBS buffer, followed by treatment in 100 μ L of $1 \times$ staining buffer containing 5.0 μ L 7-AAD (0.1 mM, Beyotime Biotechnology, Nanjing, Jiangsu, China) for 15 min at room temperature. Cell death (7-AAD⁺) was analyzed by flow cytometry (CytoFLEX LX, Beckman-Coulter).

Immunofluorescence (IF) assay

Cells were collected and plated onto slides, followed by fixation with 4% paraformaldehyde for 30 min. Permeabilization was achieved with absolute alcohol for 15 min. After blocking with 5% non-fat milk at room temperature for 2 h, cells were washed and incubated overnight with primary antibodies against NSUN2 (1:200, ab259941, Abcam). After washing, cells were incubated with a secondary antibody conjugated to FITC or PE. Cellular DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology), and fluorescence signals were detected by a laser scanning confocal microscope (Leica Stellaris 5, Leica Microsystems Inc., Deerfield, IL, USA).

mRNA half-life ($t_{1/2}$) measurement

AML cells were transduced with sh-NSUN2 or sh-NC. After 48 h of transduction, AML cells were treated with 2 μ M actinomycin D (ActD, TargetMol) at various time points. Total RNA was

extracted and analyzed by qRT-PCR to determine *FOSB* mRNA expression. The half-life of *FOSB* mRNA was calculated using GraphPad Prism.

Global m⁵C RNA methylation levels assay

Global m⁵C RNA methylation levels were measured using the MethylFlash™ m⁵C RNA Methylation ELISA Easy Kit (EpiGentek, Farmingdale, NY, USA) following the manufacturer's instructions. Briefly, 200 ng of global RNA was extracted from leukemic cells, and DNase I was used to remove DNA. RNA samples (200 ng in 2–8 µL of RNase-free water) were mixed with 200 µL of binding solution and added to assay wells. Six different m⁵C concentrations (0.05%, 0.1%, 0.2%, 0.5%, 1.0%, and 2.0%) were prepared by diluting the positive control containing 2% m⁵C and added to assay wells. After an incubation for 90 min, the binding solution was removed, and each well was washed three times with 150 µL of diluted wash buffer. Then, 50 µL of m⁵C detection complex solution was added to each well and incubated for 50 min at room temperature. Finally, 50 µL of the fluorescence development solution was added, and the incubation continued for 4 min at room temperature in the dark. Relative fluorescence units (RFU) were measured at 530ex/590em nm using the SpectraMax iD5 microplate reader (Molecular Devices, San Jose, CA, USA). To calculate the percentage of m⁵C RNA, a standard curve was generated by plotting RFU values against the positive control at each concentration. Subsequently, linear regression was applied to determine the optimal slope (RFU/1%) and the most linear portion of the standard curve (including at least four concentration points). The percentage of m⁵C in total RNA was calculated using the formula = (Sample (RFU)-negative control (RFU))/(slope*200).

m⁵C blot assay

Total RNA was extracted by TRIzol, denatured in RNA incubation buffer at 95 °C for 3 min, and

immediately cooled on ice. The denatured samples were spotted onto an Amersham Hybond-N⁺ membrane (GE Healthcare) and crosslinked by UV exposure (254 nm for 5 min). The membrane was washed with 1×standard saline citrate (SSC) buffer and blocked with 5% non-fat milk in 1×PBST for 1 h. The membrane was incubated overnight with an anti-m⁵C antibody (1:1000, 28692S, CST) at 4 °C. After washing, the membrane was incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. Finally, the membrane was washed with 1×SSC buffer for 5 min and stained with 0.02% methylene blue for internal control. Images were digitally acquired (ChemiDoc MP, USA).

Flow cytometry

Murine BM mononuclear cells were obtained by crushing femurs, tibias, and iliac bones using a pestle and mortar in staining buffer (PBS with 2% FBS), and BM cells were filtered through a 70 µm cell strainer (BD PharMingen). Peripheral blood (PB) was collected from the tail vein of the mice in EDTA-treated tubes (BD PharMingen). Before staining, red blood cells were lysed by ammonium chloride solution (BD PharMingen). BM cells from transplanted mice were harvested and stained with various antibodies diluted in staining buffer (PBS with 2% FBS) for 30 min. Murine antibodies used for the L-GMP assay included: Lineage (Lin) cocktail was prepared from biotinylated antibodies (Mac-1 and Gr-1 for myeloid cells, B220 for B cells, CD3e for T cells, Ter-119 for erythroid lineage); Streptavidin-APC-R700 (565144, BD Horizon); c-Kit-PE (553355, BD PharMingen); Sca-1-PE-Cy7 (558162, BD PharMingen); CD34-APC (560230, BD PharMingen); CD16/CD32-BV510 (101333, Biolegend); Mac-1-PE-Cy7 (552850, BD PharMingen); For HSPCs: Streptavidin-APC-R700 (565144, BD Horizon); c-Kit-PE (553355, BD PharMingen), Sca-1-PE-Cy7 (558162, BD PharMingen); CD34-APC (560230, BD PharMingen); CD135-BV421 (566292,

BD PharMingen). For blood Lin analysis: Mac-1-PE-Cy7 (552850, BD PharMingen); Gr-1-APC (553129, BD PharMingen); CD3e-BB700 (145-2C11, BD PharMingen); B220-FITC (561877, BD PharMingen). For competitive repopulation assay: CD45.2-PE (560695, BD PharMingen); CD45.1-APC-R700 (565814, BD PharMingen). Flow cytometric analysis was performed using the CytoFLEX LX (Beckman-Coulter, Brea, CA, USA), and cell sorting was conducted on the FACS Aria II (Becton Dickinson, Mountainview, CA, USA). Data were analyzed using the FlowJo software 10.0 (Becton Dickinson).

Engraftment of NOD/SCID-IL2R γ mice (NSG)

Male 10-week-old NSG mice (Shanghai Model Organisms Center, Shanghai, China) were intraperitoneally injected with busulfan (30 mg/kg, Sigma) 24 h before transplantation. A total of 1×10^6 AML cells were transplanted via tail vein injection into the NSG mice. NSG mice assignment is random. OS time was monitored from the first day of the experiment until the death.

Cell cycle analysis

Leukemic cells (2×10^6) were incubated for 30 min at room temperature in $1 \times$ PBS buffer supplemented with propidium iodide (0.04 mg/mL, Invitrogen) and RNase (100 μ g/mL, Invitrogen). Cells were analyzed by flow cytometry, and FlowJo software v10.0 (Becton, Dickinson) was used to determine the distribution of cells across the cell cycle phases.

Mitochondrial membrane potential (MMP) assay

The fluorescent carbocyanine dye (JC-1) labeling mitochondria with high membrane potential was used to assess MMP. JC-1 staining buffer was prepared by mixing the JC-1 probe and $1 \times$ buffer (Beyotime Biotechnology, Nanjing, Jiangsu, China). AML cells (about 5×10^5) were collected and incubated with JC-1 staining buffer for 20 min in a humidified atmosphere. JC-1 with red

(490ex/530em nm) representing MMP level was measured by flow cytometry.

Transmission electron microscope (TEM) assay

Leukemia cells, with or without NSUN2 knockdown, were fixed by immersion in glutaraldehyde solution, rinsed with cold 1× PBS buffer (pH 7.4), and fixed with an osmium acid solution. Increasing concentrations of acetone were used for dehydration. Finally, AML samples were embedded and polymerized for ultrathin sectioning. TEM (Hitachi H-7500, Japan) was used to examine mitochondrial morphology. The number of cristae per mitochondrion was calculated.

Competitive repopulation assay

Six-week-old *Nsun2*^{fl/fl} Cre^{ERT2} mice were intraperitoneally injected daily with tamoxifen (75 mg/kg, TAM, Sigma–Aldrich, St. Louis, MO, USA) for six days to deplete *Nsun2* expression (referred to as Δ/Δ) or with corn oil as a vehicle control (referred to as fl/fl). Mice were sacrificed one month after the final TAM injection. For competitive repopulation, BM cells from fl/fl or Δ/Δ CD45.2⁺ cells (2×10⁶) mixed with an equal number of CD45.1⁺ cells from B6.SJL mice were transplanted into lethally irradiated B6.SJL mice (CD45.1⁺, 6–8 weeks). PB samples from recipient mice were collected monthly for four months to analyze the chimerism of CD45.2/CD45.2+CD45.1 populations.

Bisulfite sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen) from MOLM-13 cells transduced with sh-NSUN2 or sh-NC. RNA was treated with the conversion solution from the RNA Bisulfite Conversion Kit (Epigentek Group Inc., San Diego, CA, USA) and incubated at 65 °C for 5 min, followed by 60 °C for 90 min. After desalting with F-Spin columns, samples were desulfonated by a desulphonation solution and a 90% ethanol mixture for 30 min at room temperature. The converted

RNA was used for cDNA synthesis, followed by PCR amplification. PCR products were inserted into the pUC18 vector and transformed into competent *Escherichia coli* (DH5 α , Vazyme Biotech, Nanjing, Jiangsu, China). The plasmid was extracted from a single clone and sequenced. Methylated cytosines were identified by retaining cytosines as methylated. However, unmethylated cytosines that were converted to uracil and read as thymidine were considered unmethylated.

Wright-Giemsa stain

Murine PB smears and BM cytopins were stained using Wright-Giemsa stain according to standard protocols for morphological analysis.¹ BM mononuclear cells ($5 \times 10^5/\text{mL}$) suspended in 100 μL of staining buffer were collected and centrifuged onto slides. Meanwhile, approximately 3 μL of PB was plated on slides. After the slides were air-dried, Wright-Giemsa staining buffer (1.0 mL) was applied for 4 min. Morphological analysis was performed by an optical microscope after the slides were washed and thoroughly dried.

Hematoxylin and eosin (H&E) stain

Spleen and liver tissues from mice were fixed in formalin and embedded in paraffin. Tissue samples were dehydrated and stained with H&E according to standard protocols.¹

Virus production and cell infection

These procedures were performed as previously documented². Briefly, HEK293T cells (4×10^6) were plated in 10 cm dishes one day prior to transfection. For retrovirus or lentivirus production, individual expression vectors with packaging plasmids (VSVG and Gappol for retroviruses, PSPA2 and MD2G for lentiviruses) were transfected into 293T cells using polyethylenimine (PEI, Sigma-Aldrich). Viruses were harvested from the supernatant and filtered through a low protein-binding polysulfone filter (0.45 μm , Millipore) 48 and 72 h after the transfection. Leukemic cells were

suspended in viral supernatant with 8 µg/mL polybrene (Sigma-Aldrich) and centrifuged at 2,000×g for 2 h. GFP⁺ cells were sorted by flow cytometry for the subsequent analysis. Alternatively, puromycin (1 µg/mL) was added to the medium for 2 to 4 days to select positive clones based on transfection efficiency.

Chromatin immunoprecipitation (ChIP) assay

The enrichment of FOSB binding to the *NSUN2* or *BCL2L1* gene promoter was assessed by ChIP-quantitative real-time PCR assay (qRT-PCR) or ChIP-Seq. Briefly, AML cells were cross-linked with 1% formaldehyde for 10 min. Nuclear extracts were prepared, and chromatin was sonicated to generate DNA fragments of 200 to 1000 bp. DNA-protein complexes were immunoprecipitated using specific antibodies against FOSB or non-relevant mouse immunoglobulin IgG (Beyotime Biotechnology, China), coupled with G-Sepharose beads (Beyotime Biotechnology, China), and incubated overnight at 4 °C. Cross-linked DNA-protein complexes were immunoprecipitated by beads and were reversed by heating at 65 °C for 4 h. DNA was extracted and purified. A standard PCR reaction was performed with primer sequences specific to the *NSUN2* or *BCL2L1* promoter. Fold enrichment was calculated using the $2^{-\Delta\Delta Ct}$ method: $\Delta Ct = Ct \text{ of the ChIP DNA} - Ct \text{ of the input DNA}$, and $\Delta\Delta Ct = \Delta Ct \text{ of the sample (immunoprecipitated DNA of NSUN2 or BCL2L1)} - \Delta Ct \text{ of the IgG control}$.

For ChIP-Seq, MOLM-13 and MV4-11 cells were overexpressed with Flag-tagged FOSB, which were cross-linked with 1% formaldehyde for 10 min. DNA-protein complexes were immunoprecipitated using specific antibodies against Flag (Anti-DYKDDDDK Affinity Beads, Smart-Lifesciences, Nanjing, Jiangsu, China), or non-relevant mouse immunoglobulin IgG (Beyotime Biotechnology, China) coupled with G-Sepharose beads (Beyotime Biotechnology,

China). DNA was extracted, purified, and subjected for sequence. The occupancy of FOSB at *NUSN2* or *BCL2L1* promoter was visualized by IGV software.

Luciferase (Luc) activity

For the Luc reporter assay, 293T cells were transfected with various concentrations of pLVX-FOSB, as well as wild-type pGL3-NSUN2-P (WT), pGL3-NSUN2-P (Mut), pGL3-BCL2L1-P (WT), pGL3-BCL2L1-P (Mut), or empty control (Vec) using polyethylenimine (PEI, Sigma-Aldrich) according to the manufacturer's instructions. The cells were also co-transfected with the pRL-TK control vector (Promega) encoding Renilla Luc for normalization of transfection efficiency. The Firefly and Renilla Luc activities were measured 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), according to the manufacturer's guidelines. The results were expressed as the relative Luc units of the Firefly/Renilla Luc ratio.

RNA immunoprecipitation (RIP)

Anti-m⁵C antibody (ab10805, Abcam), anti-m⁶A antibody (ab151230, Abcam), anti-NSUN2 antibody (ab259941, Abcam), and mouse IgG (ab190475, Abcam) were incubated with protein G-Sepharose beads (ab193259, Abcam) overnight at 4 °C with rotation. AML cells were exposed to UVC (400 mJ/cm²), and cellular extracts were incubated with conjugated m⁵C, m⁶A, NSUN2, or IgG antibody at 4 °C for IP for 2 h. Methylated RNAs were immunoprecipitated (IPed) by beads, and RNA was extracted and purified using the RNeasy kit (QIAGEN). Fragmented RNA (1/10) was used as an input control. qPCR was performed using IPed RNA or input RNA with primers (Table S2). The amplification cycle (Cq) value for the m⁵C, m⁶A, or NSUN2-IP was normalized to the Cq of the corresponding input to calculate relative enrichment.

RNA sequencing (RNA-seq) analysis

Total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and quality were measured using DS-11 spectrophotometer (DeNovix). The cDNA library was constructed using the KAPA Stranded RNA-Seq Library Preparation Kit (Illumina) as follows: Oligo (dT) magnetic beads were used to isolate poly (A) mRNA. The mRNA was then fragmented into small pieces by the fragmentation buffer. cDNA libraries were constructed by PCR amplification of suitable fragments. Sequencing was performed on the Illumina HiSeq 4000 platform, and image analysis, base calling, and error estimation were obtained by Illumina/Solexa Pipeline. StringTie (version 1.2.3) was used to reconstruct the transcriptome after the trimmed reads were mapped to the corresponding reference genome (hg38 from UCSC) by HISAT2 (version 2.0.4) using default parameters³. The software Integrative Genomics Viewer and the R packages ggplot2, pheatmap, and ggpubr were used for data visualization and analysis of differentially expressed genes (DEGs). DEGs were identified based on a Log₂fold change (FC) > 0.5 and $P < 0.05$. Gene ontology (GO) and pathway enrichment analyses were performed on DEG. Downregulated genes ($P < 0.05$, log₂FC ≤ -0.5) were subjected to GO enrichment analysis.

Methylated RNA immunoprecipitation sequencing (MeRIP-seq) analysis

MeRIP-seq was performed by Cloudseq Biotech Inc. (Shanghai, China) following the reported procedure with some modifications.⁴ Briefly, total RNA was collected, fragmented, and incubated with an anti-m⁵C monoclonal antibody (ab10805, Abcam) for 2 h at 4 °C in an IPP buffer. The mixture was subsequently incubated with protein A beads (Thermo Fisher) at 4 °C for an additional 2 h. The bound RNA was eluted from the beads in the IPP buffer and extracted by TRIzol reagent (Thermo Fisher). Purified RNA was used for RNA-seq library generation. The input sample (without immunoprecipitation) and the m⁵C IP samples were subjected to 150 bp paired-end

sequencing on an Illumina HiSeq sequencer. For m⁵C-seq analysis, raw reads for both IP and input samples were trimmed by Cutadapt (version 4.8). Spliced Transcripts Alignment to a Reference (STAR, version 2.7.11b) was used to compare the reads to the human genome (hg38 from UCSC) using default parameters.³ GO and pathway enrichment analyses were performed on the differentially methylated genes (DMG) identified by Log₂FC > 0.5 and *P* < 0.05. MeRIP-Seq, RNA-seq, and the subsequent bioinformatics analysis were provided by Cloud-Seq Biotech Ltd. Co. (Shanghai, China).

Mice housing

Nsun2^{fl/fl} inducible knockout mice were purchased from GemPharmatech Co., Ltd (Nanjing, Jiangsu, China) by Cas9-knockout technology.⁵ Cre^{ERT2} mice (strain 129S/SvEvx57BL/6) with a background of C57BL/6J were obtained from the Shanghai Model Organisms Center (Shanghai, China) and were crossed with wide-type C57/B6 for at least 10 generations.⁶ *Nsun2*^{fl/fl} and Cre^{ERT2} mice were crossed to produce *Nsun2*^{fl/fl} Cre^{ERT2}. Mice were housed under pathogen-free conditions in standard housing conditions at the animal core of the First Affiliated Hospital of Wenzhou Medical University. All procedures involving mice follow the ARRIVE guidelines and were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (WYYY-AEC-2021-298).

MLL-AF9 (MA9) and AML1-ETO9a (AE9a)-induced murine AML model

BM lineage-negative (Lin⁻) cells, collected from BM of 8-week-old *Nsun2*^{fl/fl} Cre^{ERT} mice five days after 5-fluorouracil (TargetMol) treatment, were cultured overnight in StemSpan SFEM (Stemcell Technologies) supplemented with murine SCF (50 ng/mL, PeproTech), TPO (50 ng/mL, PeproTech), and FLT3-L (50 ng/mL, PeproTech). Lin⁻ cells were transduced with MSCV-GFP-IRES-MA9 or

AE9a (Addgene, Watertown, MA, USA) via two rounds of “spinoculation” as previously described.

^{7, 8} For primary BMT in the MA9-induced murine model, GFP⁺ cells (5×10^5 for every recipient mouse) were sorted by flow cytometry 48 h after transduction. They were intravenously injected into lethally irradiated (8.0 Gy) C57BL/6J mice (Shanghai Model Organisms Center, Shanghai, China). TAM (Sigma–Aldrich) was dissolved in corn oil (Sigma–Aldrich) at 10 mg/mL. It was aliquoted and stored at -20 °C in the dark until use. The required aliquots of TAM were thawed by shaking at room temperature 2 h before injection. To induce *Nsun2* depletion in primary BMT, recipient mice were intraperitoneally injected with either corn oil or TAM (75 mg/kg body weight) once daily from days 15 to 20 after transplantation. For secondary or tertiary BMT, sorted fl/fl GFP⁺ cells (5×10^3 for every recipient mouse) from primary or secondary BMT recipients were collected and injected via tail vein into lethally irradiated (8.0 Gy) recipient mice along with normal BM cells (5×10^5 for every recipient mouse). TAM (75 mg/kg body weight) was injected once daily from days 10 to 15 after transplantation. The mice were euthanized by CO₂ inhalation when typical leukemic symptoms, i.e., hunched posture, labored breathing, and decreased activity, had been observed. BM cells, spleen, and liver were isolated for various tests. All mice were randomly assigned. Responses were then scored by an experimenter blinded to the injection condition and experimental cohort.

For primary BMT in the AE9a-induced model, TAM (75 mg/kg body weight) was injected once daily from days 41 to 46 after transplantation to induce the depletion of *Nsun2*. For secondary BMT in the AE9a-induced model, TAM (75 mg/kg body weight) was injected once daily from days 21 to 26 after transplantation to deplete *Nsun2*.

Colony formation assay

BM GFP⁺ cells were sorted from *Nsun2*-depleted or WT MA9-transduced AML mice. Murine LSK

(Lin⁻Sca-1⁺cKit⁺) cells were sorted from the BM of *Nsun2*-depleted or WT mice. BM GFP⁺ and LSK cells were plated in murine methylcellulose medium (MethoCult™ GF M3434, Stemcell Technologies) for 14 days to observe colony formation. Human AML cells with or without *NSUN2* knockdown (KD) were seeded in human methylcellulose medium (MethoCult™ H4434 Classic, Stemcell Technologies). Colony (> 40 cells) was counted 14 days after plating according to the manufacturer's protocol.

Plasmid production

The wild-type coding sequence (CDS) of *NSUN2* was amplified from mononuclear cells of a healthy donor and inserted into the retroviral vector MSCV-puro (Clontech, Palo Alto, CA, USA) between Bgl II/EcoRI (Takara Bio). The wild-type CDS of *FOSB* or *BCL2L1* was amplified from mononuclear cells of a healthy donor and inserted into the vector LVX-puro (Sigma-Aldrich) between EcoRI/NotI (Takara Bio). *NSUN2* mutants (C271A and C321A) were constructed using the site-directed mutagenesis kit (Vazyme Biotech). Gene-specific short hairpin RNAs (shRNAs) for *NSUN2*, *FOSB*, and *YBX1* were designed and cloned into vector pLKO.1-Puro (Sigma-Aldrich). A nonfunctional shRNA construct was also prepared. *FOSB*-5'-untranslated region (UTR), CDS, and 3'-UTR-1, -2, and -3 were amplified and cloned into the vector pGL-3-basic between MluI/HindIII (Takara Bio). The *FOSB*-3'-UTR-3 mutant (C3656T) was generated using the site-directed mutagenesis kit (Vazyme Biotech). Potential *FOSB*-binding motifs at the *NSUN2* or *BCL2L1* promoter were amplified and cloned into pGL-3-basic. Mutations on *FOSB*-binding motifs at the *NSUN2* or *BCL2L1* promoter were constructed by the site-directed mutagenesis kit (Vazyme Biotech). All constructs were verified by DNA sequencing.

Supplemental References

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Supplemental Table 1. The clinical characteristics of 102 AML patients for qRT-PCR

Characteristic	All patients N (%)	Relative expression of NSUN2
Overall	102	
Gender		
Male	57 (55.8)	0.69–26.53
Female	45 (44.2)	0.39–15.34
Age (range)	51 (17-75)	
FAB subtype		
M0-M1	11 (10.8)	0.92–8.66
M2	14 (13.8)	2.44–9.32
M3	7 (6.9)	0.39–5.38
M4	34 (33.3)	0.69–26.53
M5	36 (35.3)	0.72–20.35
Cytogenetics		
Normal karyotype	38 (37.2)	0.72–15.34
Complex karyotype	18 (17.6)	0.98–19.91
t(15;17)	7 (6.9)	0.39–5.38
t(8;21)	15 (14.7)	2.44–13.32
Inv(16)	11 (10.8)	1.99–26.53
t(11q23)	11 (10.8)	1.25–20.35
Not available	2 (1.9)	4.45–4.84
Genetic mutation		
WT1 mutation	12 (11.7)	1.29–20.35
FLT3 mutation	13 (12.7)	0.92–14.88
CEBPA mutation	18 (17.6)	0.39–19.91
MLL-PTD mutation	4 (3.9)	1.40–6.36
TET2 mutation	14 (13.7)	1.29–26.53
NPM1 mutation	6 (5.9)	0.72–9.32
c-KIT mutation	9 (8.8)	0.69–11.37

Supplemental Table 2. The sequences of primers

Genes	Sequences
NSUN2-F	5'-CAA GCT GTT CGA GCA CTA CTA C-3'
NSUN2-R	5'-CTC CCT GAG AGC GTC CAT GA-3'
FOSB-F	5'-TGC GCC GGG AAC GAA ATA A-3'
FOSB-R	5'-CTG AGC CCG GCA AAT CTC TC-3'
YBX1-F	5'-TGC AGC AGA CCG TAA CCA TT-3'
YBX1-R	5'-TGG TAA TTG CGT GGA GGA CC-3'
SEMA3A-F	5'-GTG CCA AGG CTG AAA TTA TCC T-3'
SEMA3A-R	5'-CCC ACT TGC ATT CAT CTC TTC T-3'
RYR1-F	5'-TGG CTC ACC TAT GCT GCT C-3'
RYR1-R	5'-GAC AGT GCG TCG TCC ATG T-3'
PDE11A-F	5'-CAC TGC TGG GTT TCA AGA CAT-3'
PDE11A-R	5'-GAA GGC ATT GTT GGT TCC CC-3'
FHAD1-F	5'-TAA AGA GCG CAG AAG GCT TTT-3'
FHAD1-R	5'-GAG TGC ATG GTG GTT GTC GAT-3'
ANKDD1A-F	5'-AGA GGC ACG TCT GTG TTT TGG-3'
ANKDD1A-R	5'-ATC TTG GCC CCT GAG TTT ACC-3'
DNAJC12-F	5'-AGC ATC CTG AAA ACC CCA AAG-3'
DNAJC12-R	5'-GGC TCG ACT CTC TTC ATT GGT-3'
INPP5A-F	5'-ACT CGG ATA CCT TAG AGA GCA C-3'
INPP5A-R	5'-TTC TTG ACC ATT TGC ACT CGG-3'
PHC2-F	5'-ACG CAT GTT ATC GAA GGG TTT-3'
PHC2-R	5'-ACC CCT GTG CAT ACT TCT TCT-3'
TMEM67-F	5'-CTT GGC TGT TTT ATG GAG ACC A-3'
TMEM67-R	5'-ACC TCC TTC TAA AGT TTG CCA C-3'
NT5DC3-F	5'-GGA GGA CTG GTG CAA TCA TCC-3'
NT5DC3-R	5'-GGT CAT TTC TCG CAT CTC CTT C-3'
DCAF4-F	5'-ACC TGC AAA GTC TGA AGA CCC-3'
DCAF4-R	5'-GGC ATA GCA GAA TGT GGG AAT C-3'
Pre-FOSB-F	5'-GGG TAA TGC GCT GCT CAA TG-3'
Pre-FOSB-R	5'-AAA GTT CTC AGC CCC GCT AC-3'
BCL2L1-F	5'-GAG CTG GTG GTT GAC TTT CTC-3'
BCL2L1-R	5'-TCC ATC TCC GAT TCA GTC CCT-3'
GAPDH-F	5'-CCG GGA AAC TGT GGC GTG ATG G-3'
GAPDH-R	5'-AGG TGG AGG AGT GGG TGT CGC TGT T-3'
β-actin-F (mouse)	5'-GGC TGT ATT CCC CTC CAT CG-3'
β-actin-R (mouse)	5'-CCA GTT GGT AAC AAT GCC ATG T-3'
Nsun2-F (mouse)	5'-TAC CAT GTT CCC ACC AAC GG-3'
Nsun2-R (mouse)	5'-GGA TTC GAA GGC ATC GCT CT-3'
Fosb-F (mouse)	5'-CGA GAA GAG ACA CTT ACC CCA-3'
Fosb-R (mouse)	5'-GTT TCC GCC TGA AGT CGA TCT-3'

Firefly-luciferase-F	5'-CAC TCT GGC GAC ATT GCC TA-3'
Firefly-luciferase-R	5'-GCT GCA GCA GGA TAG ACT CC-3'
Renilla-luciferase-F	5'-GAG AAG GGC GAG GTT AGA CG-3'
Renilla-luciferase-R	5'-TGG AAA AGA ACC CAG GGT CG-3'
m ⁵ C-RIP-F (FOSB-3'-UTR)	5'-TTT TCT CCT CCG CCT GTG TC-3'
m ⁵ C-RIP-R (FOSB-3'-UTR)	5'-TCA CAC TCT CAC ACT CGC AC-3'
*sh-NSUN2#1	5'-TGC AGT GTC CCA TCG TCT TAT-3'
*sh-NSUN2#2	5'-CAG TGG AAG GTA ATG ACG AAA -3'
*sh-NSUN2#3 (3'-UTR)	5'-GTG AGG CAA GAC CGA AGT AAA-3'
*sh-FOSB#1	5'-GCC AAC CAC AAT TCA ATG AAT-3'
*sh-FOSB#2	5'-CCT TCG TAC ACT TCT TCG TTT-3'
*sh-YBX1#1	5'-GCA GAC CGT AAC CAT TAT A-3'
*sh-YBX1#2	5'-GGT TCC CAC CTT ACT ACA T-3'
*sh-NC (Scramble)	5'-CAA ATC ACA GAA TCG TCG TAT-3'
*sh-Fosb (mouse)#1	5'-CAA CGG TCA CCG CAA TCA CAA-3'
*sh-Fosb (mouse)#2	5'-CTC TTT ACA CAC AGT GAA GTT-3'
*MSCV-NSUN2-F	5'-CCG CTC GAG ATG GGG CGG CGG TCG CGG GG-3'
*MSCV-NSUN2-R	5'-GGA ATT CTC ACC GGG GTG GAT GGA CCC-3'
*LVX-FOSB-F	5'-GGA TCT ATT TCC GGT GAA TTC ATG TTT CAG GCT TTC CCC GG-3'
*LVX-FOSB-R	5'-AGA GGG GCG GGA TCC GCG GCC GCT CAC AGA GCG AGG AGG GAG G-3'
*LVX-BCL2L1-F	5'-CAT TCT AGA ACT AGT CTC GAG ATG TCT CAG AGC AAC CGG GA-3'
*LVX-BCL2L1-R	5'-GGA TCT ATT TCC GGT GAA TTC TCA TTT CCG ACT GAA GAG TGA GC-3'
*NSUN2-P(WT)-F	5'-AGG TAC CGA GCT CTT ACG CGT GTT TCA GCA TGT TGG TCA GGA CG-3'
*NSUN2-P(WT)-R	5'-CAG TAC CGG AAT GCC AAG CTT CAA ATC GCA AAT CGC ATG AA-3'
*NSUN2-P(WT1)-F	5'-AGG TAC CGA GCT CTT ACG CGT TGG CCA CCA 5'-GTC CTA ACA AAG-3'
*NSUN2-P(WT1)-R	5'-CAG TAC CGG AAT GCC AAG CTT TGG GGG GCT TTG CAG ACT-3'
*NSUN2-P(WT2)-F	5'-AGG TAC CGA GCT CTT ACG CGT GAA TAC TCT GAC CTG AGA TTG CTT TT-3'
*NSUN2-P(WT2)-R	5'-CAG TAC CGG AAT GCC AAG CTT GCA GTC TGT GTC CTC AGT GTG C-3'
*NSUN2-P(WT3)-F	5'-AGG TAC CGA GCT CTT ACG CGT ACG GTA GGA TAT GCT TAG GAG AGA A-3'
*NSUN2-P(WT3)-R	5'-CAG TAC CGG AAT GCC AAG CTT TTT AAG CAA TGG AGA ATC TCT TTT GC-3'

*BCL2L1-P(WT)-F	5'-CTA GCT AGC CTC AGA GCA ACC GGG AGC TG-3'
*BCL2L1-P(WT)-R	5'-GAA GAT CTT CGA CAG ATG GAG AGG TCT GT-3'
*BCL2L1-P(WT1)-F	5'-CTA GCT AGC GGC CAC AGC AGC AGT TTG GA-3'
*BCL2L1-P(WT1)-R	5'-GAA GAT CTC TCA CCA ATA CCT GCA TCT-3'
*BCL2L1-P(WT2)-F	5'-CTA GCT AGC ATA CAA ATG ACC CTG TGC TT-3'
*BCL2L1-P(WT2)-R	5'-GAA GAT CTT ATA AGG TAA GGG AAA CAC C-3'
*FOSB-5'UTR-F	5'-AGG TAC CGA GCT CTT ACG CGT ATT CAT AAG ACT CAG AGC TAC GGC C-3'
*FOSB-5'UTR-R	5'-CAG TAC CGG AAT GCC AAG CTT TTC CCT GGG CAC AGG GGG C-3'
*FOSB-CDS-F	5'-AGG TAC CGA GCT CTT ACG CGT ATG TTT CAG GCT TTC CCC GG-3'
*FOSB-CDS-R	5'-CAG TAC CGG AAT GCC AAG CTT TCA CAG AGC GAG GAG GGA GG-3'
*FOSB-3'UTR-1-F	5'-AGG TAC CGA GCT CTT ACG CGT CTC TGT GAA CTC TTT AGA CAC ACA AAA C-3'
*FOSB-3'UTR-1-R	5'-CAG TAC CGG AAT GCC AAG CTT GGG ATC AAT AAG CTA AGG TCG G-3'
*FOSB-3'UTR-2-F	5'-AGG TAC CGA GCT CTT ACG CGT CCC ACA TTT CCA TGG TGT GAG-3'
*FOSB-3'UTR-2-R	5'-CAG TAC CGG AAT GCC AAG CTT GCC CCT TGA GTC TGC CAG TC-3'
*FOSB-3'UTR-3-F	5'-AGG TAC CGA GCT CTT ACG CGT GGG CTG GAA TCT CAT GAT TCC-3'
*FOSB-3'UTR-3-R	5'-CAG TAC CGG AAT GCC AAG CTT TGC GTT TTT AAG AGC TTT TTA TTG G-3'
ChIP-1F for NSUN2	5'-GGC ATC TGA CTG TGC ACT CT-3'
ChIP-1R for NSUN2	5'-AGG CTG CTT TGC TCA TGG AT-3'
ChIP-2F for NSUN2	5'-GCA CCG AAT ACT CTG ACC TGA-3'
ChIP-2R for NSUN2	5'-AGT GAG TAC TGT GTG CCT GA-3'
ChIP-3F for NSUN2	5'-GCA GTG TTG CTC TTA GGG CT-3'
ChIP-3R for NSUN2	5'-ACC AAC TTT TGC ATG GAG AGT-3'
ChIP-4F for NSUN2	5'-GGC TCA AAC AGT GTG GAT TGC-3'
ChIP-4R for NSUN2	5'-TCT CTT TTG CGT GGC TAT TGA-3'
ChIP-1F for BCL2L1	5'-TCC CCA TGG CAG CAG TAA AG-3'
ChIP-1R for BCL2L1	5'-AAA AAG GCC ACA ATG CGA CC-3'
ChIP-2F for BCL2L1	5'-GGC ATC TTT CAC CCT GGT CA-3'
ChIP-2R for BCL2L1	5'-CCT ACC CTC ACA GGT TTG GG-3'
NSUN2-RIP-F1 (FOSB-3'-UTR)	5'-GTG CGA GTG TGA GAG TGT GA-3'
NSUN2-RIP-R1 (FOSB-3'-UTR)	5'-CCA CAA GTA CAG CAT GGG GT-3'

NSUN2-RIP-F2 (FOSB-3'- 5'-TTT TCT CCT CCG CCT GTG TC-3'
UTR)
NSUN2-RIP-R2 (FOSB-3'- 5'-TCA CAC TCT CAC ACT CGC AC-3'
UTR)

*Primers for construction of plasmids.

RIP: RNA immunoprecipitation.

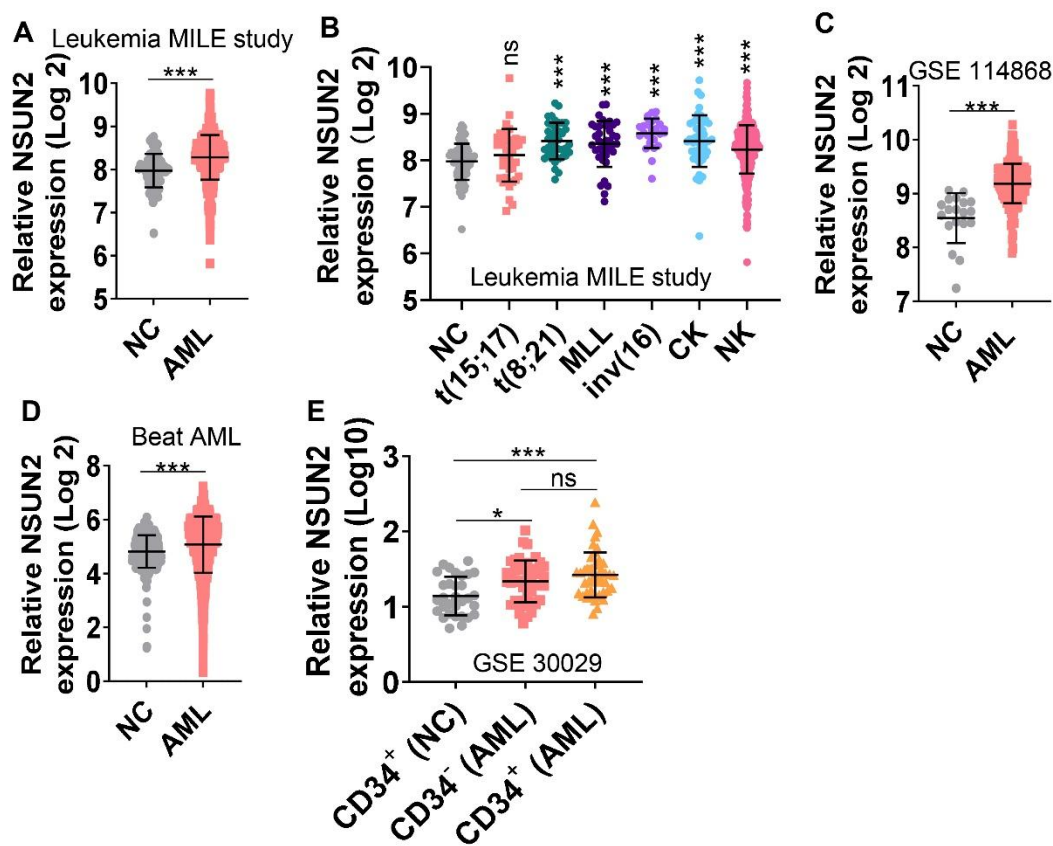
ChIP: Chromatin immunoprecipitation.

Supplemental Table 3. Limiting dilution assay of MLL-AF9-transduced *Nsun2*^{fl/fl} and *Nsun2*^{Δ/Δ} AML mice

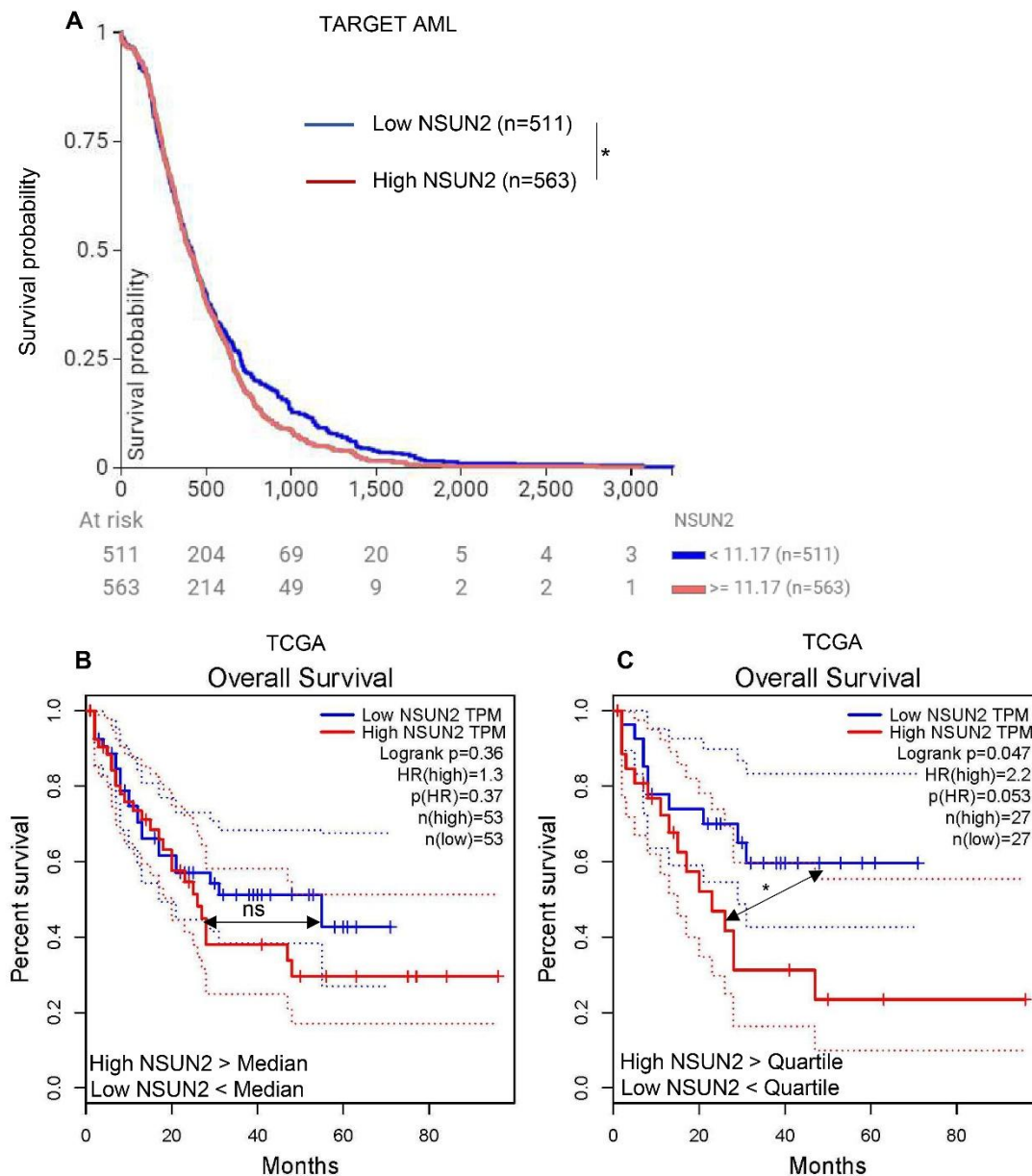
Dose	fl/fl (response/total)	Δ/Δ (response/total)	<i>P</i> value
50	4/6	1/6	
100	5/6	3/6	
200	6/6	5/6	
LSC frequency	1 in 49	1 in 144	0.021

The numbers of response mice mean that the recipient mice develop full-blown leukemia and die within six months after transplantation.

Supplemental Figures and legend

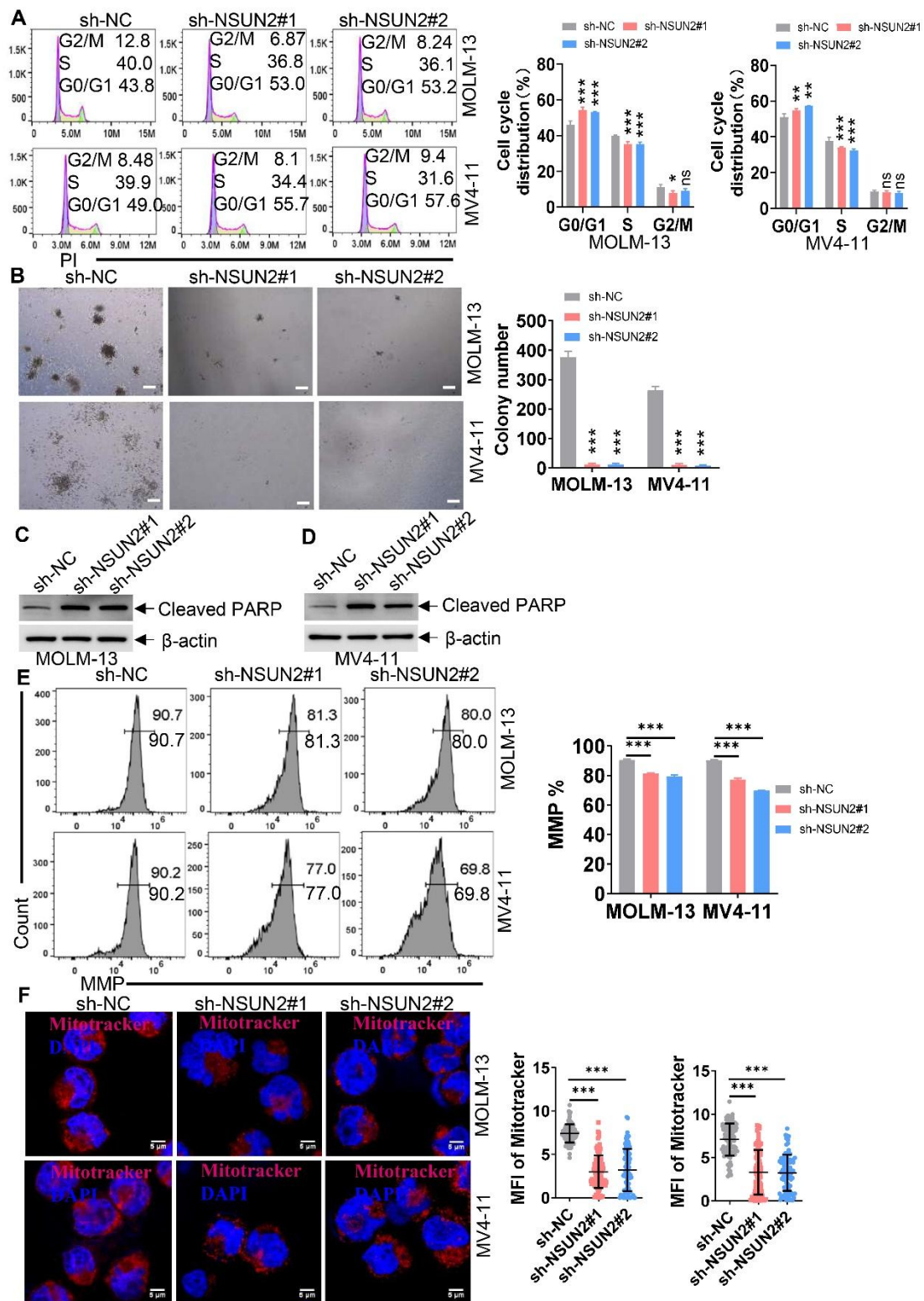


Supplemental Figure 1: NSUN2 expressions are higher in primary AML samples compared with normal controls (NC). (A and B) *NSUN2* expressions were analyzed using the BloodSpot database. (C and D) *NSUN2* expressions were assessed in NC and primary AML samples using the GSE 114868 (C) and Beat AML database (D). (E) *NSUN2* expressions were determined in CD34⁺ HSPCs, CD34⁺ AML, and CD34⁺ AML cells according to GSE 30029 database. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with NC; ns = not significant.



Supplemental Figure 2. Analyzing the outcome in AML patients according to different NSUN2 expressions. (A) Overall survival (OS) was evaluated in AML patients with higher (above the median) versus lower (below the median) expression of *NSUN2* in the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) AML database (https://target.nci.nih.gov/dataMatrix/TARGET_DataMatrix.html). (B) OS was evaluated in AML patients with higher (above the median) versus lower (below the median) expression of *NSUN2* in the TCGA database. (C) OS was assessed in AML patients with higher (above the quartile) versus

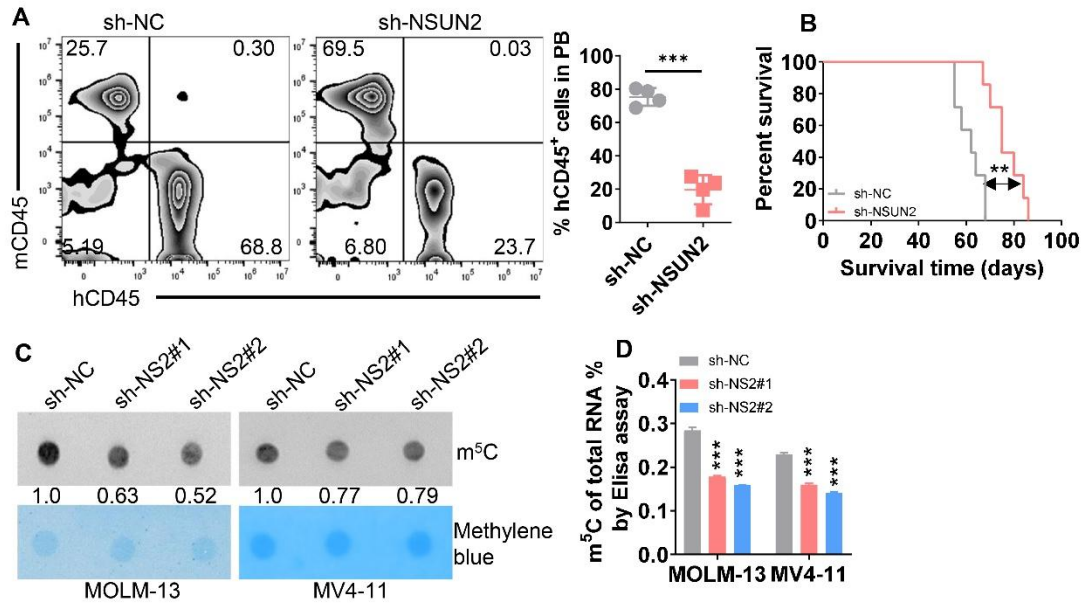
lower (below the quartile) expression of *NSUN2* in the TCGA database. * $P < 0.05$; ns = not significant.



Supplemental Figure 3. NSUN2 knockdown (KD) inhibits survival and affects mitochondrial function in AML cells. (A) AML cells were transduced with sh-NSUN2 or sh-NC for 48 h. Cells were stained with PI solution, and cell cycle distribution was assessed using flow cytometry.

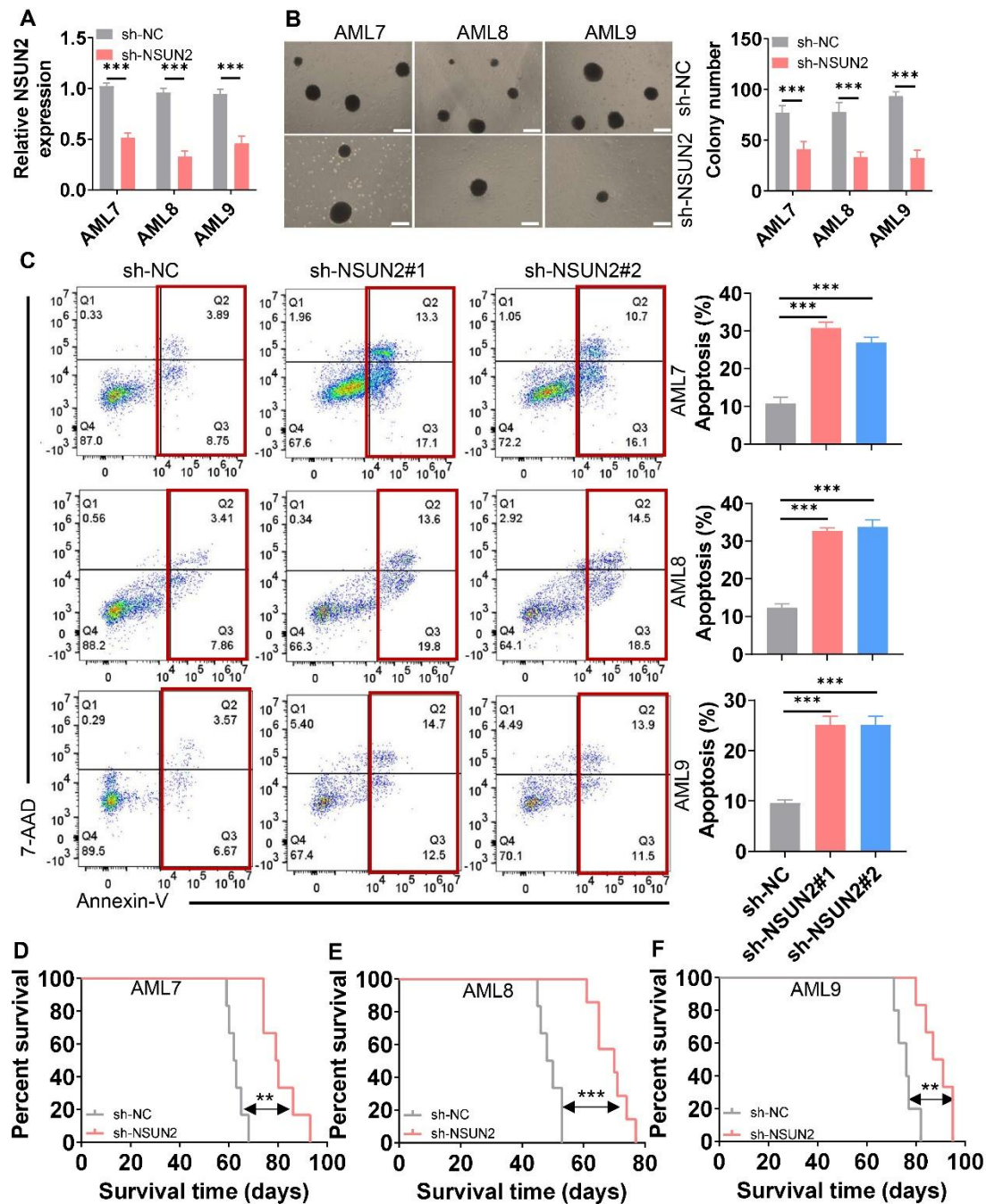
Representative plots (left) and statistical analysis of G0/G1, S, and G2/M phases (right) are shown.

(B) AML cells transduced with sh-NSUN2 (2×10^3 /dish) or sh-NC (2×10^3 /dish) were plated in methylcellulose medium with puromycin (1 μ g/mL) for 14 days to count colony formation. Representative plots (left) and statistical analysis of colonies (right) are shown. Bar scales represent 100 μ m. (C and D) Cleaved PARP protein levels were measured in AML cells transduced with sh-NSUN2 or sh-NC for 48 h, followed by puromycin (1 μ g/mL) treatment for an additional 48 h. $n = 3$ technical replicates. (E) Mitochondrial membrane potential (MMP) assay was measured by JC-1 probe staining in leukemic cells transduced with sh-NSUN2 or sh-NC. Statistical analysis of MMP (left) and the representative plots (right) are shown. (F) The mitochondria were stained by MitoTracker Red CMXRos in red. Relative mitochondrial mass was calculated by MFI of MitoTracker staining. The representative images (left) and statistical analysis of MFI of MitoTracker staining (right, $n = 50$ per group) are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus sh-NC. ns = not significant.



Supplemental Figure 4. NSUN2 KD inhibits survival in NSG mice and decreases m⁵C levels.

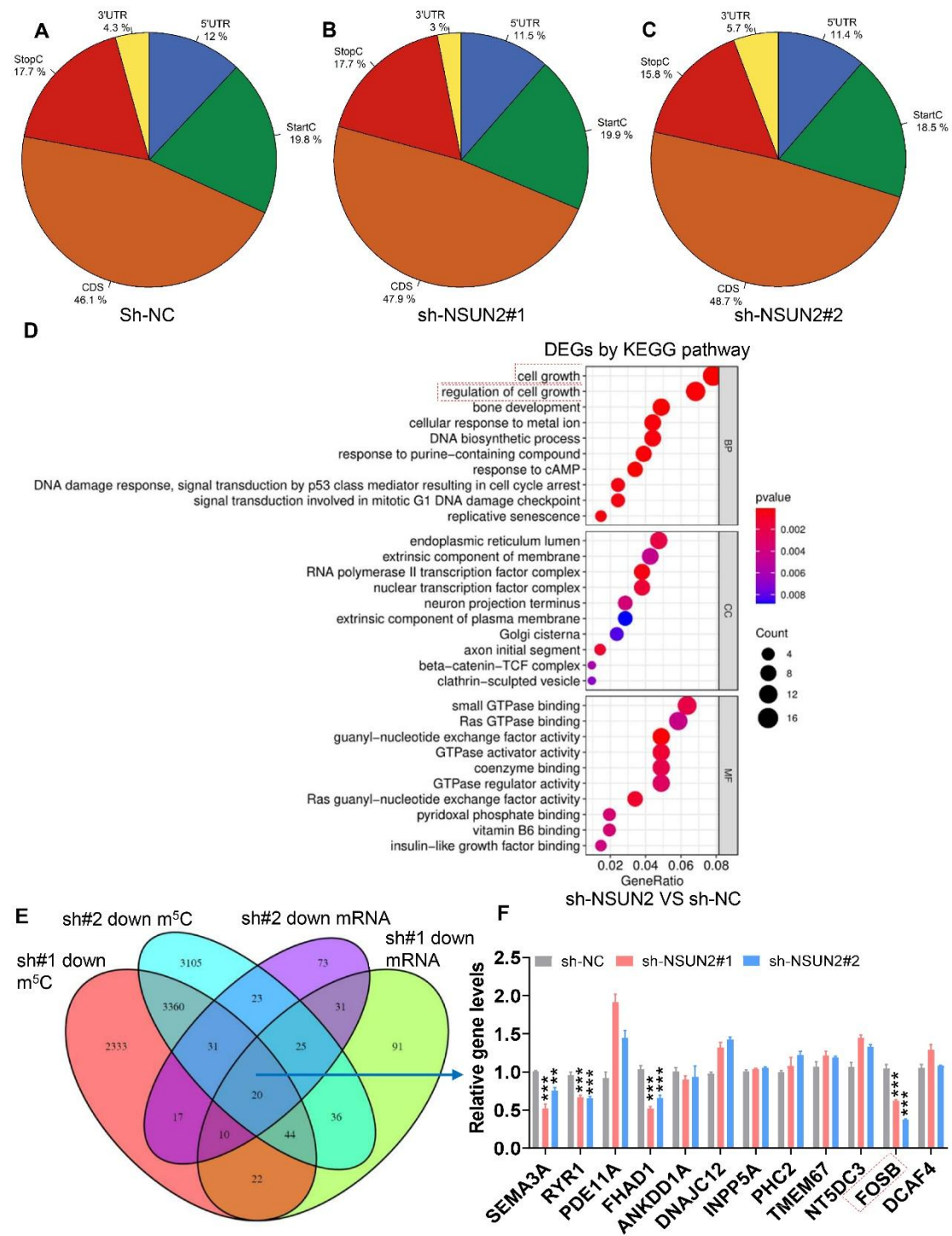
(A) The frequency of human CD45/murine CD45 (hCD45/mCD45) was measured in PB from MOLM-13-sh-NC (n = 4) and MOLM-13-sh-NSUN2#1 (n = 4)-xenografted NSG mice. Representative plots (left) of hCD45 and mCD45 staining and statistical analysis of hCD45/hCD45+mCD45 cells (right) are shown. (B) OS was determined in MOLM-13-sh-NC (n = 7) and MOLM-13-sh-NSUN2#1 (n = 7)-transplanted NSG mice. (C and D) m⁵C levels in total RNA were measured by m⁵C blot (C) and ELISA assay (D) in AML cells transduced with sh-NC or sh-NS2 for 48 h, followed by puromycin (1 µg/mL) treatment for an additional 48 h. ***P* < 0.01; ****P* < 0.001 versus sh-NC.



Supplemental Figure 5. NSUN2 KD inhibits survival and induces apoptosis in AML patient

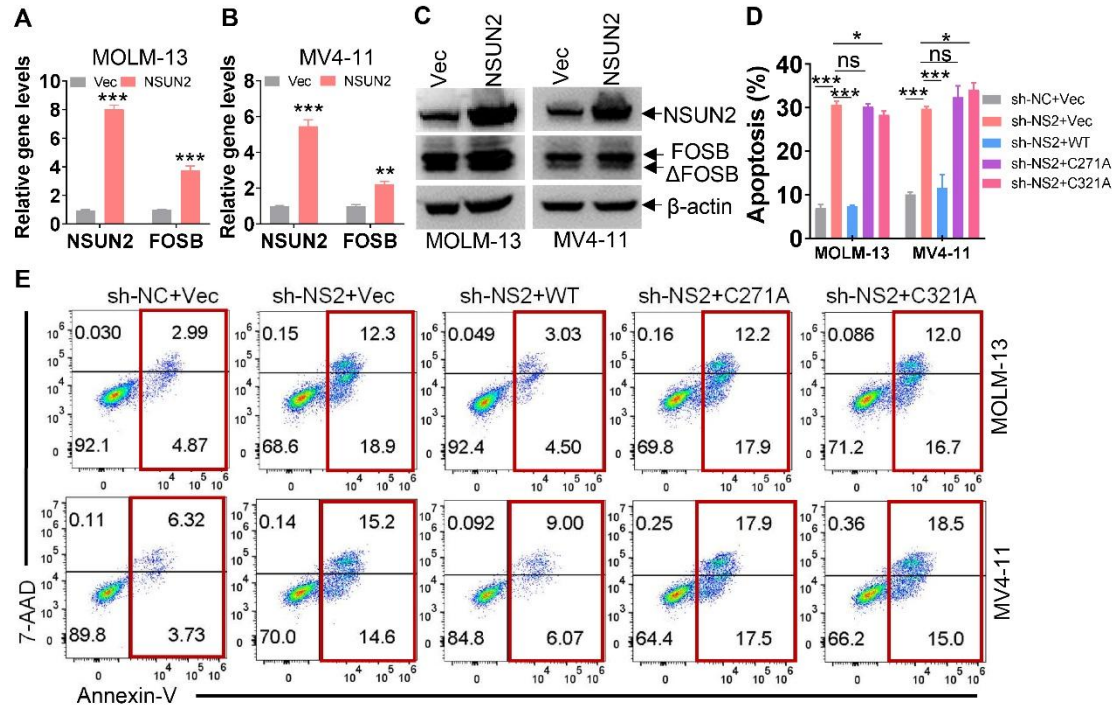
specimens. (A) *NSUN2* transcript levels were measured in CD34⁺ cells from three AML patients after transduction with sh-NC or sh-NSUN2#1 for 48 h. (B) Primary CD34⁺ cells after transduction with sh-NC or sh-NSUN2#1 were plated in methylcellulose medium for 14 days for colony formation (2×10^3 /dish). Representative plots (left) and statistical analysis of colony counts (right) are shown. Bar scales represent 200 μ m. (C) BM cells from three AML patients were transduced

with sh-NC or sh-NSUN2#1 for 48 h. Apoptosis was measured by Annexin V/7-AAD staining. Representative plots (left) and statistical analysis of Annexin V⁺/7-AAD⁺ + Annexin V⁺/7-AAD⁻ cells (right) are shown. (D–F) OS was assessed in NSG mice transplanted with primary cells transduced with sh-NC or sh-NSUN2#1 from AML#7 (n = 6 for sh-NC and n = 6 for sh-NSUN2#1), AML#8 (n = 6 for sh-NC and n = 7 for sh-NSUN2#1), and AML#9 (n = 5 for sh-NC and n = 6 for sh-NSUN2#1). ** $P < 0.01$; *** $P < 0.001$ versus sh-NC.

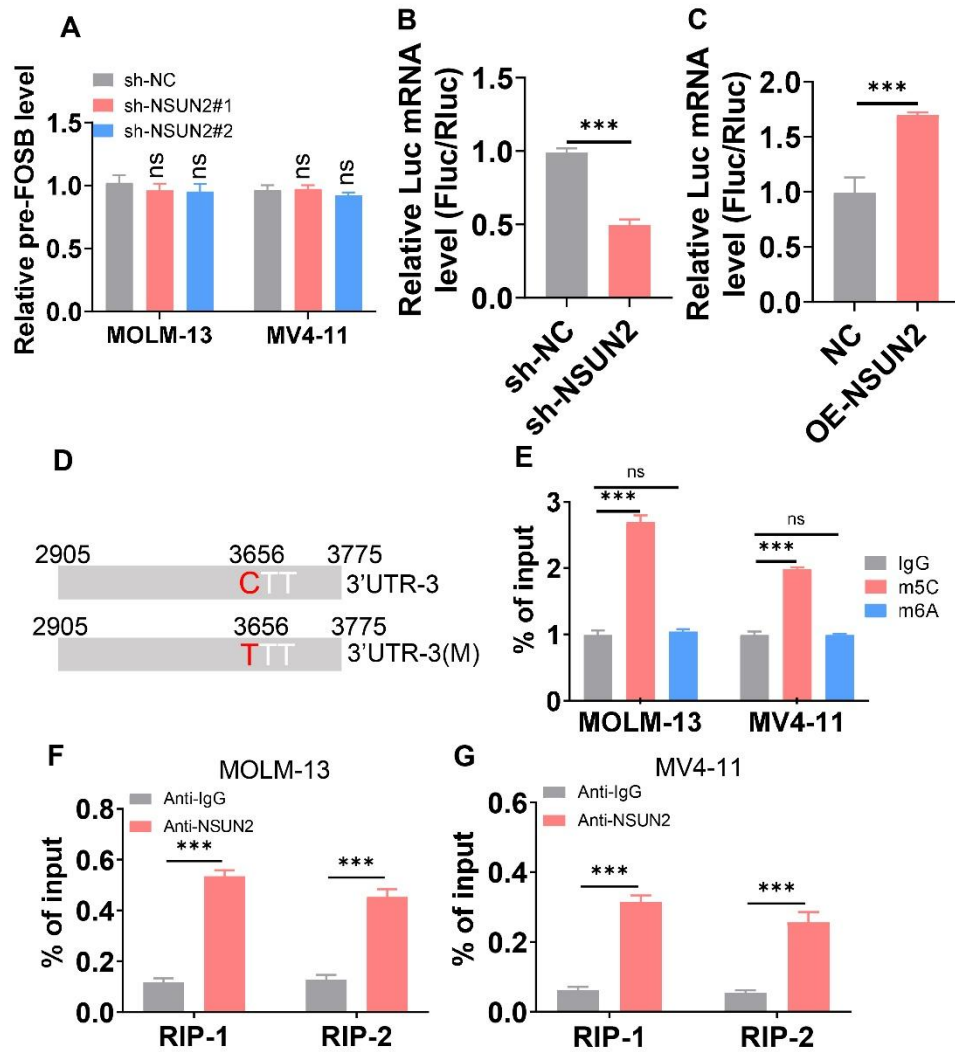


Supplemental Figure 6. Methylated RNA immunoprecipitation sequencing (MeRIP-seq) and RNA-seq analysis identify potential NSUN2 targets. MeRIP-seq and RNA-seq were performed in MOLM-13 cells transduced with sh-NC or sh-NSUN2. (A–C) Distribution of different m⁵C sites across the indicated transcript regions. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG)

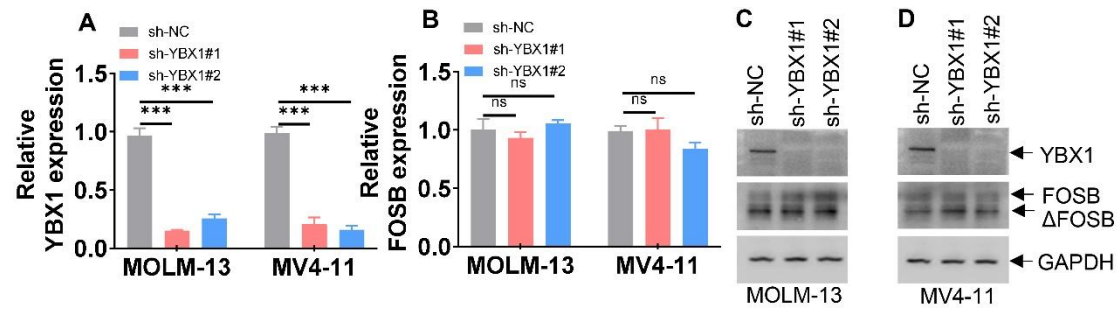
pathway analysis revealed enriched signal pathways. (E) Venn diagram showing the overlap between differentially methylated genes (DMGs) and differentially expressed genes (DEGs), as determined by MeRIP-seq and RNA-seq. (F) The transcript levels of potential NSUN2 targets were measured by qRT-PCR. $**P < 0.01$; $***P < 0.001$ versus sh-NC.



Supplemental Figure 7. NSUN2 positively regulates FOSB expression and promotes leukemogenesis in an m⁵C-dependent manner. (A–C) NSUN2 and FOSB transcript and protein levels were measured in leukemic cells overexpressing blank control (Vec) or NSUN2. (D and E) AML cells transduced with sh-NC were overexpressed with Vec, and AML cells transduced with sh-NSUN2#3 (3'-UTR) were overexpressed with Vec, wild-type (WT) NSUN2, or its two catalytically inactive mutants (C271A or C321A). Apoptosis was measured by Annexin V/7-AAD staining. n = 3 or more independent biological replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant.

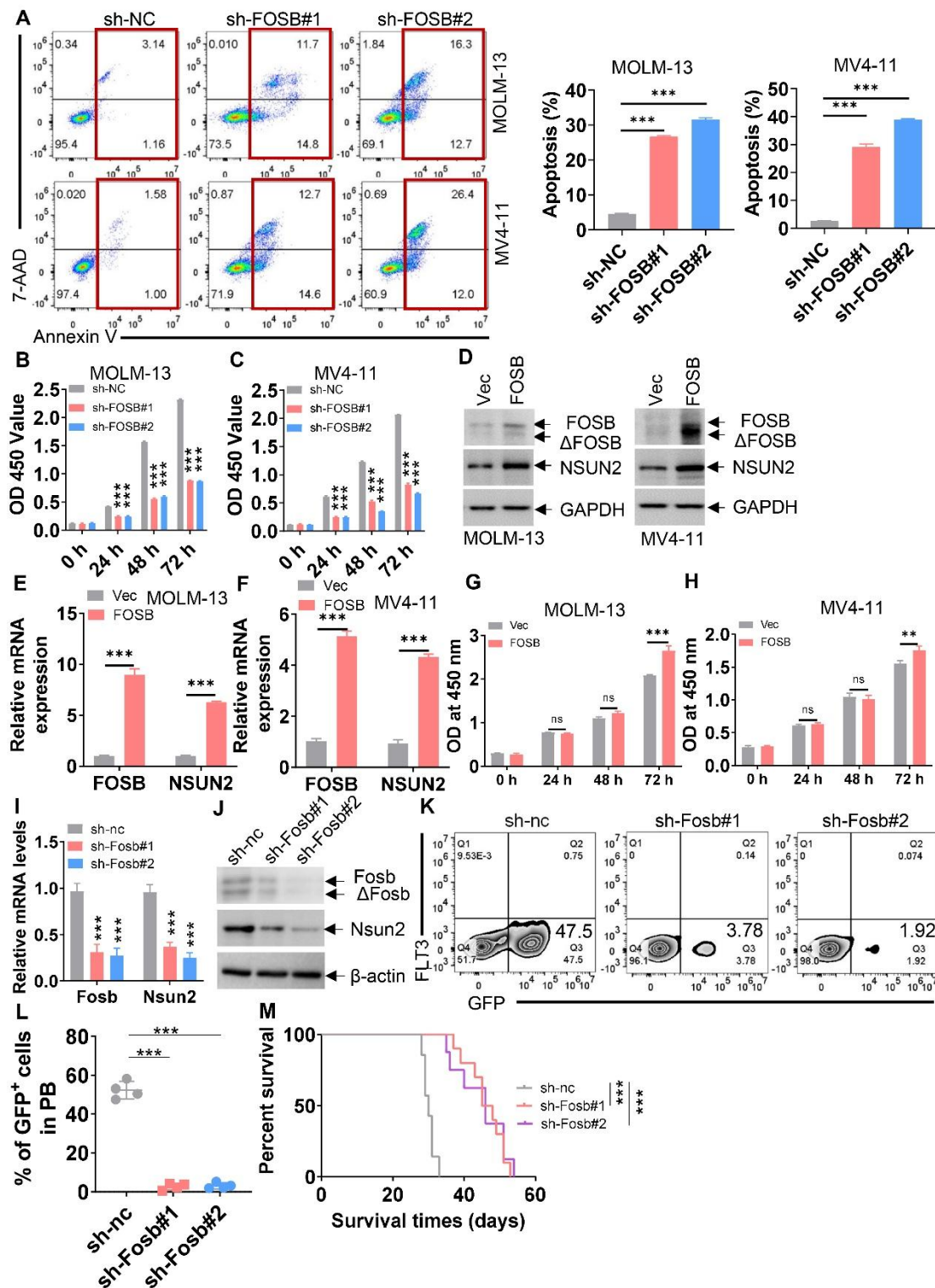


Supplemental Figure 8. NSUN2 stabilizes *FOSB* mRNA by methylating its 3'UTR. (A) Pre-*FOSB* transcript levels were measured in AML cells transduced with sh-NSUN2 or sh-NC. (B and C) pGL3-*FOSB*-3'UTR-3 was transfected into 293T cells with KD of NSUN2 or OE of NSUN2. Relative Luc mRNA levels (Firefly/Renilla) were measured in each cell lysate. (D) Schematic presentation of m⁵C methylation at position 3656 of *FOSB*-3'UTR-3, where C at position 3656 was mutated to T. (E) Enrichment of endogenous m⁵C or m⁶A modification in *FOSB* mRNA in AML cells. (F and G) Enrichment of endogenous *FOSB* mRNA when RIP was performed by anti-NSUN2 or anti-IgG antibody in AML cells. *** $P < 0.001$. ns = not significant.



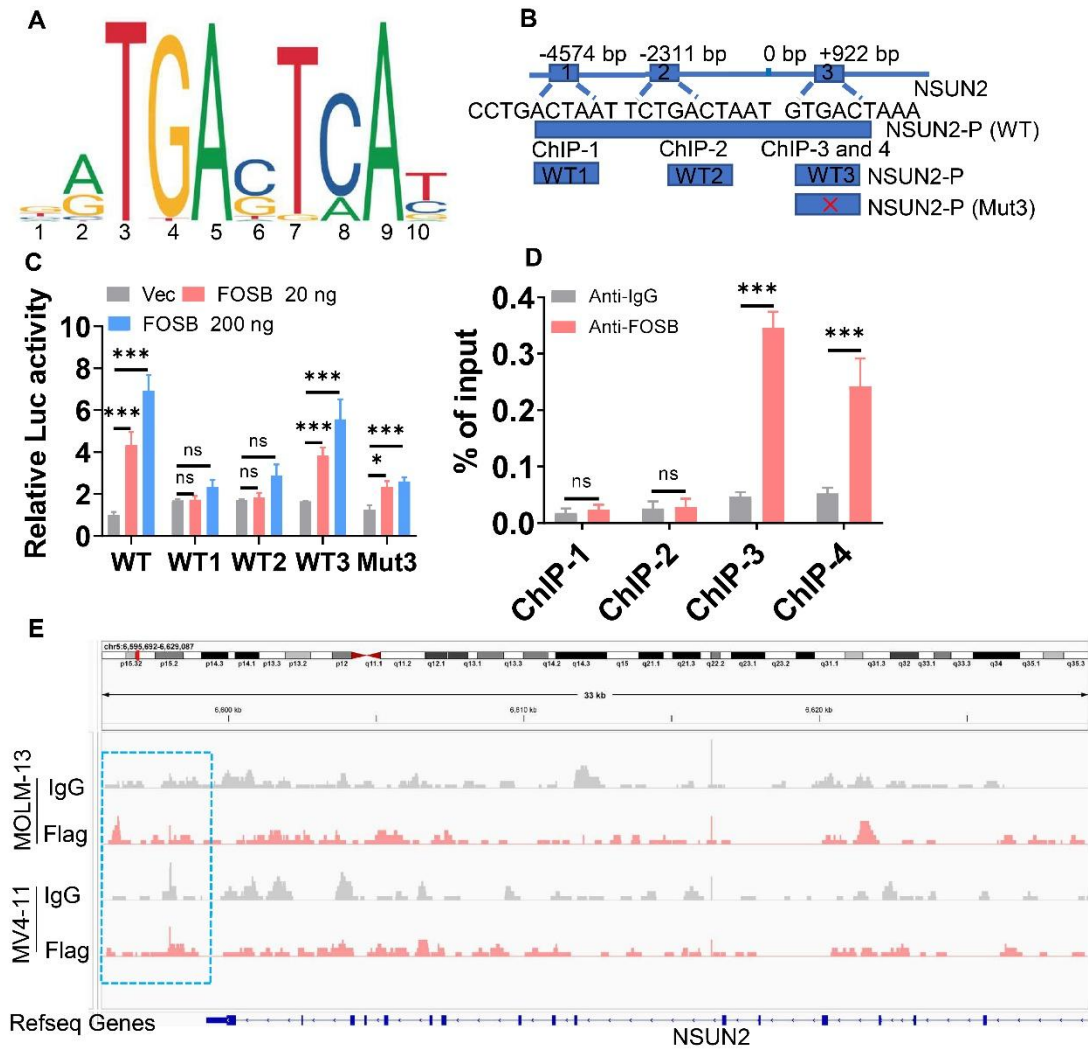
Supplemental Figure 9. KD of *YBX1* does not affect FOSB expression in leukemic cells. (A–D)

The transcript (A and B) and protein (C and D) expressions of YBX1 and FOSB were measured in MOLM-13 and MV4-11 cells, which were transduced with sh-NC or sh-YBX1 for 48 h, followed by puromycin (1 μ g/mL) treatment for an additional 48 h. *** $P < 0.001$ versus sh-NC. ns = not significant.



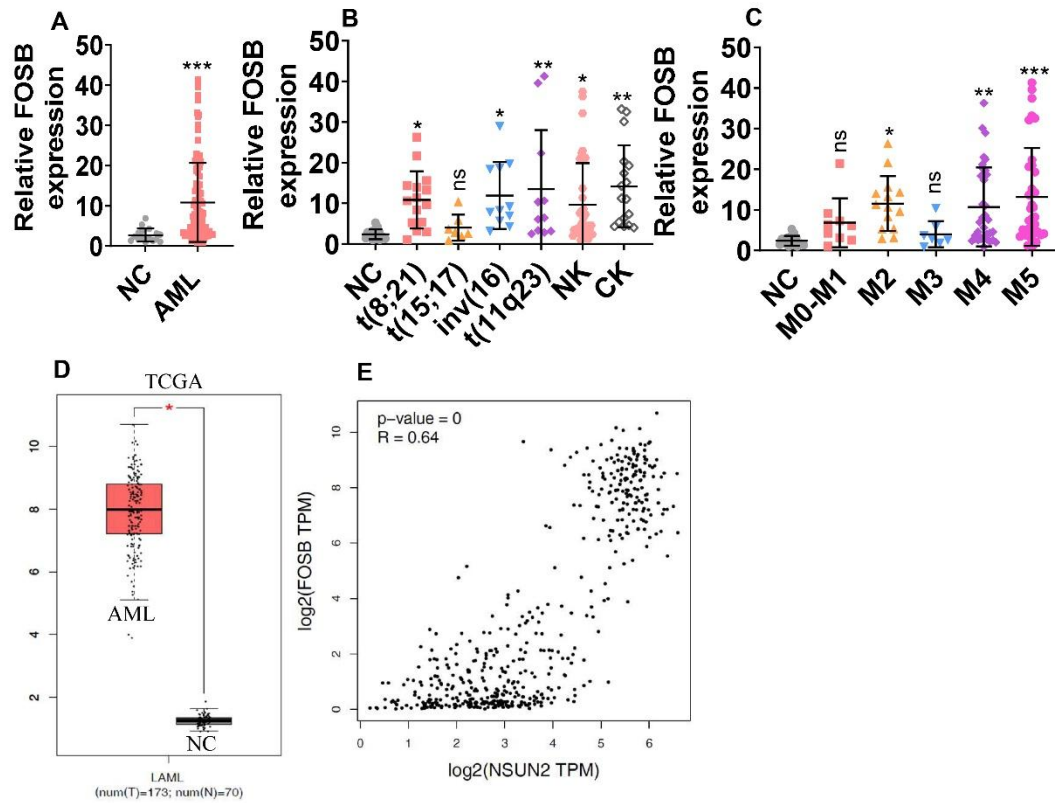
Supplemental Figure 10. FOSB facilitates leukemogenesis. (A) Apoptosis was measured in AML cells after transduction with sh-FOSB or sh-NC for 48 h, followed by puromycin (1 μ g/mL) treatment for an additional 48 h. (B and C) Cell proliferation was measured by CCK-8 assay in MOLM-13 and MV4-11 cells at the indicated times after transduction with sh-FOSB or sh-NC for

48 h. (D–F) The protein and transcript levels of FOSB and NSUN2 were measured in AML cells, which were overexpressed with FOSB or negative control (Vec) for 48 h. (G and H) Cell proliferation was measured by CCK-8 assay in MOLM-13 and MV4-11 cells at indicated times after overexpressing FOSB or Vec for 48 h. (I and J) BM GFP⁺ cells isolated from MA9-transformed leukemic mice were transduced with sh-nc or sh-Fosb for 48 h. The transcript (I) and protein expressions (J) of Fosb and Nsun2 were measured in GFP⁺ cells with sh-nc or sh-Fosb. (K and L) Equal GFP⁺ cells transduced with sh-nc or sh-Fosb (1×10^4 GFP⁺ cells per mouse) were xenografted into recipient mice. GFP⁺ cells were measured in PB from mice transplanted with GFP⁺ cells with sh-nc (n = 4), sh-Fosb#1 (n = 4), or sh-Fosb#2 (n = 4). Representative flow cytometry plots (K) and statistical analysis (L) are shown. (M) OS was assessed in recipient mice xenografted with GFP⁺ cells with sh-nc (n = 7), sh-Fosb#1 (n = 10), or sh-Fosb#2 (n = 9). ** $P < 0.01$; *** $P < 0.001$. ns = not significant.

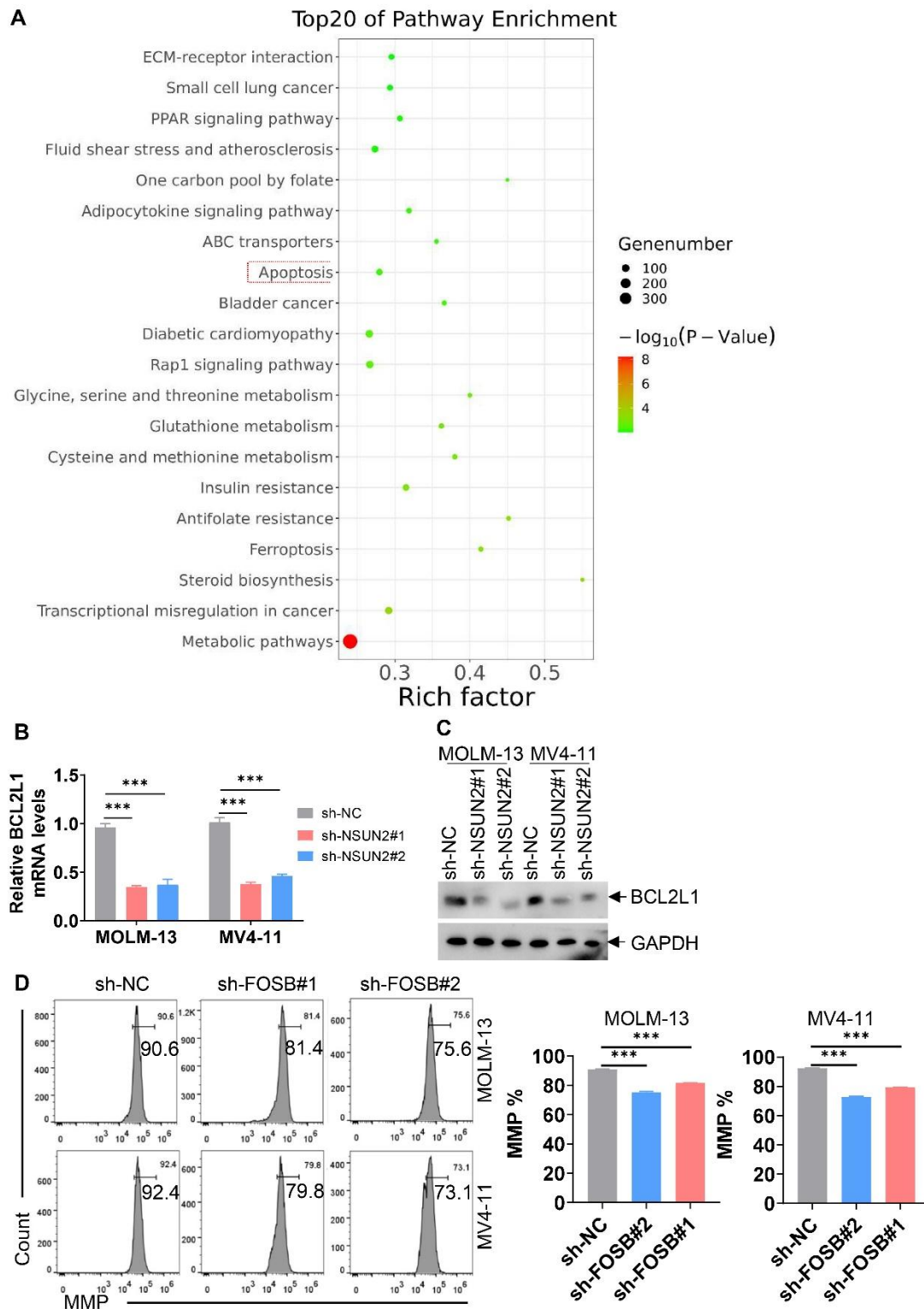


Supplemental Figure 11. FOSB activates NSUN2 expression by directly binding to NSUN2 promoter. (A) Predicted FOSB-binding motif in NSUN2 promoter using JASPAR (<https://jaspar.elixir.no/>). (B) Three potential FOSB-binding sequences in the NSUN2 promoter are shown. The potential binding motif (GTGACTAA) in WT3 was mutated to Mut3. (C) Luc activity was measured in 293T cells, which were transfected with blank vector or different doses of vector overexpressing FOSB, along with co-transfection of pGL3-NSUN2-P (WT1-3) or Mut3 for 48 h. (D) Soluble chromatin from MOLM-13 cells was immunoprecipitated (IPed) with anti-FOSB or anti-IgG. IPed DNA was analyzed by qPCR using four different primers for ChIP assays. (E) MOLM-13 and MV4-11 cells overexpressing Flag-tagged FOSB were IPed by anti-IgG or anti-Flag

antibody. IPed DNA were subjected to sequence. A FOSB-binding peak was identified in *NSUN2* promoter. n = 3 or more independent biological replicates. * $P < 0.05$; *** $P < 0.001$. ns = not significant.

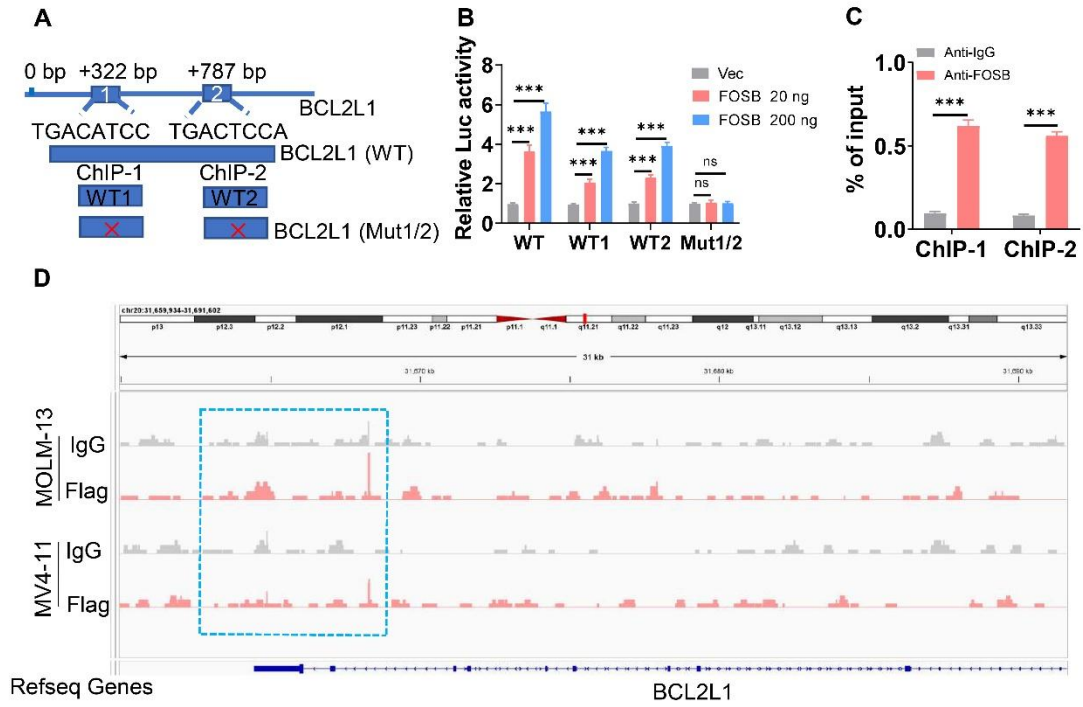


Supplemental Figure 12. *FOSB* expression is higher in AML patient samples compared with NC samples. (A) The transcript expression of *FOSB* was measured in BM cells from 102 untreated AML patients and 18 NC samples by qRT-PCR. (B) The expression of *FOSB* was analyzed in 102 untreated AML patients with different chromosomal translocations compared with 18 NC samples. (C) The expression of *FOSB* was analyzed in 102 untreated AML patients according to FAB subtypes (M0–M5) compared with 18 NC samples. (D) *FOSB* expression was assessed in the TCGA database. (E) The relationship between *NSUN2* and *FOSB* mRNA expressions was analyzed using GEPIA (Gene Expression Profiling Interactive Analysis). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus NC samples. ns = not significant.



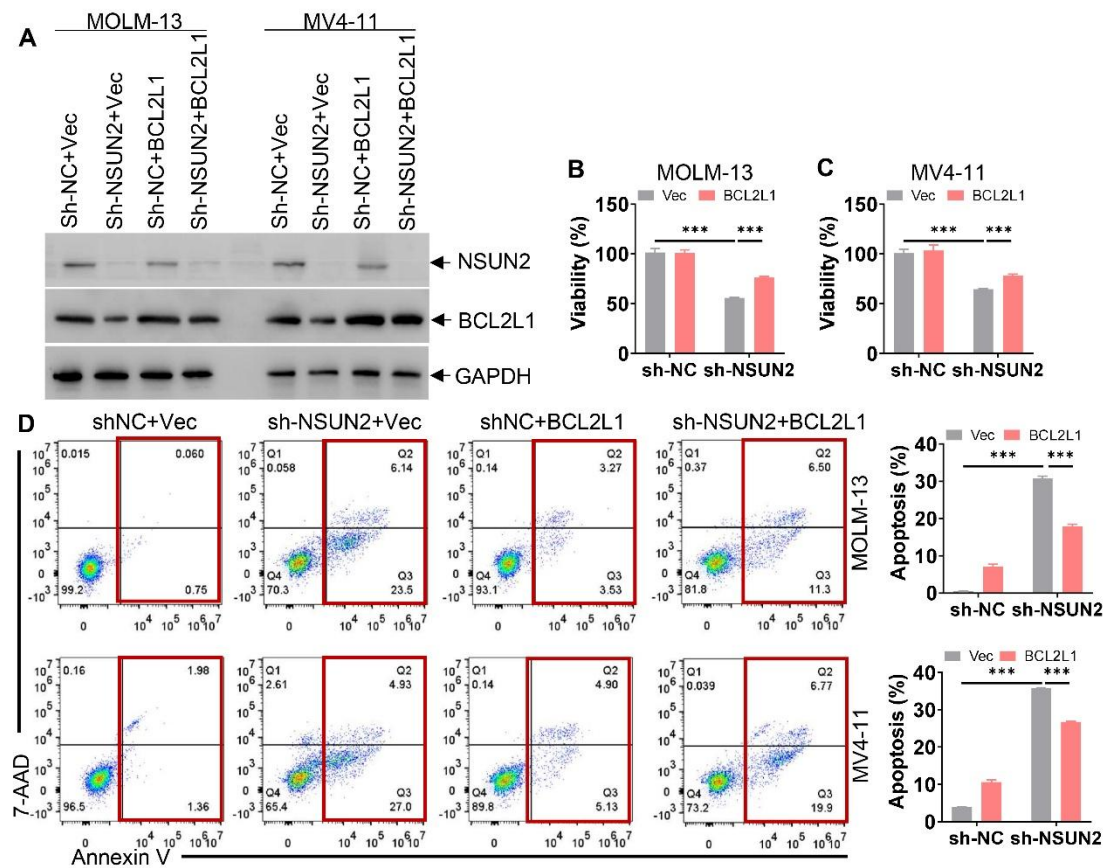
Supplemental Figure 13. NSUN2 KD inhibits BCL2L1 expression and FOSB KD affects mitochondrial function in AML cells. (A) MOLM-13 cells transduced with sh-NC or sh-FOSB#1 (sh-FOSB) were subjected for RNA-seq. Apoptosis-associated genes were significantly enriched by

pathway enrichment analysis. (B and C) BCL2L1 transcript and protein levels were measured in MOLM-13 and MV4-11 cells after sh-NSUN2 or sh-NC transduction for 48 h, followed by puromycin (1 µg/mL) treatment for an additional 48 h. (D) MMP assay was measured by JC-1 probe staining in leukemic cells transduced with sh-FOSB or sh-NC. The representative images (left) and statistical analysis of MMP (right) are shown. *** $P < 0.001$.



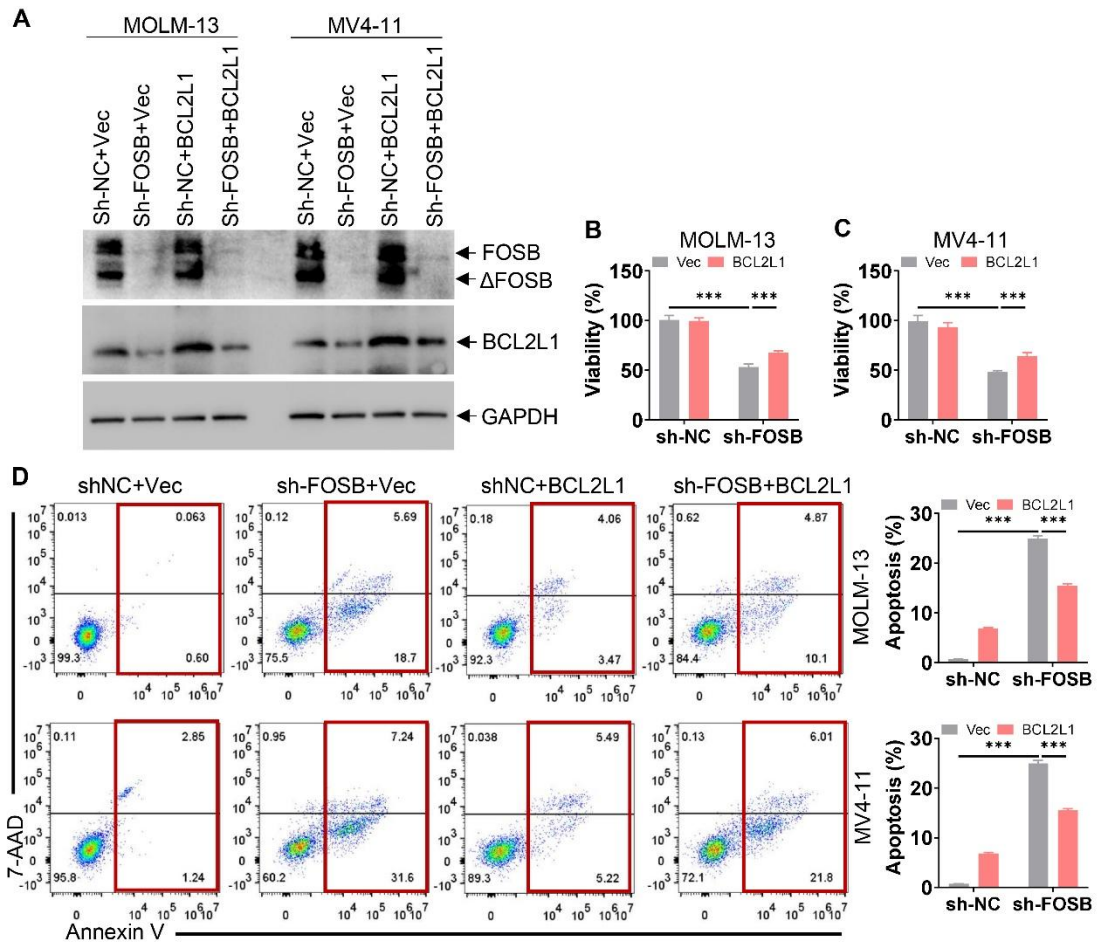
Supplemental Figure 14. FOSB activates *BCL2L1* expression by direct binding to *BCL2L1*

promoter. (A) Two potential FOSB-binding sequences in the *BCL2L1* promoter. The potential binding motifs in WT1 and WT2 were mutated to Mut1/2. (B) Luc activity was measured in 293T cells, which were transfected with blank vector or different doses of vector overexpressing FOSB, along with co-transfection of pGL3-BCL2L1-WT, WT1, WT2, or Mut1/2 for 48 h. (C) ChIP-qPCR showing FOSB binding to the *BCL2L1* promoter in MOLM-13 cells. (D) MOLM-13 and MV4-11 cells overexpressing Flag-tagged FOSB were IPed by anti-IgG or anti-Flag antibody. IPed DNA were subjected to sequence. A FOSB-binding peak was identified in the *BCL2L1* promoter. $n = 3$ or more independent biological replicates. *** $P < 0.001$. ns = not significant.



Supplemental Figure 15. BCL2L1 overexpression rescues the NSUN2 KD-induced phenotype.

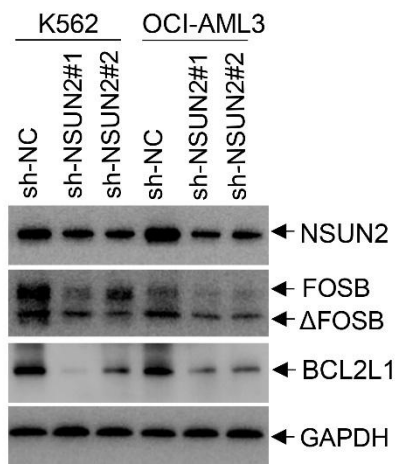
Leukemic cells were transduced with sh-NC or sh-NSUN2 for 48 h and overexpressed with blank vector (Vec) or vector overexpressing BCL2L1 for another 48 h. NSUN2 and BCL2L1 protein levels (A), viability (B and C), and apoptosis (D) were measured. *** $P < 0.001$.



Supplemental Figure 16. BCL2L1 overexpression rescues the FOSB KD-induced phenotype.

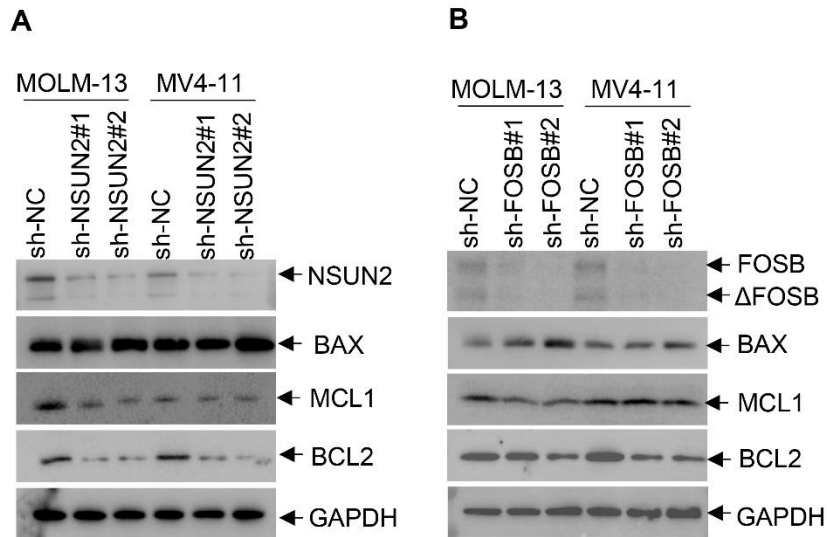
Leukemic cells were transduced with sh-NC or sh-FOSB for 48 h and overexpressed with blank vector (Vec) or vector overexpressing BCL2L1 for another 48 h. FOSB and BCL2L1 protein levels (A), viability (B and C), and apoptosis (D) were measured. *** $P < 0.001$.

A

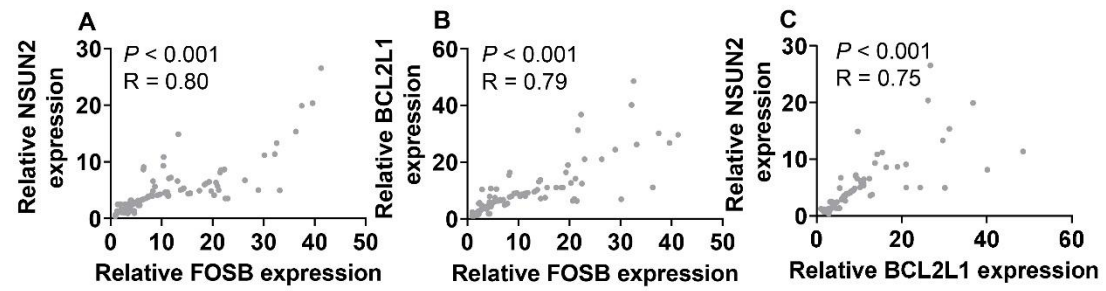


Supplemental Figure 17. NSUN2 KD decreases FOSB and BCL2L1 protein levels in K562 and

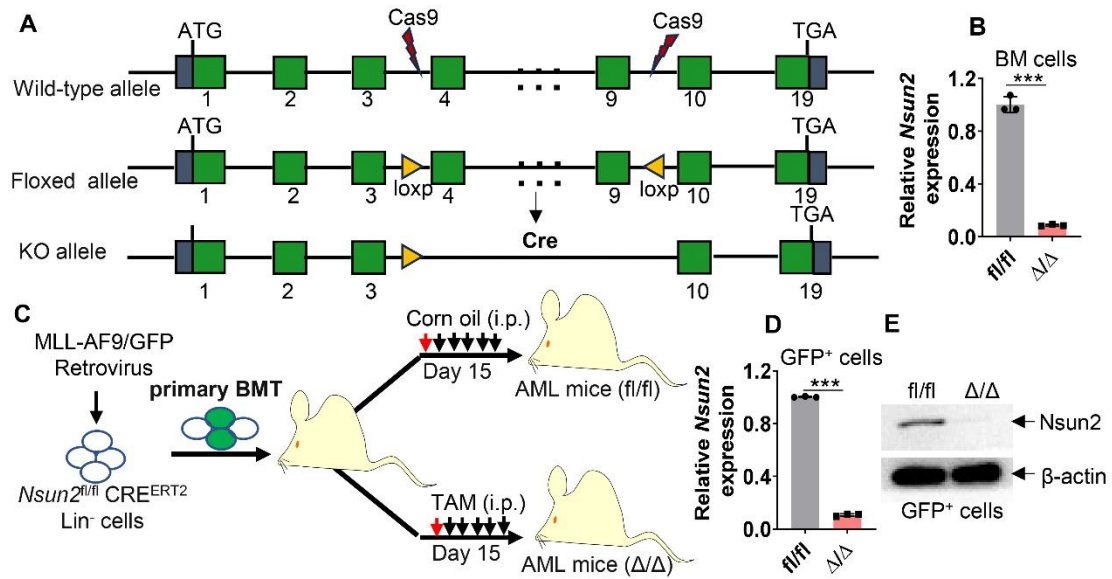
OCI-AML3 cells. (A) NSUN2, FOSB, and BCL2L1 protein levels were measured in K562 and OCI-AML3 cells after transduction with sh-NSUN2 or sh-NC for 48 h, followed by puromycin (1 $\mu\text{g/mL}$) treatment for an additional 48 h.



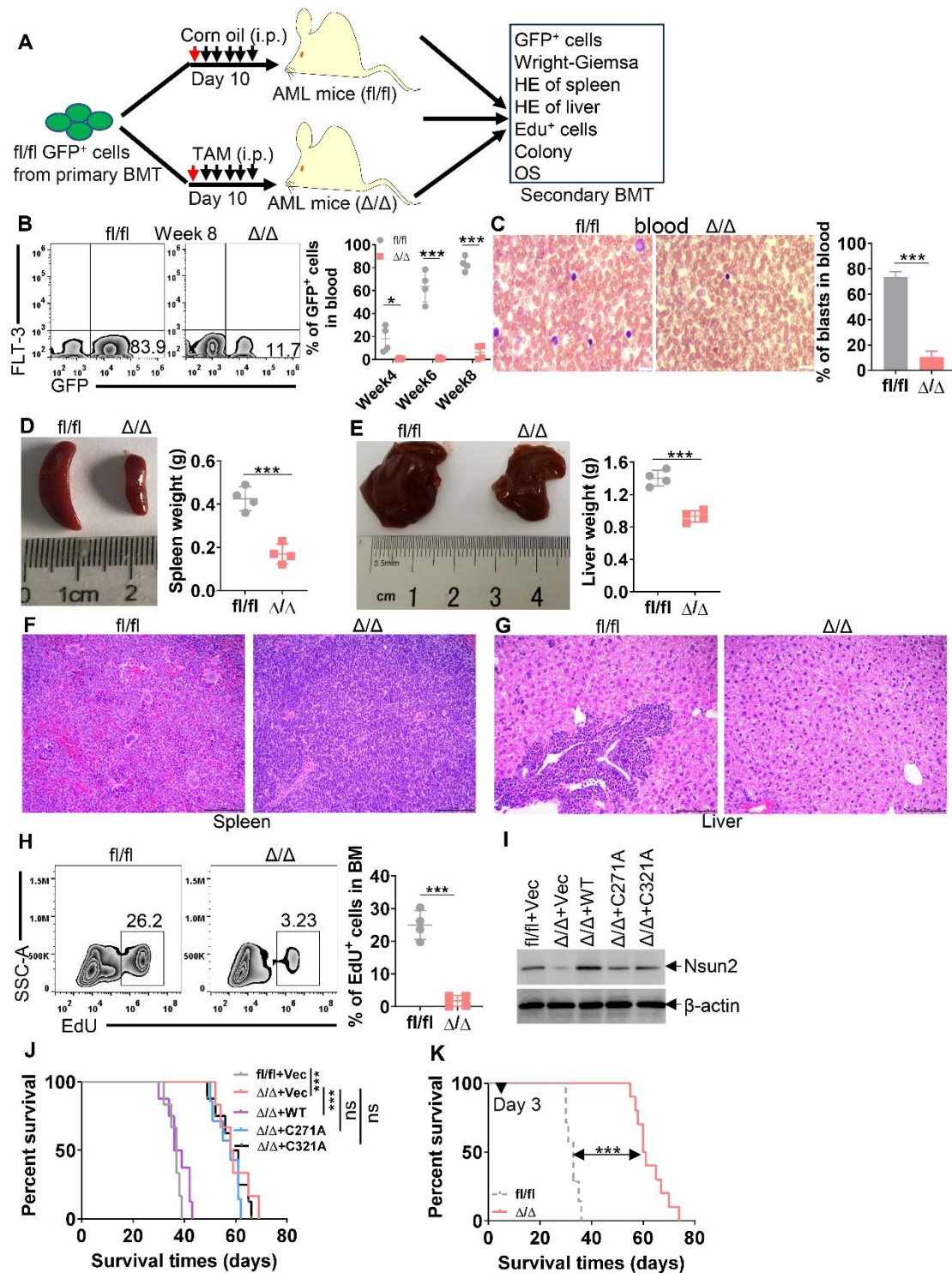
Supplemental Figure 18. NSUN2-FOSB axis regulates BCL-2 family members. (A) The indicated protein levels were measured in MOLM-13 and MV4-11 cells after transduction with sh-NSUN2 or sh-NC for 48 h, followed by puromycin (1 μ g/mL) treatment for an additional 48 h. (B) The indicated protein levels were measured in MOLM-13 and MV4-11 cells after transduction with sh-FOSB or sh-NC for 48 h, followed by puromycin (1 μ g/mL) treatment for an additional 48 h.



Supplemental Figure 19. The relationship between *NSUN2*, *FOSB*, and *BCL2L1* mRNA levels in primary AML patient samples. (A–C) *NSUN2* vs *FOSB* (A), *NSUN2* vs *BCL2L1* (B), and *FOSB* vs *BCL2L1* mRNA levels (C) were measured and analyzed in 102 untreated AML patients in-house.

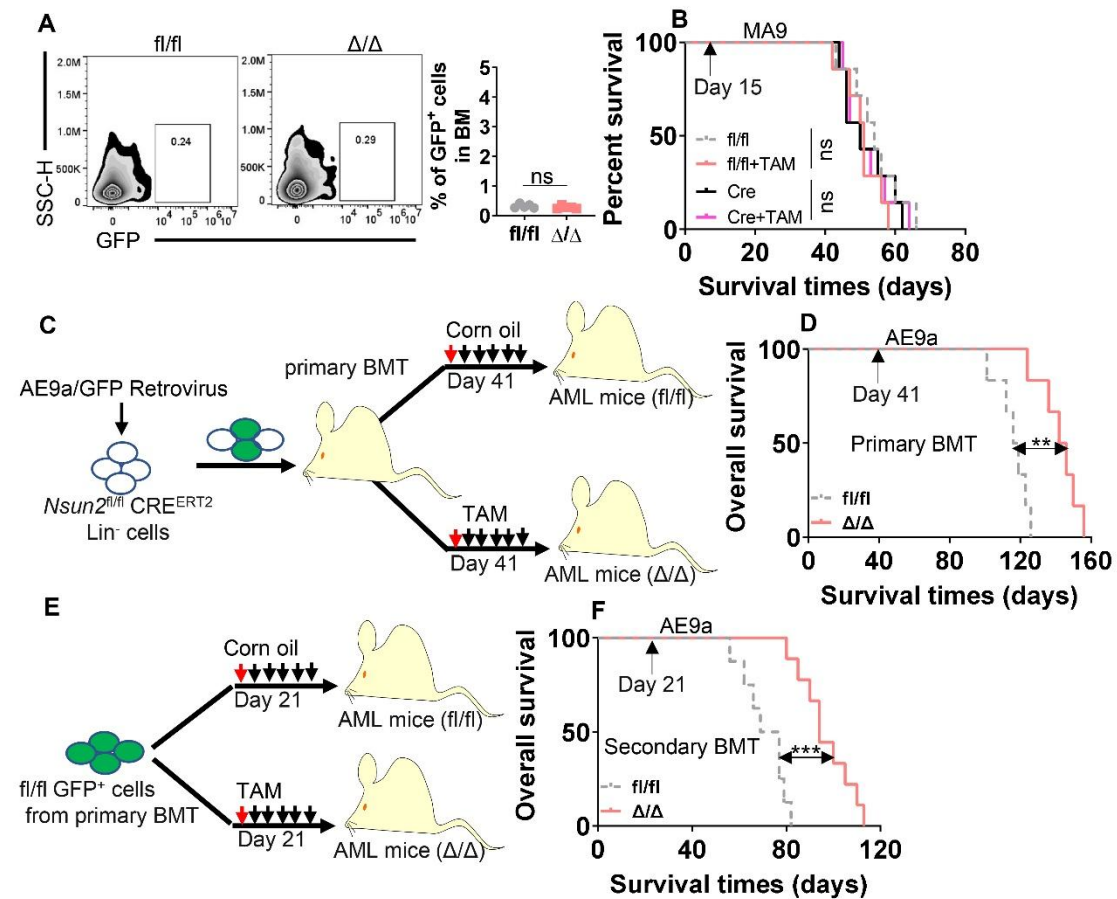


Supplemental Figure 20. TAM treatment depletes *Nsun2* in *Nsun2*^{fl/fl} Cre^{ERT2} mice. (A) Schematic representation of the *Nsun2*^{fllox/fllox} mice produced by the Cas9 system. (B) Six-week-old *Nsun2*^{fl/fl} Cre^{ERT2} mice were treated with TAM to deplete *Nsun2* expression (referred to as Δ/Δ) or with corn oil as a vehicle control (referred to as fl/fl). Mice were sacrificed one month after the last TAM injection, and BM cells from fl/fl (n = 3) and Δ/Δ (n = 3) mice were isolated to measure *Nsun2* transcript levels by qRT-PCR. (C) Experimental scheme for the MA9-transformed leukemic mouse model. Corn oil or TAM was injected on days 15 – 20 during primary BM transplantation (BMT). (D and E) *Nsun2* transcript and protein expressions were measured by qRT-PCR (D, n = 3 for fl/fl and n = 3 for Δ/Δ) and Western blot (E) in BM GFP⁺ fl/fl and Δ/Δ leukemic cells, respectively. A two-tailed Student's t-test was used to compare differences between two groups. ****P* < 0.001.



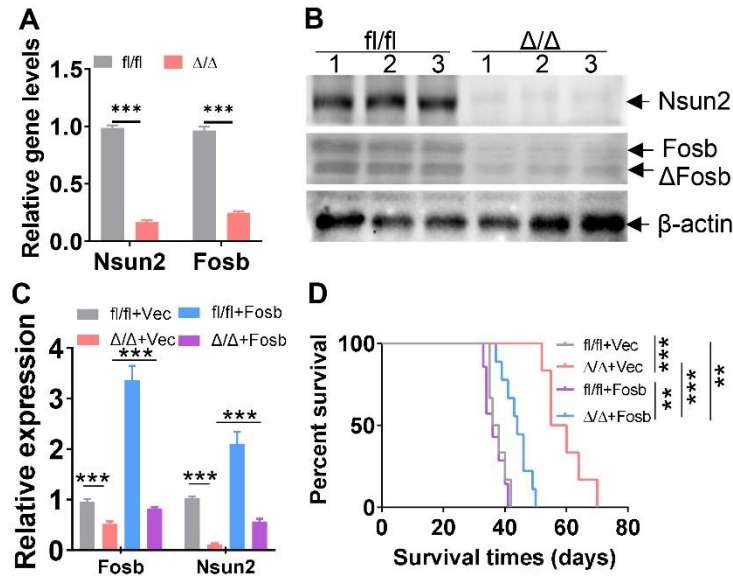
Supplemental Figure 21. The anti-leukemogenesis effects of *Nsun2* knockout in the MA9-transformed murine AML model. (A) Experimental scheme for the MA9-transformed murine AML model. Corn oil or TAM was injected in secondary BMT on days 10–15. (B) The frequency of GFP⁺ cells was measured in peripheral blood (PB) from fl/fl (n = 4) and Δ/Δ (n = 4) leukemic

mice at 4, 6, and 8 weeks after BMT. Representative flow cytometry plots (left) and statistical analysis (right) are shown. (C) Representative images of PB smears from fl/fl (n = 3) and Δ/Δ (n = 3) mice (left). Statistical analysis of the average percentage of leukemic cells in PB is shown (right). Scale bar represents 20 μm for PB. (D and E) The representative images of the spleen (left, D) and liver (left, E), and statistical analysis of the spleen (right, D) and liver (right, E) weights from fl/fl (n = 4) and Δ/Δ (n = 4) leukemic mice. (F and G) H&E staining of the spleen (F) and liver (G) tissues from fl/fl and Δ/Δ leukemic mice. Scale bars = 100 μm . (H) EdU staining was performed in BM GFP⁺ cells from fl/fl (n = 4) and Δ/Δ (n = 4) leukemic mice. Representative flow cytometry plots (left) and statistical analysis are shown (right). A two-tailed Student's t-test was used to compare differences between two groups. (I and J) Fl/fl cells were overexpressed with Vec, and Δ/Δ cells were overexpressed with Vec, wild-type (WT) Nsun2, or its two mutants. The protein levels of Nsun2 were measured by Western blot (I). All these groups of cells were xenografted in mice (n = 6 for fl/fl+Vec; n = 6 for Δ/Δ +Vec; n = 8 for Δ/Δ +WT; n = 7 for Δ/Δ +C271A; n = 8 for Δ/Δ +C271A), and OS was calculated (J). (K) GFP⁺ cells from primary BMT were transplanted into recipient mice. Corn oil or TAM was injected on days 3–8 in recipient mice. OS was calculated in fl/fl (n = 7) and Δ/Δ (n = 10) leukemic mice. Kaplan-Meier survival curves were generated to analyze OS time. * P < 0.05; *** P < 0.001. ns = not significant.

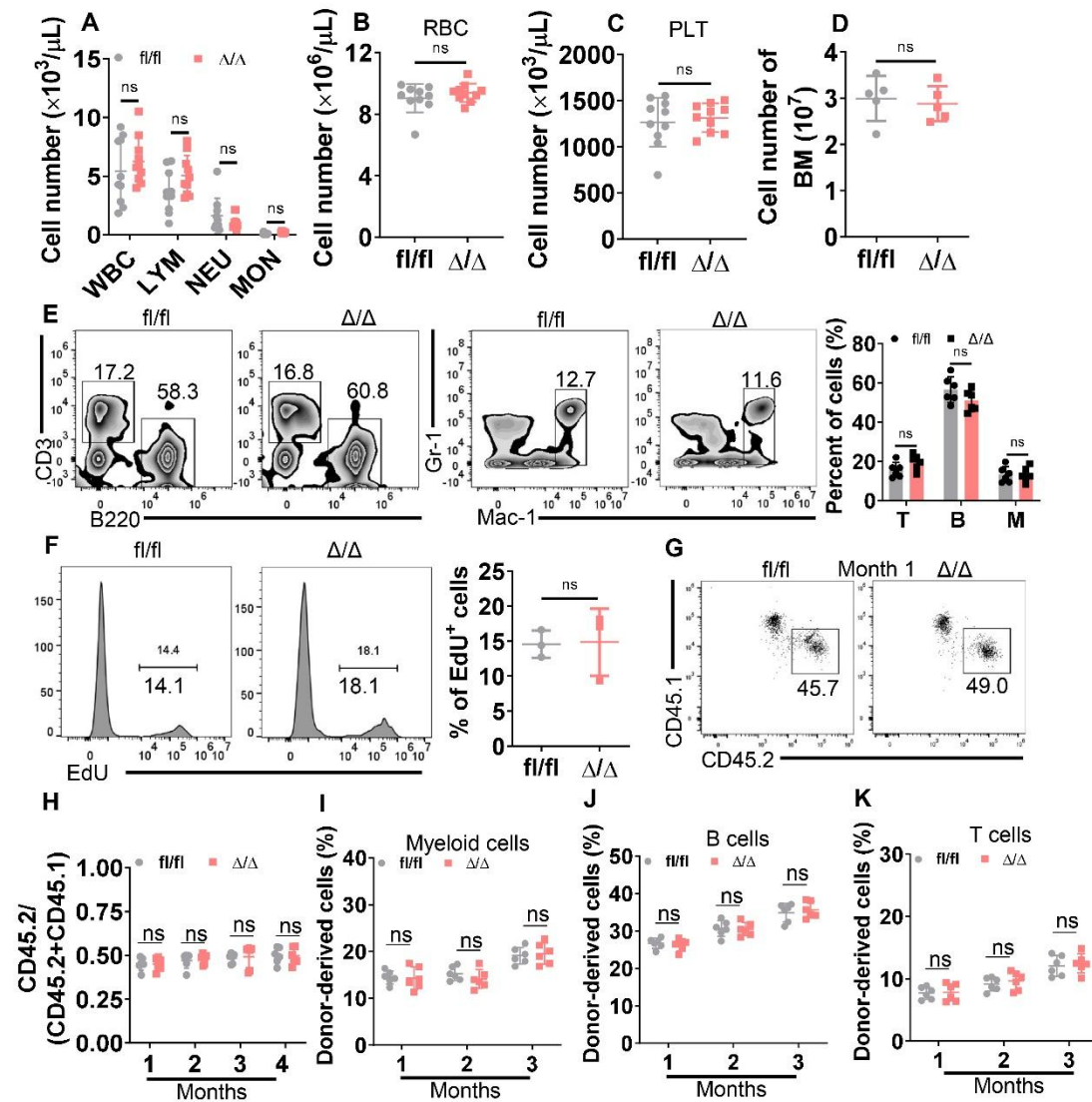


Supplemental Figure 22. The effect of homing ability, flox/flox, or Cre^{ERT2} on the survival of the MA9-induced model and the anti-leukemia effect of *Nsun2* knockout in AML1-ETO9a (AE9a)-induced murine AML model. (A) BM GFP⁺ cells from secondary BMT of mice receiving fl/fl or Δ/Δ cells were injected into the lethally irradiated mice. Homed cells (GFP⁺) were measured in BM at 16 h after transplantation. Representative plots (left) and statistical analysis of GFP⁺ cells are shown (right). (B) BM Lin⁻ cells from *Nsun2*^{fl/fl} or Cre^{ERT2} mice were transduced with MA9 and transplanted into lethally irradiated mice treated with or without TAM (days 15 – 20). OS was calculated in four groups (n = 7 for fl/fl; n = 7 for fl/fl+TAM; n = 8 for Cre; n = 8 for Cre+TAM). (C and D) Experimental scheme for the AE9a-transformed mouse model in primary BMT. Corn oil or TAM was injected on days 41–46 after transplantation (C). Kaplan-Meier curve showing the effect of *Nsun2* depletion in the primary BMT (n = 6 for fl/fl; n = 6 for Δ/Δ) (D). (E and F)

Experimental scheme for the AE9a-transformed mouse model in secondary BMT. Corn oil or TAM was injected on days 21–26 after transplantation (E). Kaplan-Meier curve showing the effect of *Nsun2* depletion in secondary BMT (n = 8 for fl/fl; n = 10 for Δ/Δ) (F). Kaplan-Meier survival curves were generated to analyze OS time. $**P < 0.01$; $***P < 0.001$. ns = not significant.



Supplemental Figure S23. Fosc expression is lower in Δ/Δ than fl/fl cells, and overexpression of Fosc rescues the extended OS induced by *Nsun2* knockout. (A and B) Murine *Nsun2* and *Fosb* transcript (A) and protein (B) levels were measured in BM fl/fl (n = 3) and Δ/Δ (n = 3) GFP⁺ cells from MA9-transduced leukemic mice. (C and D) GFP⁺ fl/fl and Δ/Δ cells were isolated from MA9-transformed mice and transduced with blank Vec or vector overexpressing *Fosb*. Transcript levels of *Fosb* and *Nsun2* were measured (C). Equal numbers of cells (1×10^4 GFP⁺ cells for per mouse) from the four groups were xenografted into recipient mice for calculating OS (n = 6 for fl/fl+Vec; n = 6 for Δ/Δ +Vec; n = 7 for fl/fl+Fosb; n = 9 for Δ/Δ +Fosb) (D). ***P* < 0.01; ****P* < 0.001.

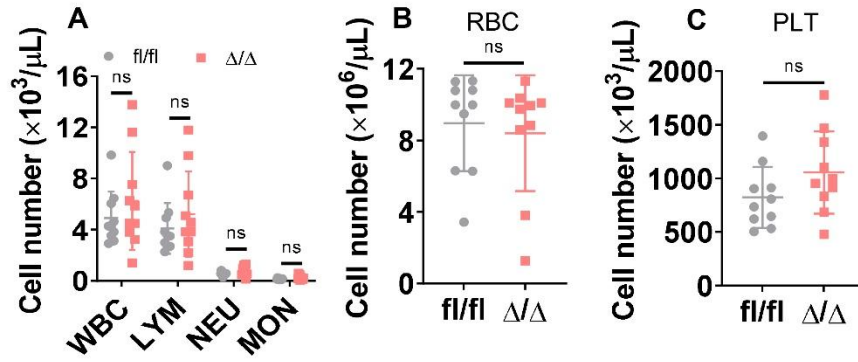


Supplemental Figure 24. *Nsun2* depletion does not affect normal hematopoiesis. Six-week-old

Nsun2^{fl/fl} Cre^{ERT2} mice were treated with TAM to deplete *Nsun2* expression (Δ/Δ) or corn oil as a vehicle control (fl/fl). Mice were sacrificed one month after the last TAM injection. (A–C) Blood count analysis was performed in the PB from fl/fl (n = 10) and Δ/Δ (n = 10) mice. A for WBC, LYM, NEU, and MON; B for RBC; C for PLT. (D) Total BM cell numbers were counted in fl/fl (n = 5) and Δ/Δ (n = 5) mice. (E) Mature lineage cells were analyzed in the PB from fl/fl (n = 5) and Δ/Δ (n = 5) mice. T cells (CD3⁺B220⁺); B cells (CD3⁺B220⁺); M cells (Mac-1⁺Gr-1⁺ or Mac-1⁺Gr-1⁻). (F) EdU staining was performed in BM cells from fl/fl (n = 3) and Δ/Δ (n = 3) mice.

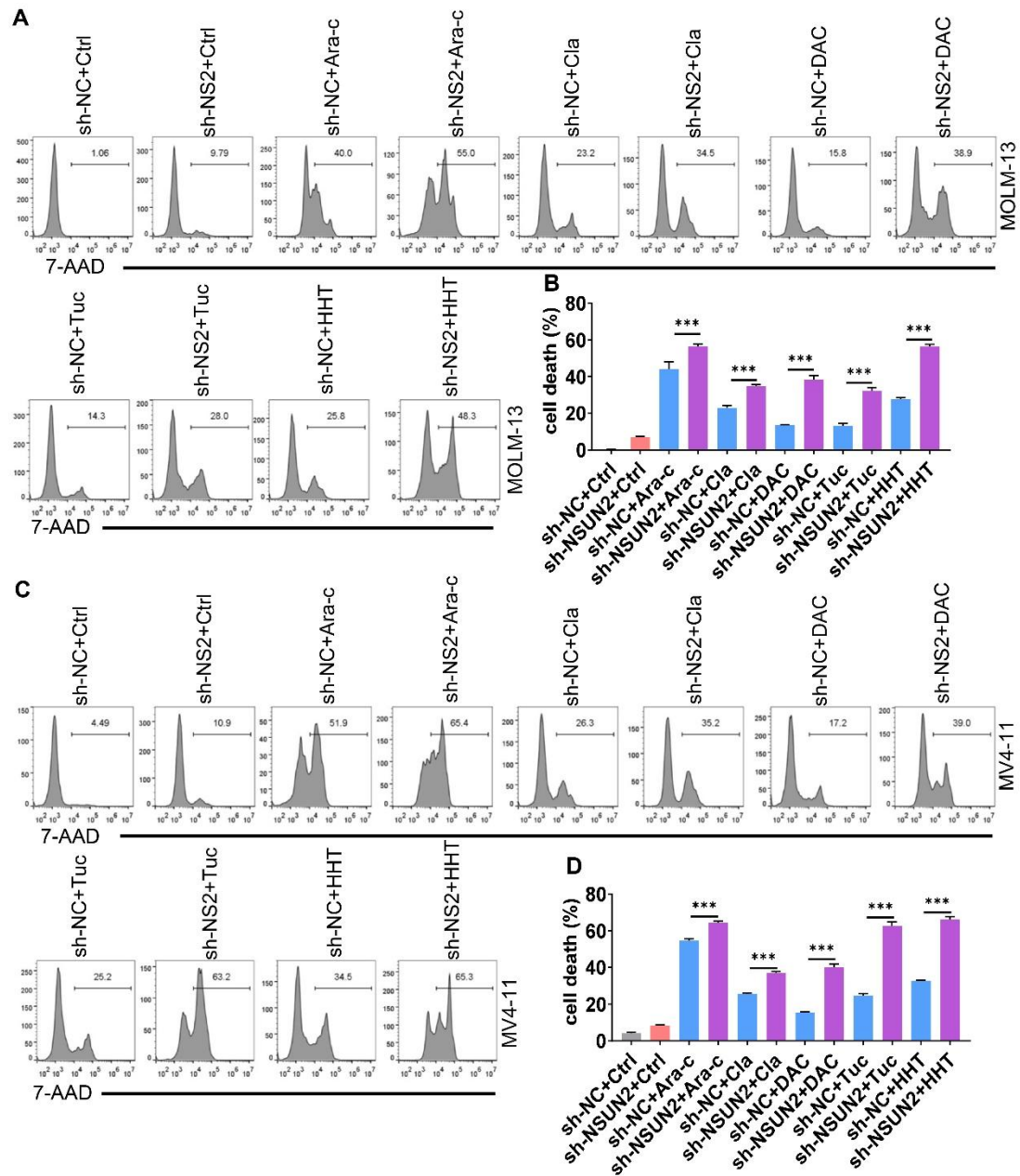
Representative flow cytometry plots (left) and statistical analysis of EdU⁺ cells (right) are shown.

(G–K) Total BM cells from fl/fl or Δ/Δ mice (CD45.2) mixed with an equal number of CD45.1 BM cells were transplanted into CD45.1 mice. Representative plots (G) and statistical analysis of CD45.2/(CD45.2+CD45.1) populations at different months after BMT (n = 6 for fl/fl and Δ/Δ) (H). Mature lineage cells including myeloid cells (I), B cells (J), and T cells (K) were analyzed in the PB from CD45.1 mice (n = 6 for fl/fl and Δ/Δ). ns = not significant.

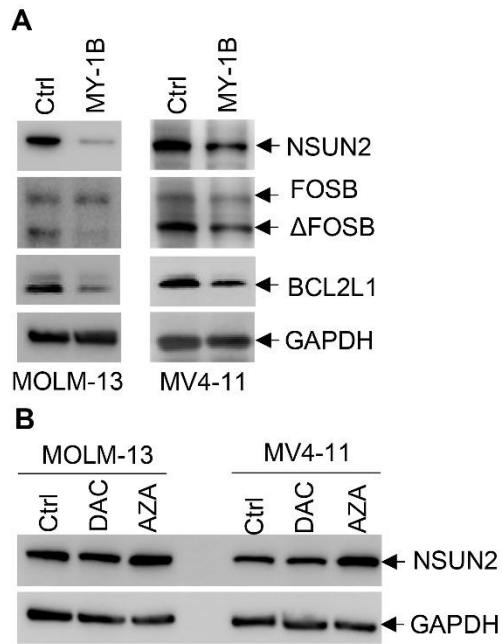


Supplemental Figure 25. *Nsun2* depletion does not affect normal hematopoiesis in elderly mice.

Elderly *Nsun2*^{fl/fl} Cre^{ERT2} mice (> 12 months) were treated with TAM to deplete *Nsun2* expression (Δ/Δ) or corn oil as a vehicle control (fl/fl). Mice were sacrificed one month after the last TAM injection. (A–C) Blood count analysis was performed in the PB from fl/fl (n = 10) and Δ/Δ (n = 10) mice. A for WBC, LYM, NEU, and MON; B for RBC; C for PLT. ns = not significant.



Supplemental Figure 26. NSUN2 KD facilitates cytotoxicity of chemotherapeutic drugs. (A–D) Cell death was measured by 7-AAD staining using flow cytometry in MOLM-13 and MV4-11 cells, which were transduced with sh-NC or sh-NSUN2 for 48 h, followed by treatment with cytarabine (1 μ M, Ara-C), cladribine (0.5 μ M, Cla), decitabine (1 μ M, DAC), tucidinostat (1 μ M, Tuc), and homoharringtonine (0.002 μ M, HHT) for 24 h. Representative plots (A and C) and statistical analysis of 7-AAD⁺ cells (B and D) are shown. *** $P < 0.001$.



Supplemental Figure 27. NSUN2 inhibitor MY-1B regulates FOSB-BCL2L1 axis and hypomethylation agents do not regulate NSUN2 protein level. (A) The protein levels of NSUN2, FOSB, and BCL2L1 were measured in MOLM-13 and MV4-11 cells, which were treated with or without MY-1B (5 μ M) for 48 h. (B) The protein level of NSUN2 was measured in MOLM-13 and MV4-11 cells, which were treated with or without DAC (1 μ M) or AZA (1 μ M) for 72 h.