NSUN2-FOSB reciprocity facilitates leukemogenesis in an m^{5}C-dependent manner by increasing BCL2L1 expression

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Abstract

NOP2/Sun RNA methyltransferase family member 2 (NSUN2) catalyzes 5-methylcytosine (m^{5}C) modifications on RNA to regulate mRNA stability. However, its roles in normal hematopoiesis and leukemogenesis remain poorly understood. Here, we show that NSUN2 is markedly upregulated in primary acute myeloid leukemia (AML) patients' samples compared with normal hematopoietic cells. NSUN2 knockdown impaired AML cell proliferation, induced apoptosis, and reduced colony formation. Genetic ablation of Nsun2 in an MLL-AF9-transformed murine AML model substantially impaired leukemia stem cell self-renewal and prolonged overall survival, while sparing normal hematopoiesis, highlighting NSUN2 as a potential therapeutic target. Notably, wild-type NSUN2, but not catalytically inactive mutants, restored leukemia stem cell function and leukemogenesis in NSUN2-deficient AML cells, indicating that these effects are m^{5}C-dependent. Mechanistically, NSUN2 stabilized FosB proto-oncogene (FOSB) mRNA via m^{5}C modification at nucleotide 3656 in the 3′ untranslated region, thereby upregulating FOSB expression. In turn, FOSB transcriptionally activated NSUN2, forming a feedforward regulatory loop. Furthermore, FOSB promoted expression of the anti-apoptotic regulator B-cell lymphoma-2-like protein 1 (BCL2L1) by directly binding to its promoter. In conclusion, these findings uncover a novel NSUN2-FOSB-BCL2L1 axis that drives AML leukemogenesis in an m^{5}C-dependent manner, suggesting the therapeutic potential of targeting this pathway.

Introduction

Chemical modifications on RNA, including N^6-methyladenosine (m^{6}A) and 5-methylcytosine (m^{5}C), play critical roles in regulating gene expression at the post-transcriptional level.[1] m^{5}C modification occurs on multiple types of eukaryotic RNA, including ribosomal RNA, transfer RNA, and messenger RNA (mRNA), and has been increasingly recognized on mRNA, where it modulates RNA stability and translation efficiency.[2] For example, m^{5}C modification of *p16* mRNA enhances its stability.[3] Thus, dysregulated m^{5}C modulation of mRNA has critical implications for processes such as tumorigenesis, protein synthesis, and cell proliferation.[4]

AML is a fatal hematologic malignancy characterized by blocked differentiation and impaired cell death, driven by clonal expansion of hematopoietic stem and progenitor cells (HSPC).[5] Emerging evidence indicates that aberrant m^{5}C modification contributes to the initiation and progression of AML.[6]

NOP2/Sun RNA methyltransferase family member 2 (NSUN2) is a methyltransferase that catalyzes m^{5}C modifications of various RNA, including transfer RNA, mRNA, and ribosomal RNA.[7] Elevated NSUN2 expression promotes tumor prolif-

eration, metastasis, and progression through m^{5}C-mediated stabilization of target mRNA.[8] For instance, NSUN2 enhances esophageal squamous cell carcinoma progression by stabilizing *GRB2* mRNA via m^{5}C modification.[9] Recent studies suggest that NSUN2 also supports proliferation and survival in AML.[6,10] However, its precise roles in normal hematopoiesis and leukemogenesis remain unclear.

In this study, we demonstrate that NSUN2 knockdown (KD) impairs AML survival and leukemia stem cell (LSC) self-renewal while sparing normal hematopoiesis. Mechanistically, NSUN2 methylates FosB proto-oncogene (*FOSB*) mRNA, and FOSB reciprocally regulates NSUN2 expression, establishing an NSUN2-FOSB regulatory circuit. Furthermore, FOSB increases B-cell lymphoma-2-like protein 1 (BCL2L1) expression by directly binding to its promoter. Our findings reveal the therapeutic potential of targeting the NSUN2-FOSB-BCL2L1 axis in AML.

Methods

Leukemic cell lines, primary acute myeloid leukemia samples, and normal hematopoietic cells

Human leukemic cell lines were obtained from the American Type Cell Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified incubator with 5% CO_2 at 37°C. Human bone marrow mononuclear cells were isolated from patients with *de novo* AML using density gradient centrifugation (Invitrogen). Primary CD34$^+$ cells from AML patients were isolated by the EasySep™ human CD34$^+$ selection kit (StemCell Technologies, Vancouver, Canada). Normal CD34$^+$ cells isolated from healthy donors were used as normal controls. All procedures involving human participants adhered to the ethical standards set by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (KY2022-R110) and to the Helsinki Declaration of 1975, as revised in 2000. The clinical characteristics of AML patients are summarized in *Online Supplementary Table S1*.

Methylated RNA immunoprecipitation

Details of methylated RNA immunoprecipitation (MeRIP) are provided in the *Online Supplementary Methods*. Quantitative polymerase chain reaction (PCR) was performed using m^{5}C- or m^{6}A-IPed RNA or input RNA with primers (*Online Supplementary Table S2*).

Limiting dilution assays

Nsun2-depleted or wild-type (WT) green fluorescence protein (GFP)$^+$ cells were isolated from MLL-AF9 (MA9)-induced secondary bone marrow transplant (BMT) recipients. Three doses of donor cells (*Online Supplementary Table S3*) were transplanted into lethally irradiated mice (N=6 for each group). Recipient survival was monitored for 6 months after BMT. LSC frequency was evaluated using extreme limiting dilution assay (ELDA) software.[11]

Data availability

All RNA-sequencing data are publicly available on the Gene Expression Omnibus under accession numbers GSE 252880, GSE 253074, and GSE 308401.

Other procedures

Details of CCK-8 measurements, apoptosis assays, m^{5}C blotting, and other experiments are provided in the *Online Supplementary Methods.*

Statistical analysis

Data from at least three independent experiments *in vitro* were expressed as the mean ± standard deviation. A Mann-Whitney-Wilcoxon test was used to compare differences in two independent subgroups in clinical samples. Generally, a two-tailed Student *t* test was used to compare differences between two groups. One-way analysis of variance followed by the Tukey test was performed to compare three or more groups. Kaplan-Meier survival curves were generated to analyze overall sorvival, and *P* values were calculated using the log-rank test. Fisher exact tests were applied to compare categorical variables. All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant when $P<0.05$.

Results

NSUN2 expression is elevated in acute myeloid leukemia cells compared with normal controls

To investigate *NSUN2* expression in AML, we measured *NSUN2* levels in 102 patients with *de novo* AML (*Online Supplementary Table S1*) and 18 healthy volunteers as normal controls (NC). *NSUN2* expression was significantly higher in AML patients than in NC (Figure 1A), and *NSUN2* levels were elevated in AML cells with specific chromosomal translocations (Figure 1B) and French-American-British subtypes (Figure 1C) compared with those in NC. *NSUN2* expression was also higher in 12 CD34$^+$ AML cells than in seven CD34$^+$ NC samples (Figure 1D). To further validate these findings, we analyzed public datasets. *NSUN2* expression was consistently higher in AML cells than in NC according to BloodSpot (*Online Supplementary Figure S1A, B*),[12] GSE 114868 (*Online Supplementary Figure S1C*),[13] and the Beat AML database (*Online Supplementary Figure S1D*). *NSUN2* expression was also elevated in both CD34$^-$ and CD34$^+$ AML cells in comparison to that in CD34$^+$ NC in the GSE30029 (*Online Supplementary Figure S1E*).[14] Furthermore, NSUN2 protein level was higher in six primary AML samples than in two NC samples (Figure 1E), and its level was also elevated in six CD34$^+$ AML compared to that in two CD34$^+$

NC samples (Figure 1E). Similarly, the mean fluorescence intensity of NSUN2 was markedly higher in two primary AML samples than in the two NC samples (Figure 1F).

We next assessed whether NSUN2 expression correlates with patients' outcome. The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) AML database revealed that AML patients with higher *NSUN2* levels had shorter overall survival ($P<0.05$) (*Online Supplementary Figure S2A*). However, NSUN2 expression above the median did not predict poor prognosis in The Cancer Genome Atlas (TCGA) database (*Online Supplementary Figure S2B*). Only AML patients with NSUN2 expression above the upper quartile had inferior survival (*Online Supplementary Figure S2C*).

NSUN2 facilitates the survival of acute myeloid leukemia cells

To investigate the functional role of NSUN2 in AML, we decreased NSUN2 expression using two short hairpin (sh) RNA (Figure 2A, B). NSUN2 KD markedly reduced cell proliferation (Figure 2C), induced G0 cell cycle arrest (*Online Supplementary Figure S3A*), impaired colony formation (*Online Supplementary Figure S3B*), and increased apoptosis (Figure 2D). Poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3, was prominently cleaved in *NSUN2* KD cells (*Online Supplementary Figure S3C, D*). Because apoptosis is often initiated via mitochondrial pathways, we examined mitochondrial function.[15] NSUN2 KD decreased mitochondrial membrane potential in AML cells (*Online Supplementary Figure S3E*) and reduced mitochondrial mass compared with that in sh-NC cells (*Online Supplementary Figure S3F*). Transmission electron microscopy revealed that NSUN2 KD caused irregular mitochondrial swelling, disrupted and blurred cristae, and reduced cristae density in MOLM-13 cells (Figure 2E). MOLM-13 cells with or without NSUN2 KD were xenografted in NSG mice. NSUN2 KD substantially reduced the leukemic burden, as evidenced by decreased human $CD45^+$ cells in peripheral blood (*Online Supplementary Figure S4A*) and extended overall survival in NSG mice (*Online Supplementary Figure S4B*). Since NSUN2 is the main enzyme catalyzing m^5C modification,[2] we measured the global m^5C level. NSUN2 KD reduced global m^5C levels, as shown by m^5C blotting (*Online Supplementary Figure S4C*) and enzyme-linked immunosorbent assay (*Online Supplementary Figure S4D*). We further evaluated the effects of NSUN2 KD in primary

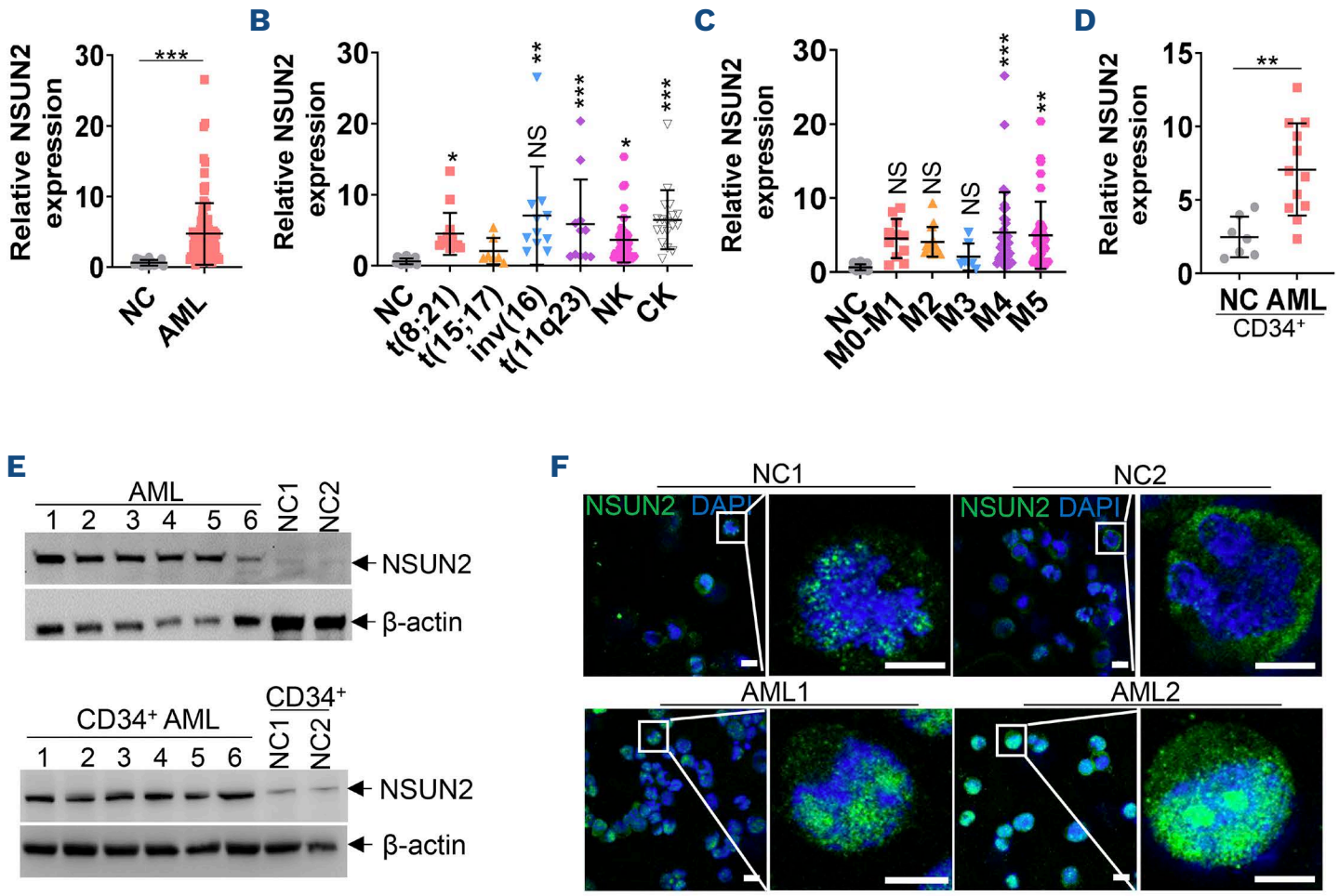

Figure 1. NSUN2 expression is higher in primary acute myeloid leukemia samples than in normal controls. (A) The transcript expression of *NSUN2* was measured by quantitative real-time polymerase chain reaction (qRT-PCR) in bone marrow cells from 102 untreated acute myeloid leukemia (AML) patients in-house and 18 healthy volunteers as normal controls (NC). (B, C) *NSUN2* expression in the same AML cohort, stratified by chromosomal translocation (B) and French-American-British subtype (C), compared with NC. (D) *NSUN2* expression was measured by qRT-PCR in 12 $CD34^+$ AML cells *versus* seven normal $CD34^+$ hematopoietic stem and progenitor cells. (E) NSUN2 protein levels were measured in two NC and six AML samples, and in two $CD34^+$ NC and six $CD34^+$ AML samples. (F) NSUN2 expression was determined in two NC and two primary AML samples by immunofluorescence assay. Bar scales represent 10 μm. Data are expressed as the mean ± standard deviation of three or more independent biological replicates. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with NC; NS: not significant. NK: normal karyotype; CK: complex karyotype.

AML patients' samples. KD efficiency is shown in *Online Supplementary Figure S5A*. In three independent specimens, NSUN2 KD reduced colony formation (*Online Supplementary Figure S5B*), induced apoptosis (*Online Supplementary*

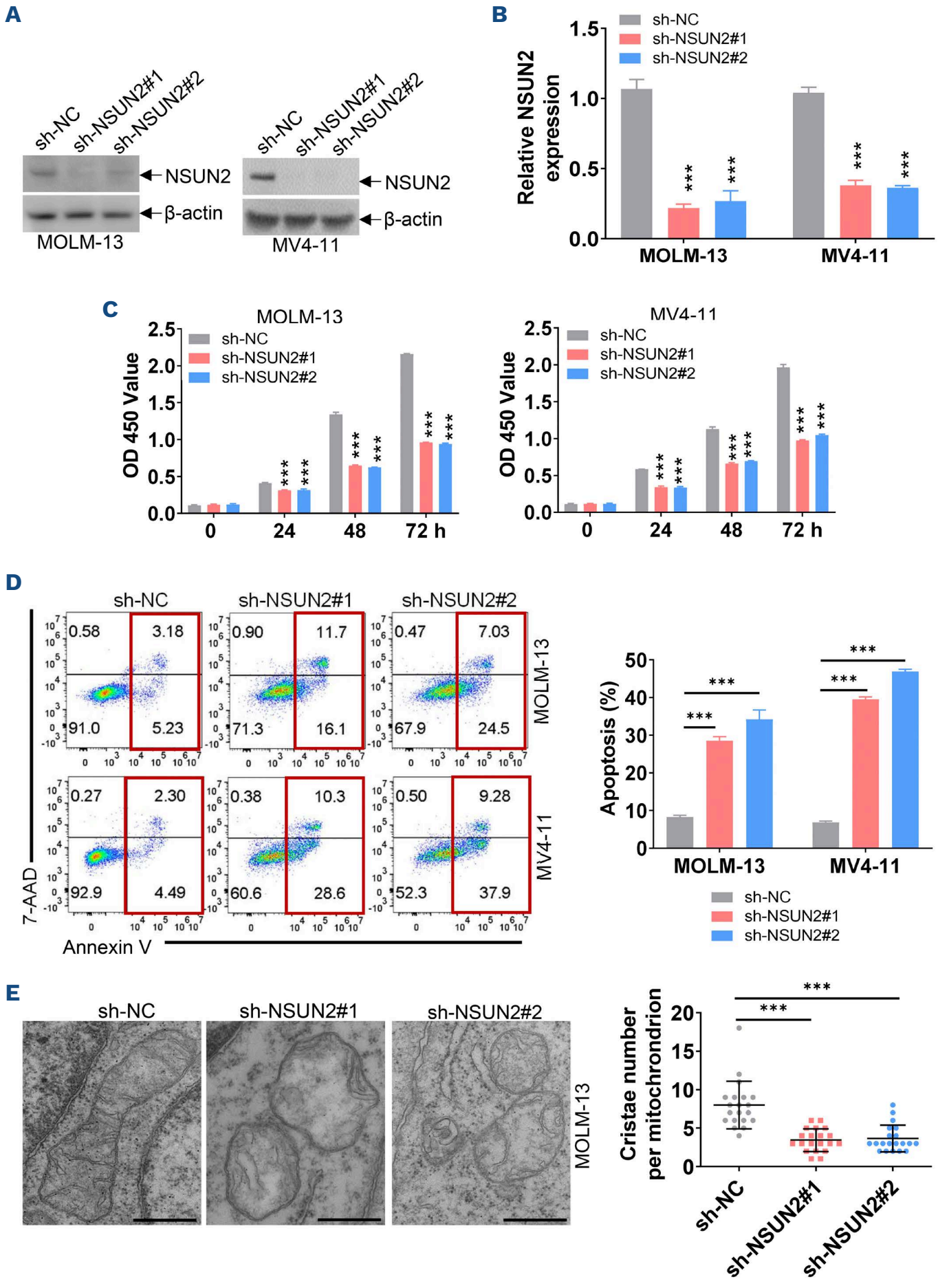

Figure 2. NSUN2 knockdown impairs survival and induces apoptosis in acute myeloid leukemia cell lines. (A, B) NSUN2 protein and transcript levels were measured in MOLM-13 and MV4-11 cells after transduction with sh-NSUN2 or sh-NC for 48 h, followed by puromycin (1 μg/mL) treatment for an additional 48 h. (C) A CCK-8 assay was performed in acute myeloid leukemia (AML) cells at the indicated times after transduction with sh-NSUN2 or sh-NC for 48 h. (D) Apoptosis was measured in AML cells after transduction with sh-NSUN2 or sh-NC for 48 h, followed by puromycin (1 μg/mL) treatment for an additional 48 h. Annexin V/7-AAD staining was used to assess early and late apoptosis. The representative plots (left) and statistical analysis of annexin V^+/7-AAD^+ + annexin V^+/7-AAD^- cells (right) are shown. (E) Representative transmission electron microscopy images for mitochondria (left) and quantitation of mitochondrial cristae (right) are shown in MOLM-13 cells transduced with sh-NSUN2 (NS2) or sh-NC (N=20/per group). Bar scales represent 500 nm. N=3 or more independent technical replicates. ***P<0.001 *versus* sh-NC.

Figure S5C), and prolonged overall survival in NSG mice (*Online Supplementary Figure S5D-F*).

NSUN2 positively regulates FOSB expression and facilitates leukemogenesis in an m^{5}C-dependent manner

To identify the specific m^{5}C sites regulated by NSUN2, we performed MeRIP-sequencing and RNA-sequencing to analyze the differentially methylated genes and differentially expressed genes in NSUN2 KD *versus* sh-NC cells. m^{5}C sites were predominantly enriched in the coding sequence rather than in the 5' untranslated region (UTR) and 3'UTR (*Online Supplementary Figure S6A-C*). Cell growth and regulation of cell growth were enriched in NSUN2 KD compared to sh-NC cells according to analysis of the Kyoto Encyclopedia of Genes and Genomes (*Online Supplementary Figure S6D*). Because NSUN2-mediated m^{5}C modification enhances gene expression,[16] we analyzed the shared downregulated differentially methylated genes and differentially expressed genes. Only 20 such genes were identified in the Venn diagram (absolute $\log_2$-fold change >0.5, P<0.05) (*Online Supplementary Figure S6E*). Among these, we measured 12 genes associated with cell proliferation and found that *SEMA3A, RYR1, FHAD1*, and *FOSB* were consistently decreased in *NSUN2* KD cells compared with sh-NC cells (*Online Supplementary Figure S6F*). We prioritized FOSB for further study given its role as a member of the activator protein-1 family and its critical function in cell survival.[17] Consistently, *NSUN2* KD reduced FOSB transcript and protein expressions (Figure 3A, B), whereas NSUN2 overexpression (OE) increased them (*Online Supplementary Figure S7A-C*).

To investigate whether NSUN2 facilitates leukemogenesis and increases FOSB expression in an m^{5}C catalytic activity-dependent manner, we reintroduced WT NSUN2 or two enzyme-dead mutants (C271A or C321A) into NSUN2 KD AML cells.[18] WT NSUN2, but not its mutants, restored the reduction in FOSB expression (Figure 3C), the decrease in colony formation (Figure 3D, E), and the increase in apoptosis (*Online Supplementary Figure S7D, E*) induced by NSUN2 KD.

NSUN2 maintains *FOSB* mRNA stability via 3'UTR methylation

We next examined whether NSUN2 regulates *FOSB* mRNA stability. NSUN2 KD markedly shortened the half-life of *FOSB* mRNA (Figure 4A) without affecting pre-*FOSB* transcript levels (*Online Supplementary Figure S8A*), indicating post-transcriptional regulation. To map the m^{5}C-modified sites on *FOSB* mRNA, we constructed pGL-3-based vectors containing FOSB 5'UTR, coding sequence, or individual 3′UTR fragments (Figure 4B). NSUN2 KD reduced Luciferase (Luc) driven by the 3′UTR-3 fragment, but not by other regions (Figure 4C). Conversely, NSUN2 OE increased activity of 3′UTR-3 (Figure 4D), and this effect required intact catalytic activity, as WT but not mutant NSUN2 enhanced reporter activity (Figure 4E). Parallel effects were observed at the Luc mRNA level (*Online Supplementary Figure S8B, C*). In MOLM-13 cells expressing sh-NC, 12 of 13 clones (92.3%) were methylated at 3656 of *FOSB* 3′UTR (Figure 4F), compared with only five of 13 (38.4%) in sh-NSUN2 cells (Figure 4F). Mutation of C3656 to T (3′UTR-3M) (*Online Supplementary Figure S8D*) abolished NSUN2-dependent regulation, as KD or OE of NSUN2 no longer altered reporter activity (Figure 4C, D, column 7). To exclude contributions from m^{6}A, we performed RNA immunoprecipitation (RIP). *FOSB* mRNA was enriched by anti-m^{5}C but not anti-m^{6}A antibody, confirming that FOSB undergoes m^{5}C rather than m^{6}A modification (*Online Supplementary Figure S8E*).

To determine whether there is a physical interaction between NSUN2 protein and *FOSB* mRNA, a RIP-PCR assay was performed. Anti-NSUN2 but not anti-IgG antibody significantly enriched *FOSB* mRNA, confirming that NSUN2 protein binds to *FOSB* mRNA (*Online Supplementary Figure S8F, G*).

YBX1 knockdown does not regulate FOSB expression

Given that YBX1 binds to m^{5}C-modified transcripts and enhances their stability,[19] we tested its involvement. However, YBX1 KD had no effect on FOSB expression (*Online Supplementary Figure S9A-D*), suggesting that FOSB stability is maintained through a YBX1-independent mechanism.

FOSB rescues the anti-leukemic effects induced by NSUN2 knockdown and FOSB transcriptionally activates NSUN2 expression

To explore whether FOSB mediates an oncogenic function of NSUN2, FOSB was overexpressed in AML cells transduced with sh-NSUN2 or sh-NC. OE of FOSB (Line 3 *vs*. 1, Figure 5A) blocked proliferation defects (Figure 5B) and apoptosis (Figure 5C) induced by NSUN2 KD. Interestingly, OE of FOSB also increased NSUN2 expression (Line 3 *vs*. 1, Figure 5A). Conversely, FOSB KD markedly reduced NSUN2 transcript and protein levels (Figure 5D, E), induced apoptosis (*Online Supplementary Figure S10A*), and inhibited proliferation (*Online Supplementary Figure S10B, C*). FOSB OE increased NSUN2 expression (*Online Supplementary Figure S10D-F*) and slightly promoted proliferation (*Online Supplementary Figure S10G, H*). In murine MA9$^+$ leukemic cells, Fosb KD reduced Nsun2 expression (*Online Supplementary Figure S10I, J*), decreased GFP$^+$ leukemic burden in peripheral blood (*Online Supplementary Figure S10K, L*), and prolonged overall survival (*Online Supplementary Figure S10M*).

Given the role of FOSB as a transcription factor,[20] we next investigated whether it directly activates NSUN2 transcription. Motif prediction (JASPAR) identified a TGA(C/G)T(C/A)A consensus sequence with three potential binding sites in the *NSUN2* promoter (*Online Supplementary Figure S11A, B*).[21] OE of FOSB increased the Luc activity of WT and WT3, but not WT1 or WT2 constructs (*Online Supplementary Figure S11C*). Mutation of the WT3 (Mut3) motif largely abolished

this effect (*Online Supplementary Figure S11C*, column 5), indicating that WT3 contains the FOSB-binding motifs. Chromatin immunoprecipitation (ChIP)-PCR confirmed FOSB binding to WT3 but not WT1 or WT2 (*Online Supplementary Figure S11D*). Moreover, ChIP-sequencing was performed in AML cells overexpressing Flag-tagged FOSB, which were IPed by anti-IgG or anti-Flag antibody. Anti-Flag antibody had higher occupancy at the *NSUN2* promoter than anti-IgG antibody (*Online Supplementary Figure S11E*).

Consistent with this reciprocal loop, FOSB was elevated in AML compared with NC samples in both our cohort (*Online Supplementary Figure S12A-C*) and in TCGA (*Online Supplementary Figure S12D*), and positively correlated with NSUN2 expression in the Gene Expression Profiling Interactive Analysis (GEPIA) database (*Online Supplementary Figure S12E*).[22]

FOSB transcriptionally activates the expression of BCL2L1

To identify additional FOSB targets, we performed RNA-sequencing in MOLM-13 cells with or without FOSB KD. Pathway analysis revealed enrichment of apoptosis-related signatures (*Online Supplementary Figure S13A*). Heatmap analysis showed downregulation of the anti-apoptotic regulator BCL2L1 (BCL-XL) and upregulation of apoptotic executors in FOSB KD cells (Figure 6A). KD of FOSB decreased BCL2L1 mRNA and protein levels (Figure 6B). Consistently, NSUN2 KD reduced BCL2L1 mRNA and protein levels (*Online Supplementary Figure S13B, C*). Moreover, OE of FOSB increased BCL2L1 expression (Figure 6C). Functionally, FOSB KD reduced mitochondrial mass (Figure 6D) and mitochondrial membrane potential (*Online Supplementary Figure S13D*). Promoter analysis identified two potential FOSB-binding

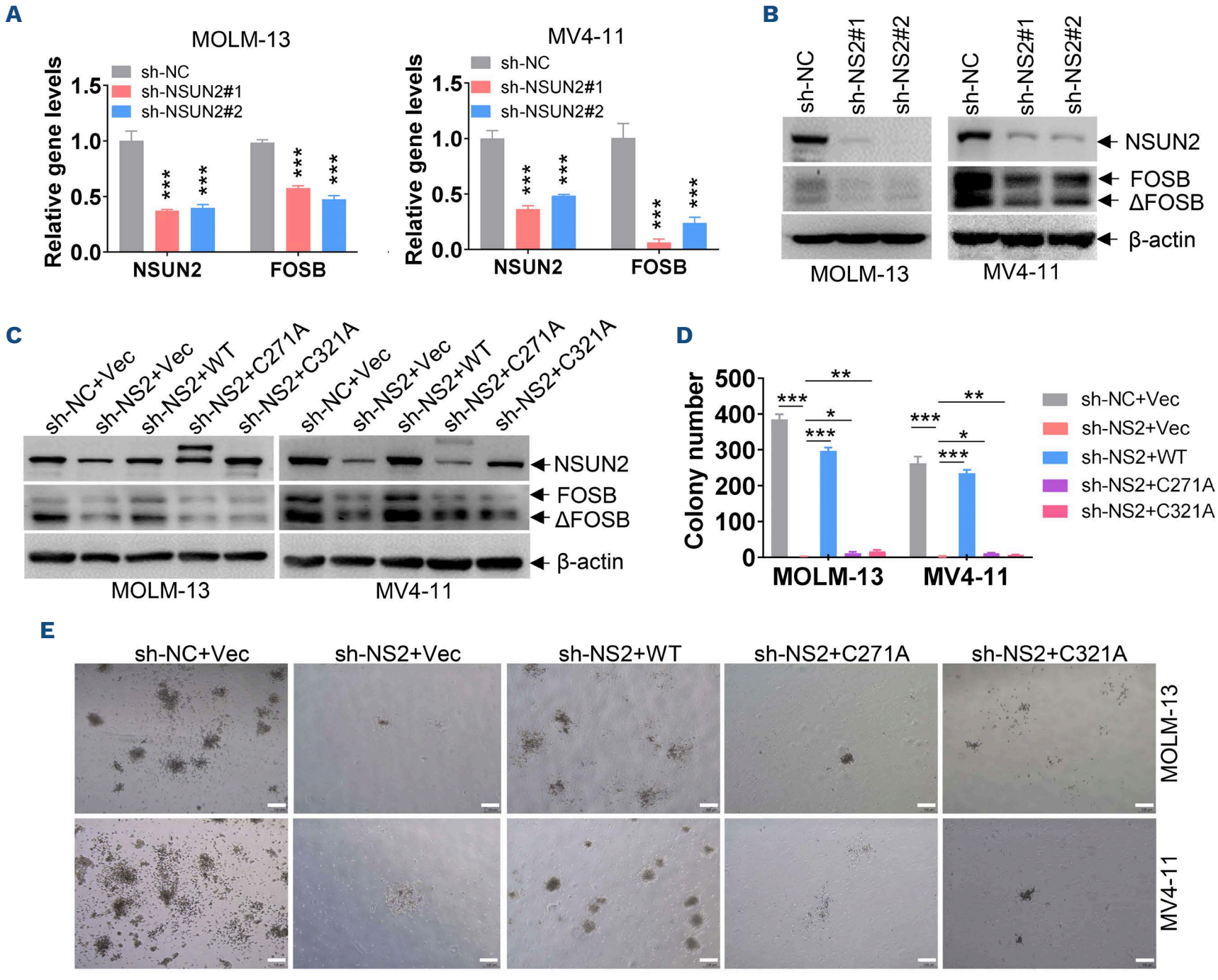

Figure 3. NSUN2 positively regulates FOSB expression and promotes leukemogenesis in an m^{5}C-dependent manner. (A, B) NSUN2 and FOSB transcript and protein levels were measured in leukemic cells transduced with sh-NSUN2 or sh-NC. (C-E) Acute myeloid leukemia (AML) cells transduced with sh-NC were overexpressed with vector (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). Cells were analyzed for NSUN2 and FOSB protein levels (C) and colony formation (2×10^3/dish for each group) (D, E). N=3 or more independent biological replicates. *P<0.05; **P<0.01; ***P<0.001; NS: not significant.

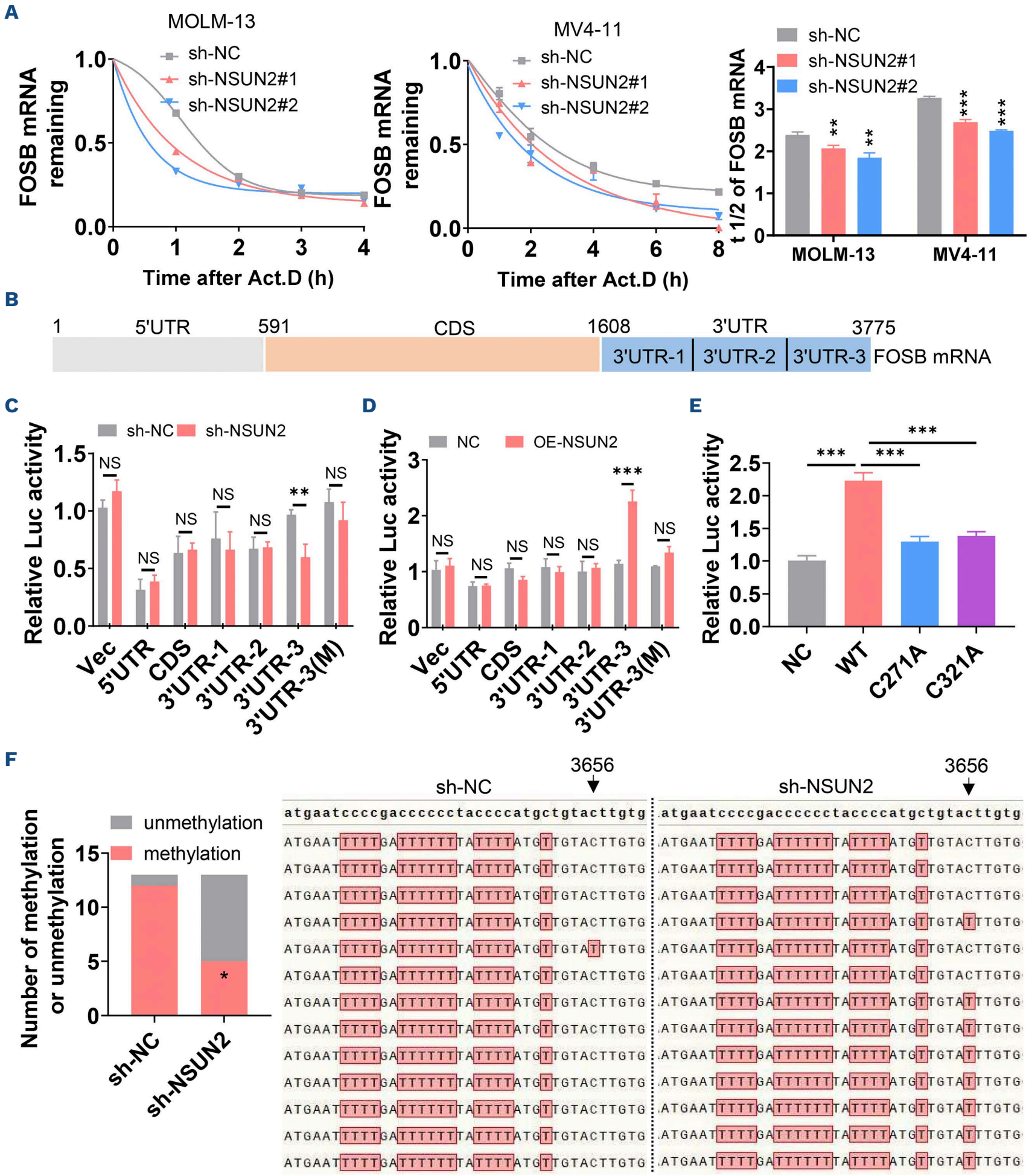

Figure 4. NSUN2 stabilizes FOSB mRNA by methylating its 3'UTR. (A) The half-lives of *FOSB* mRNA were measured in acute myeloid leukemia (AML) cells transduced with sh-NSUN2 or sh-NC for 48 h, followed by actinomycin D (Act. D, 2 μM) treatment for the indicated times. (B) Schematic presentation of different fragments of *FOSB* mRNA used in the luciferase (Luc) reporter assays. (C, D) Blank pGL3 (Vec) and pGL3 vectors carrying the indicated *FOSB* mRNA sequences were transfected into 293T cells, followed by knockdown of NSUN2 (C) or overexpression of NSUN2 (D). Relative Luc activity (Firefly/Renilla) was measured in each cell lysate 48 h after transfection. (E) 293T cells were transfected with blank vector (NC), wild-type (WT) NSUN2, or its two mutants, together with pGL3-*FOSB*-3'UTR-3. Luc activity was measured in each cell lysate. (F) Bisulfite sequencing of FOSB-3'UTR-3 in MOLM-13 cells with or without NSUN2 knockdown. Methylation frequency was calculated as methylation/(methylation + unmethylation) (left). The arrow indicates base C (position 3656), which was not converted to T after bisulfite treatment, indicating the m^5C modification. Representative sequences show m^5C modification at C3656 (right). N=3 or more independent biological replicates. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with sh-NC. NS: not significant.

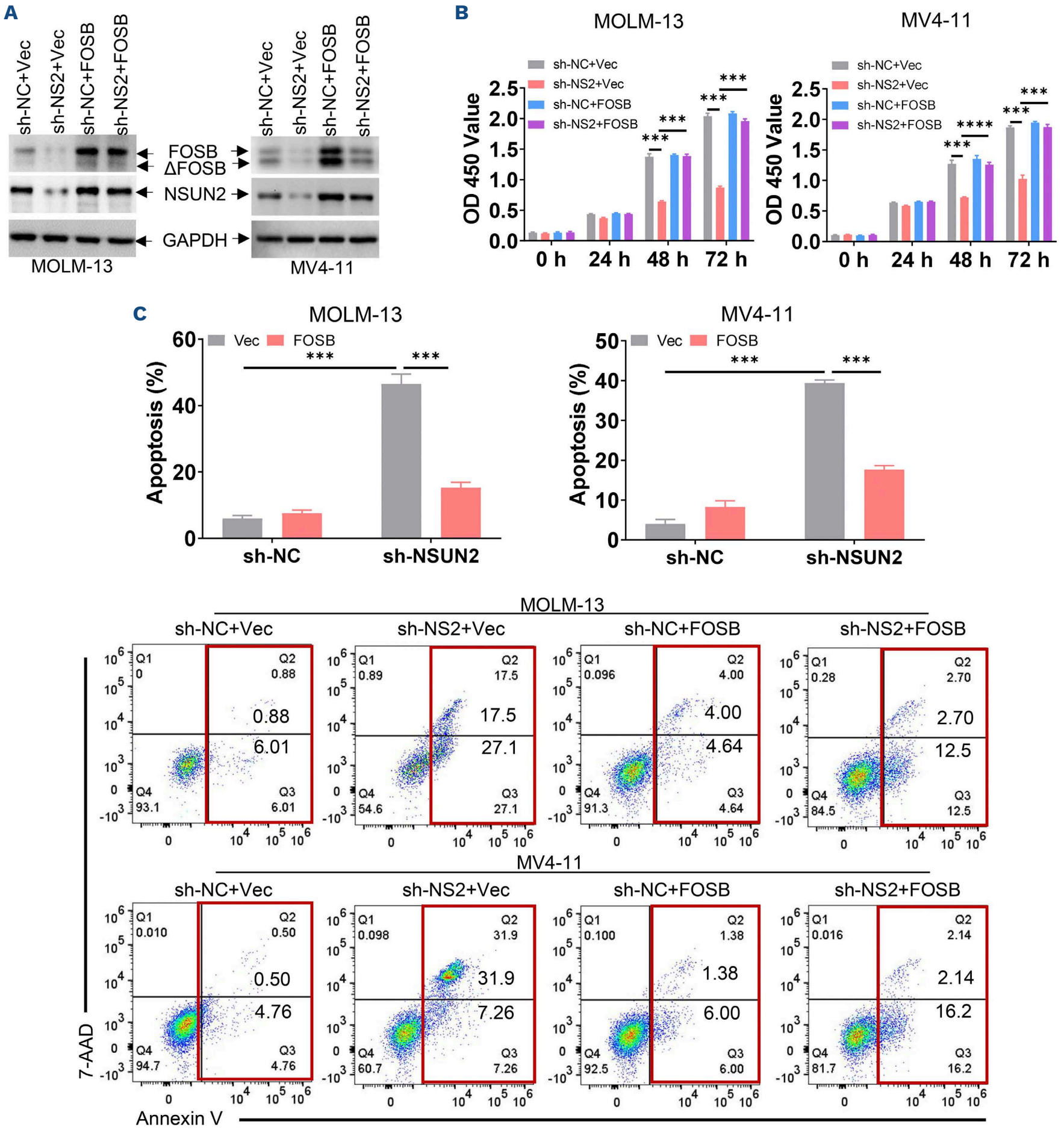

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Figure 5. Overexpression of FOSB rescues NSUN2 knockdown-induced anti-leukemia effects, and FOSB activates NSUN2 expression. (A-C) Leukemic cells were transduced with sh-NC or sh-NSUN2 for 48 h and overexpressed with blank vector (Vec) or vector overexpressing FOSB for another 48 h. NSUN2 and FOSB protein levels (A), cell proliferation (B), and apoptosis (C) were measured. (D, E) The transcript and protein levels of FOSB and NSUN2 were measured in acute myeloid leukemia cells transduced with sh-FOSB or sh-NC for 48 h, followed by puromycin (1 μg/mL) treatment for an additional 48 h. N=3 or more independent biological replicates. **P*<0.05; ***P*<0.01; ****P*<0.001.

motifs in the *BCL2L1* promoter (*Online Supplementary Figure S14A*). FOSB OE increased Luc activity of WT, WT1, and WT2 constructs, but not Mut1/2 (*Online Supplementary Figure S14B*). ChIP-PCR confirmed FOSB occupancy at both motifs (*Online Supplementary Figure S14C*). ChIP-sequencing was performed in AML cells overexpressing Flag-tagged FOSB, which were IPed by anti-IgG or anti-Flag antibody. Anti-Flag antibody presented higher occupancy at the *BCL2L1* promoter than anti-IgG antibody (*Online Supplementary Figure S14D*). To determine whether BCL2L1 is an essential target of the NSUN2-FOSB axis, BCL2L1 was overexpressed in AML cells with or without *NSUN2* KD. OE of BCL2L1 (*Online Supplementary Figure S15A*) partially blocked the decreased viability (*Online Supplementary Figure S15B, C*) and increased apoptosis (*Online Supplementary Figure S15D*) induced by NSUN2 KD. We further overexpressed BCL2L1 in AML cells with or without FOSB KD. Similarly, BCL2L1 overexpression (*Online Supplementary Figure S16A*) partially rescued the decrease in viability (*Online Supplementary Figure S16B, C*) and the increase in apoptosis (*Online Supplementary Figure S16D*) induced by FOSB KD.

To explore whether the NSUN2-FOSB-BCL2L1 axis is also operative in AML cells driven by other common genetic lesions, we knocked down NSUN2 expression in K562 cells bearing the *BCR-ABL* fusion gene[23] and OCI-AML3 cells carrying mutant *NPM1*.[24] NSUN2 KD decreased FOSB and BCL2L1 protein levels in these two cells (*Online Supplementary Figure S17A*). Furthermore, we explored whether the NSUN2-FOSB axis regulates other BCL-2 family members. NSUN2 and FOSB KD both decreased BCL2 protein level, but had little effect on MCL1 and BAX expression (*Online Supplementary Figure S18A, B*). However, we did not find FOSB-recognizing motif (TGA(C/G)T(C/A)A) in the *BCL2* promoter by Motif prediction (JASPAR, *data not shown*). Therefore, FOSB probably regulates BCL2 expression indirectly, and BCL2 downregulation in FOSB KD cells might be a by-product of apoptosis.

To better understand the clinical relevance of our findings, we analyzed the correlation between NSUN2, FOSB, and BCL2L1 mRNA levels within the same cohort. Positive correlations exist between *NSUN2 versus FOSB, NSUN2 versus BCL2L1*, and *FOSB versus BCL2L1* mRNA levels (*Online Supplementary Figure S19A-C*).

Nsun2 knockout attenuates progression of acute myeloid leukemia and inhibits leukemic stem cell self-renewal in MA9-induced murine leukemia

To investigate the role of Nsun2 in leukemogenesis, we generated conditional knockout mice (Nsun2$^{fl/fl}$ Cre^{ERT2}, hereafter fl/fl) (*Online Supplementary Figure S20A*).[25] Tamoxifen injection achieved efficient Nsun2 deletion in bone marrow cells (Δ/Δ) (*Online Supplementary Figure S20B*). MA9-transduced bone marrow Lin$^-$ cells from fl/fl mice were transplanted into recipients (*Online Supplementary Figure S20C*), and tamoxifen treatment at days 15-20 after BMT depleted Nsun2 in GFP$^+$ AML cells (*Online Supplementary Figure S20D, E*). In secondary BMT (*Online Supplementary Figure S21A*), tamoxifen-induced Nsun2 loss (days 10-15) markedly reduced GFP$^+$ leukemic cells in peripheral blood (*Online Supplementary Figure S21B, C*) and bone marrow (Figure 7A, B), decreased spleen and liver weights (*Online Supplementary Figure S21D, E*), and limited leukemic infiltration in the spleen and liver (*Online Supplementary Figure S21F, G*). Proliferation was suppressed, as EdU$^+$ cells were about 9-fold lower in Δ/Δ than fl/fl samples (*Online Supplementary Figure S21H*). Importantly, Nsun2 deletion significantly reduced LSC activity: the frequency of leukemia granulocyte-monocyte progenitors[26] decreased by >50% (Figure 7C), and extreme limiting dilution assays showed a ~75% reduction in functional LSC (1 in 48 *vs*. 1 in 144, *P*<0.05) (Figure 7D; *Online Supplementary Table S3*).[11,27] Nsun2 deficiency almost eliminated colony formation (Figure 7E), and prolonged overall survival across primary, secondary, and tertiary transplants (Figure 7F-H). Restoration of WT Nsun2, but not enzymatically inactive mutants (C271A, C321A) (*Online Supplementary Figure S21I*), reversed the extended survival (*Online Supplementary Figure S21J*), indicating that the catalytic activity of Nsun2 is required for leukemogenesis.

Our results indicate that Nsun2 is essential for AML maintenance. To further investigate the role of Nsun2 in the initiation of AML, we injected tamoxifen 3 days after transplantation in secondary BMT. Tamoxifen treatment markedly extended overall survival during AML initiation (*Online Supplementary Figure S21K*).

Nsun2 deletion did not impair bone marrow homing (*Online Supplementary Figure S22A*), and control experiments excluded confounding effects of floxed alleles or Cre^{ERT2} (*Online Supplementary Figure S22B*). In the AML1-ETO9a (A/E9a)-driven M2 AML model, Δ/Δ cells similarly conferred prolonged overall survival in both primary and secondary BMT (*Online Supplementary Figure S22C-F*), confirming that Nsun2 is broadly required for AML maintenance.

Nsun2 knockout decreases Fosb in murine acute myeloid leukemia cells and overexpression of Fosb rescues leukemogenesis

Given that NSUN2 regulates FOSB in human AML, we ex-

amined Fosb expression in murine Δ/Δ leukemic cells. Nsun2 deletion reduced Fosb transcript and protein levels (*Online Supplementary Figure S23A, B*). Fosb OE restored Nsun2 expression (*Online Supplementary Figure S23C*) and reversed the extension of overall survival conferred by Nsun2 deletion (*Online Supplementary Figure S23D*). These

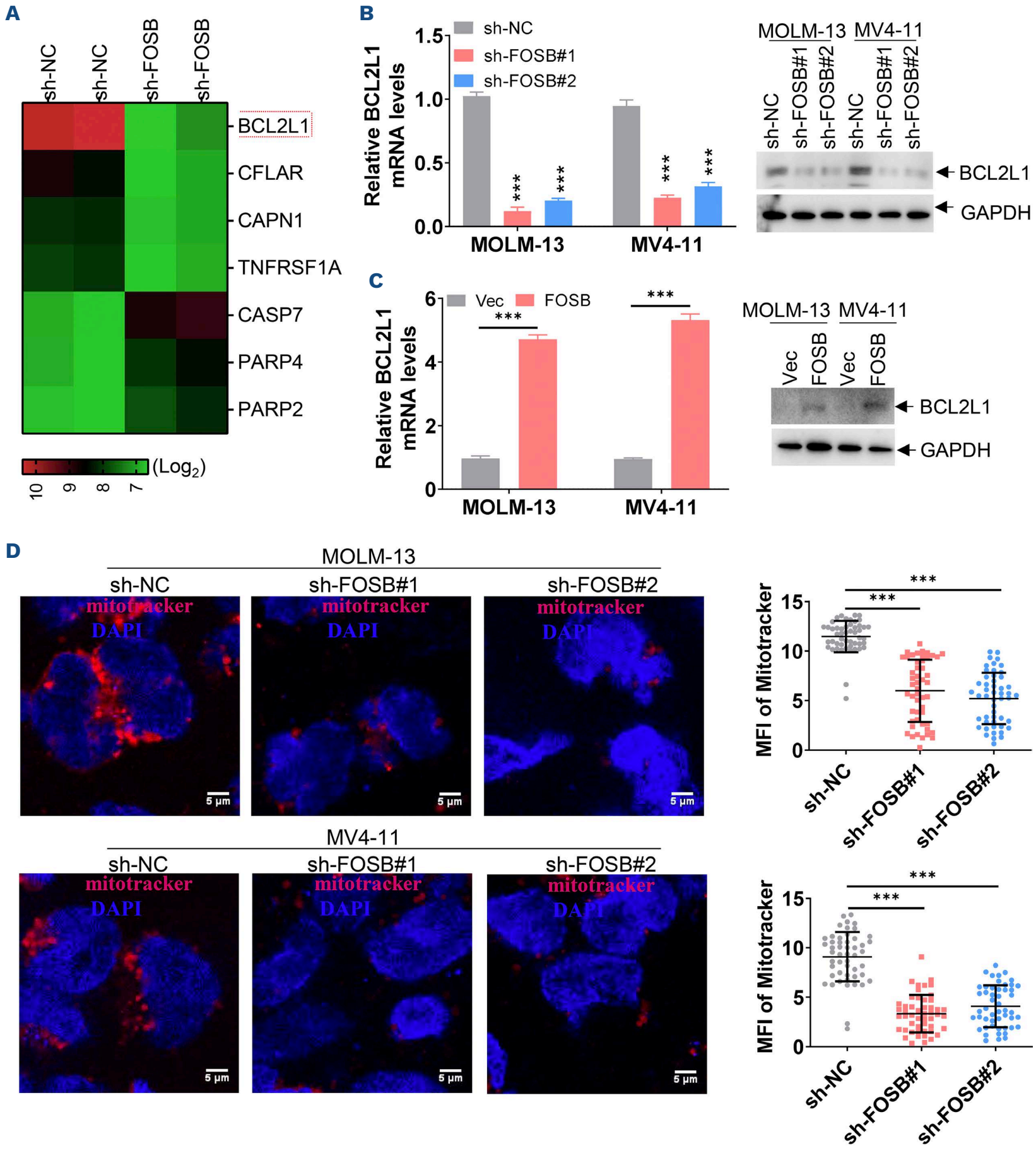

Figure 6. FOSB activates BCL2L1 expression and affects mitochondrial function. (A) MOLM-13 cells transduced with sh-NC or sh-FOSB#1 (sh-FOSB) were subjected to RNA-sequencing. Apoptosis-associated genes, including *BCL2L1*, were visualized by heatmap assay. (B) BCL2L1 transcript and protein levels were measured in MOLM-13 and MV4-11 cells after sh-FOSB or sh-NC transduction for 48 h, followed by puromycin (1 μg/mL) treatment for an additional 48 h. (C) BCL2L1 transcript and protein levels were measured in acute myeloid leukemia (AML) cells, which were overexpressed with FOSB or blank control (Vec) for 48 h. (D) The mitochondria were stained red by MitoTracker Red CMXRos in AML cells after transduction with sh-FOSB or sh-NC. Mitochondrial mass was analyzed by mean fluorescence intensity (MFI) of MitoTracker staining. The representative images (left) and statistical analysis of MFI of MitoTracker staining (right, N=50 per group) are shown. Bar = 50 nm. N=3 or more independent biological replicates. ***P<0.001 *versus* sh-NC cells.

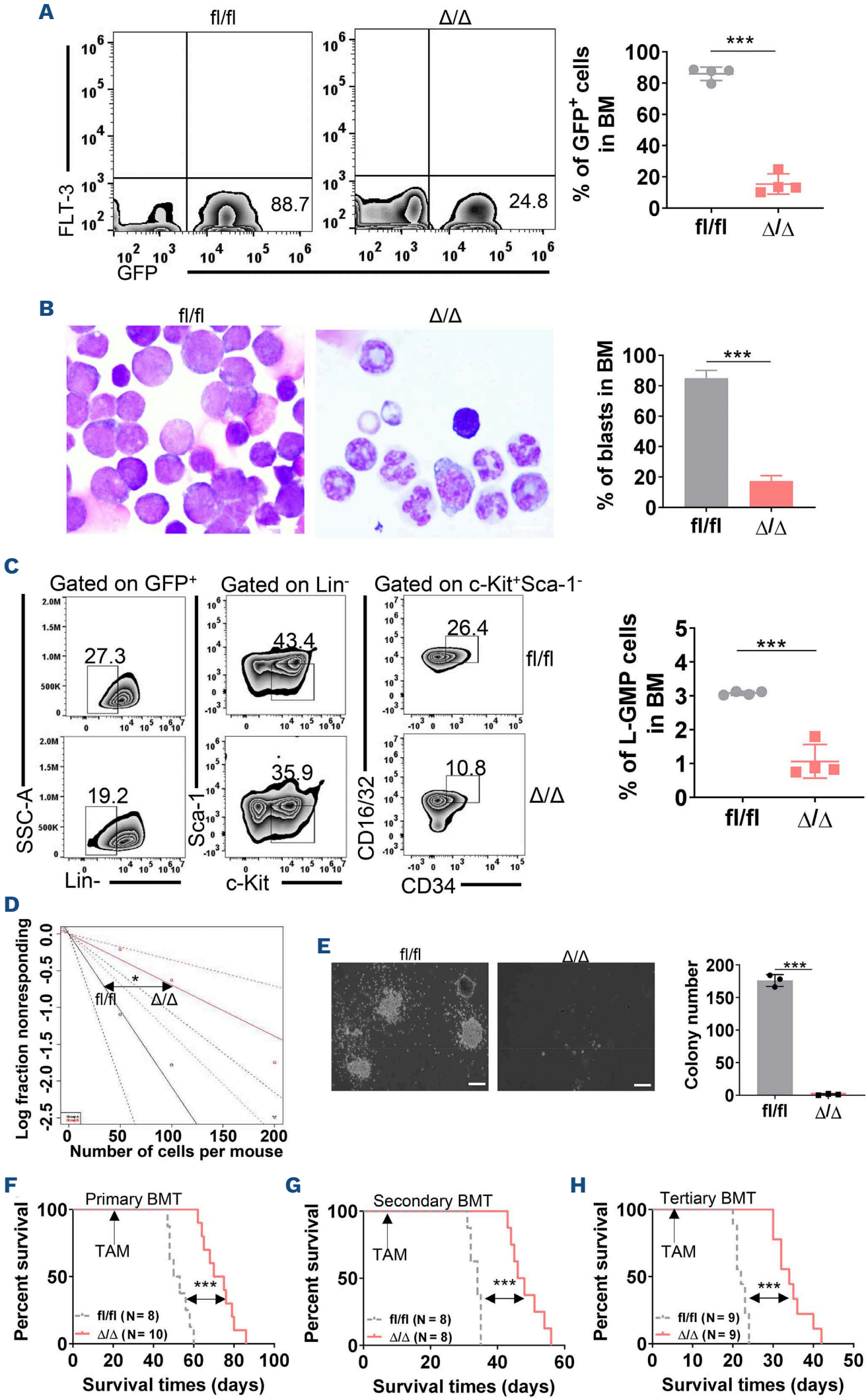

Figure 7. Nsun2 facilitates leukemia stem cell survival and self-renewal in a murine acute myeloid leukemia model. (A) The frequency of green fluorescence protein (GFP)⁺ cells was measured in bone marrow (BM) from fl/fl (N=4) and Δ/Δ (N=4) leukemic mice. The representative flow cytometry plots (left) and statistical analysis (right) are shown. (B) Representative images of BM smears from fl/fl (N=3) and Δ/Δ (N=3) mice (left). Statistical analysis of the average percentage of leukemic cells in BM is shown (right). Scale bar =10 μm. (C) Leukemia stem cell (LSC) frequency was measured in BM GFP⁺ cells from fl/fl (N=4) and Δ/Δ (N=4) leukemic mice. (D) Extreme limiting dilution assay was conducted in secondary bone narrow transplant (BMT) mice (fl/fl, N=6;

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Δ/Δ, N=6). (E) GFP$^+$ fl/fl (1,000 cells per dish) or Δ/Δ cells (1,000 cells per dish) were sorted from BM for colony assay. Representative images of colonies (left) and statistical analysis of colony number (right). A two-tailed Student *t* test was used to compare differences between two groups. (F-H) Overall survival was calculated in the primary (F), secondary (G), and tertiary BMT (H). Kaplan-Meier survival curves were generated to analyze overall survival time. N=3 or more independent biological replicates. **P*<0.05; ****P*<0.001. TAM: tamoxifen.

findings establish Fosb as a critical downstream effector of Nsun2 in murine AML.

Nsun2 depletion does not affect normal hematopoiesis

To investigate the role of NSUN2 in steady-state hematopoiesis, we first performed cell counts in peripheral blood in fl/fl and Δ/Δ mice. Four weeks after the last tamoxifen injection, total blood cell counts and lineage distributions were comparable between the two groups (*Online Supplementary Figure S24A-C*). Similarly, total bone marrow cellularity showed no difference (*Online Supplementary Figure S24D*), and the proportions of T, B, and myeloid cells in peripheral blood were indistinguishable (*Online Supplementary Figure S24E*). Additionally, *Nsun2* depletion did not affect the frequency of EdU$^+$ cells (*Online Supplementary Figure S24F*). We then explored whether *Nsun2* depletion affects the frequency and function of HSPC. The frequencies of distinct HSPC subsets, including long-term hematopoietic stem cells were similar between fl/fl and Δ/Δ mice (Figure 8A). Functional assays further demonstrated that Nsun2-deficient LSK (Lin$^-$c-Kit$^+$Sca-1$^+$) cells formed colonies at comparable numbers and distributions relative to controls (Figure 8B). Finally, a competitive repopulation assay confirmed that Nsun2 deletion did not impair self-renewal or lineage output: the proportions of donor-derived total CD45.2$^+$ cells (*Online Supplementary Figure S24G, H*), as well as myeloid (*Online Supplementary Figure S24I*), B (*Online Supplementary Figure S24J*), and T lineages (*Online Supplementary Figure S24K*) in peripheral blood, were equivalent between fl/fl and Δ/Δ groups.

We also monitored over a longer term (>12 months) to rule out potential delayed effects on hematopoietic stem cell function. Total blood cell counts and lineage distributions were comparable between the two groups (*Online Supplementary Figure S25A-C*).

NSUN2 knockdown sensitizes acute myeloid leukemia cells to chemotherapy

Finally, we investigated whether *NSUN2* KD enhances the cytotoxicity of chemotherapeutic drugs. NSUN2 KD and sh-NC cells were treated with or without various chemotherapeutic drugs, and cell death was measured by 7-amino-actinomycin D staining. NSUN2 KD potentiated the cytotoxic effects of cytarabine, cladribine, decitabine, tucidinostat, and homoharringtonine in MOLM-13 and MV4-11 cells (*Online Supplementary Figure S26A-D*), suggesting that NSUN2 KD sensitizes AML cells to chemotherapy.

The NSUN2 inhibitor MY-1B inhibits the FOSB-BCL2L1 axis[10]

Next, we explored whether the NSUN2 inhibitor MY-1B phenocopies the genetic KD of NSUN2. MY-1B treatment markedly decreased FOSB and BCL2L1 protein levels (*Online Supplementary Figure S27A*), suggesting that NSUN2 is a therapeutic target. However, two well-known hypomethylation agents, decitabine and 5-azacytidine, did not regulate NSUN2 protein levels (*Online Supplementary Figure S27B*).

Discussion

Our study uncovers a previously unrecognized mechanism by which m^{5}C RNA modification promotes leukemogenesis, offering both mechanistic insights and therapeutic opportunities. NSUN2 increases FOSB expression by enhancing *FOSB* mRNA stability, while FOSB transcriptionally activates NSUN2, establishing an NSUN2-FOSB reciprocal loop (Figure 8C). In addition, FOSB transcriptionally activates BCL2L1 expression to facilitate cell survival (Figure 8D). Together, these findings identify the NSUN2-FOSB-BCL2L1 axis as a potential therapeutic target in AML.

m^{5}C modification has been implicated in RNA stability, protein translation, and nuclear export, thereby contributing to oncogene activation across multiple tumor types.[7,28] In AML, OE of NSUN2 WT, but not its mutants, fully rescues leukemogenesis in NSUN2-deficient AML cells, confirming that its oncogenic effect is m^{5}C-dependent. Prior studies have shown that m^{5}C modifications frequently stabilize target mRNA.[3,6] For example, m^{5}C modification on the 3'-UTR of *PKM2* mRNA facilitates its stability.[29] Our data extend this paradigm by showing that m^{5}C modification of FOSB mRNA similarly promotes its stability. Nevertheless, m^{5}C can also influence translation efficiency or nuclear export,[30] suggesting that its biological consequences may vary with cellular context.

YBX1 has been considered the principal "reader" of m^{5}C-modified transcripts, stabilizing oncogenic mRNA in several cancers.[19] YBX1 binds to m^{5}C-modified androgen receptor (*AR*) mRNA and stabilizes it in prostate cancer cells.[31] However, YBX1 recognizes m^{6}A-modified *BCL2* mRNA in an m^{6}A but not m^{5}C-dependent manner in AML cells.[32] In our study, YBX1 did not regulate FOSB expression, indicating that YBX1 is not the reader of m^{5}C-modified FOSB, and that the binding specificity of YBX1 may depend on transcript context and modification type. Identification of the m^{5}C reader(s) responsible for stabilizing *FOSB* mRNA

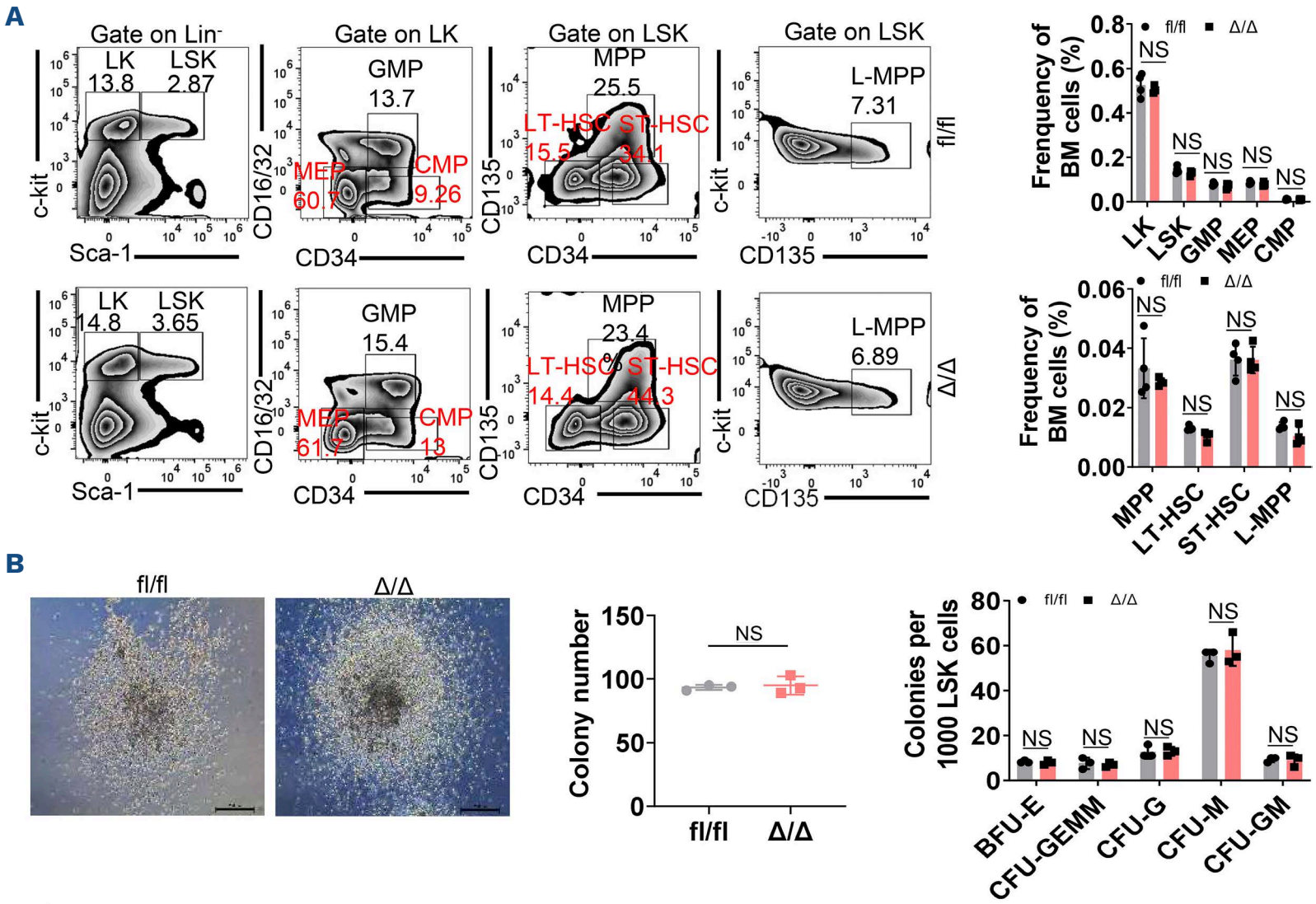

Figure 8. *Nsun2* depletion does not affect normal hematopoiesis. Six-week-old *Nsun2*$^{fl/fl}$ Cre^{ERT2} mice were treated with tamoxifen to deplete *Nsun2* expression (Δ/Δ) or corn oil as a vehicle control (fl/fl). Mice were sacrificed 1 month after the last tamoxifen injection. (A) Representative plots showing the gating strategy for different cell populations: LK cells (Lin$^-$cKit$^+$Sca-1$^-$), LSK cells (Lin$^-$cKit$^+$Sca-1$^+$), granulocyte-monocyte progenitors (GMP: Lin$^-$cKit$^+$Sca-1$^-$CD34$^+$CD16/32$^+$), megakaryocyte-erythroid progenitors (MEP: Lin$^-$cKit$^+$Sca-1$^-$CD34$^-$CD16/32$^+$), common myeloid progenitors (CMP: Lin$^-$cKit$^+$Sca-1$^-$CD34$^+$CD16/32$^-$), multipotent progenitors (MPP: Lin$^-$cKit$^+$Sca-1$^+$CD34$^+$CD135$^+$), long-term hematopoietic stem cells (LT-HSC: Lin$^-$cKit$^+$Sca-1$^+$CD34$^-$CD135$^-$), short-term hematopoietic stem cells (ST-HSC: Lin$^-$cKit$^+$Sca-1$^+$CD34$^+$CD135$^-$), and leukemia multipotent progenitors (L-MPP: Lin$^-$cKit$^+$Sca-1$^+$CD135$^+$) from fl/fl (N=4) and Δ/Δ mice (N=4). (B) Bone marrow Lin$^-$c-Kit$^+$Sca-1$^+$ cells were isolated from fl/fl and Δ/Δ mice and were plated in methylcellulose medium for colony formation (1,000 cells/dish). Representative images, statistical analysis of colony number, and classification of burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte, erythroid, macrophage, and

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megakaryocyte (CFU-GEMM), colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), and colony-forming unit-granulocyte and macrophage (CFU-GM). NS = not significant. (C, D) A schematic diagram showing how the NSUN2-FOSB regulatory loop facilitates leukemogenesis. NSUN2 increases FOSB expression by enhancing *FOSB* mRNA stability through accumulating m^5C modifications at *FOSB* 3'-UTR. In turn, FOSB promotes NSUN2 expression by binding to the *NSUN2* promoter, forming the NSUN2-FOSB regulatory loop (C). FOSB increases the expression of BCL2L1 through binding to the *BCL2L1* promoter. The NSUN2-FOSB regulatory loop enhances survival and inhibits apoptosis by upregulating BCL2L1 (D).

remains an important avenue for future investigation.

NSUN2 expression itself is subject to transcriptional regulation by multiple factors.[9,33] Although FOSB has been extensively characterized as an immediate-early gene,[34] its function in AML has been unclear. We show that FOSB KD inhibits cell proliferation and induces apoptosis, establishing FOSB as an unexpected oncogenic driver in AML. This is consistent with the report that FOSB acts as an oncogene in piperlongumine-treated breast cancer cells.[35] Our data further demonstrate that FOSB directly activates NSUN2 transcription, suggesting that stress-induced FOSB activation may drive persistent upregulation of both FOSB and *NSUN2* through a reinforcing positive feedback loop in AML.[17]

We also identified BCL2L1 as a novel transcriptional target of FOSB. BCL2L1 is a critical anti-apoptotic regulator whose inhibition induces apoptosis and mitochondrial dysfunction.[36] Its overexpression contributes to resistance to BCL2 inhibitors in chronic lymphocytic leukemia and resistance to bortezomib in mantle cell lymphoma.[37,38] BCL2L1 upregulation is also a hallmark of myeloproliferative neoplasm-derived AML, in which its inhibition confers therapeutic benefit.[39] Thus, the FOSB-BCL2L1 axis represents an attractive therapeutic vulnerability in AML.

FOSB transcriptionally activates BCL2L1 expression by binding directly to its promoter, and NSUN2 positively regulates BCL2L1 expression. It is, therefore, reasonable that NSUN2 indirectly regulates BCL2L1 expression through FOSB. NSUN2 KD induces apoptosis in AML cells, and induces ferroptosis in AML and tumor cells,[10, 40-42] suggesting a complicated function of NSUN2. In addition, NSUN2 KD sensitizes AML cells to chemotherapy, likely by inducing apoptosis or ferroptosis. Notably, NSUN2 is dispensable for normal hematopoiesis. Therefore, NSUN2 is a promising therapeutic target in AML.

Considering that NSUN2 is highly expressed in AML cells, it is reasonable that AML cells with high NSUN2 expression rely on m^5C modification to maintain rapid proliferation and survival. Therefore, AML cells are more susceptible to the depletion of *NSUN2* compared with normal HSPC cells. Consistent with this, Nsun2 depletion does not affect normal hematopoiesis and the self-renewal ability of HSPC. However, it is possible that HSPC rely more on other m^5C methyltransferases such as DNMT2 (encoded by tRNA aspartic acid methyltransferase 1), because double knockout of Nsun2 and DNMT2 in mice impairs cellular differentiation, while single knockout does not have detectable effects.[4]

The Target AML database demonstrates that high NSUN2 levels in AML patients are associated with poor outcomes. However, NSUN2 expression levels alone in AML do not predict prognosis, and the differences in overall survival were only observed in AML with SRSF2 P95H mutation according to the Beat AML database.[43] In contrast, TCGA data revealed a consistent association between high NSUN2 levels and poor prognosis.[6] We speculate that differences among enrolled AML patients account for this discrepancy. Mechanistically, we mapped an m^5C modification site (C3656) within the FOSB 3′UTR that mediates mRNA stabilization. Although KD or OE of NSUN2 did not alter Luc activity of constructs containing the 5′UTR or coding sequence, low-level modifications at these regions cannot be excluded. Moreover, standard bisulfite sequencing cannot distinguish m^5C from related modifications (hm^5C or m^4C) and is prone to RNA degradation, potentially leading to underestimation of modification sites. The development of more precise m^5C-mapping methods will be critical to refining our understanding of m^5C biology in leukemia.

In conclusion, our study identifies the NSUN2-FOSB positive feedback loop as a driver of leukemogenesis that spares normal hematopoiesis. Disrupting this circuit by inhibiting NSUN2 or blocking the NSUN2-FOSB-BCL2L1 signaling axis offers a promising therapeutic strategy for AML.

Disclosures

No conflicts of interest to disclose.

Contributions

ZB, YG, CY and ZY were responsible for writing the protocol and report, conducting the search, and screening potentially eligible studies. LM, MC, WS and QQ performed the experiments and analyzed data. ZX, YR, CY and XY contributed to the design of the review protocol, writing the report, arbitrating potentially eligible studies, providing human specimens, and data analysis. HF, SZ, SF and YY performed the in vivo AML model studies. CQ, ZM and GM designed the experiments and wrote the manuscript.

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Data-sharing statement

All RNA-sequencing data are publicly available on the Gene Expression Omnibus under accession numbers GSE 252880, GSE 253074, and GSE 308401. All datasets generated or analyzed during this study have been included in this manuscript. The data are available from the corresponding author on reasonable request.

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