Supplemental Information

Stabilization of NF-κB-Inducing Kinase Suppresses
MLL-AF9-Induced Acute Myeloid Leukemia

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Figure S1. Stabilization of NIK impairs colony-forming ability of MLL-AF9 transformed cells, Related to Figure 1. (A) Number of colonies formed in methylcellulose using MLL-AF9–transduced HSPCs from control (Con) or NIKVavCre mice (caNIK) (n = 3). The same number of cells per well were used for the second plating. (B) Scheme of serial plating for establishing immortalized leukemia cells. (C) Number of colonies formed in methylcellulose using fully transformed MLL-AF9 cells from the forth plating in the absence (Con) or presence of 400 nM 4-OHT (n = 3). (D) Representative colony pictures from control or 4-OHT-treated methylcellulose cultures. (E) Liquid culture (1 × 10⁶/25 cm flask) using fully transformed MLL-AF9 leukemia cells in the absence or presence of 400 nM 4-OHT (n = 4). (F) Colony numbers of MLL-AF9–transformed ERT2 cells (200/48-well) in the presence or absence of 4-OHT (n = 4). (G) Kaplan-Meier survival curve of lethally irradiated congenic recipients (CD45.1⁺) receiving MLL-AF9–transduced ERT2 cells with or without tamoxifen treatment (n = 6 mice in each group). **p < 0.01.
Figure S2. Stabilization of NIK has a broad anti-myeloid leukemia role, Related to Figure 1. (a, b) Number of colonies formed in methylcellulose using sorted vector (Vec)- or NIK mutant Δ78–84 (ΔNIK)-transduced mouse BCR-ABL+ chronic myelogenous leukemia (CML) cells (Lin−Kit+Sca1+, A) or BCR-ABL+NUP98-HOXA9+ AML cells (Lin−Sca1+, B) (n = 3 each). ΔNIK, a human NIK mutant lacking amino acids 78–84 (Δ78–84) of WT NIK and thus resistant to TRAF3-mediated degradation. (C) De-identified human AML with approximately 70% (AML1), 85% (AML2) blasts, and 90% blasts (AML3, therapy-related AML) and CML (5% blasts, CD34-enriched) cells were transduced with vector or ΔNIK expressing lentiviral viruses, and the transduced cells were sorted for in vitro colony assays. **p < 0.01. (D and E) NIK protein is almost undetectable in normal BM and AML cells. (D) Western blots for detection of NIK protein using whole cellular lysates extracted from WT (Con) or caNIK (NIK) BM cells. (E) Western blots for detection of NIK, RelA, and RelB proteins using whole cellular lysates extracted from normal, AML, or bcCML BM cells. Con 1 and 2, negative staging BM cells from patients with Hodgkin lymphoma and B lymphoblastic leukemia, respectively. AML1, newly diagnosed AML BM with 70% blasts; AML2, relapsed AML with 50% blasts; bcCML, blast crisis CML with 22% blasts. In addition, B cells isolated from NIK<sup>fl</sup>CD19Cre spleen were used as positive controls (Posi).
Figure S3. Stabilization of NIK alters the expression of genes regulating leukemia stem cells, Related to Figure 2. (A) Relative expression of selected genes from RNAseq in GFP ± Lin Kit+Sca1+CD16−CD34+ leukemia stem cells (n = 3, **p < 0.01). (B) Selected pathways that were enriched based on the tamoxifen-treated transcriptomes. The x-axis of the bar chart is the normalized enrichment score (NES) and the dot is the -log10 (FDR q-value). It should be noted that the MLL-AF9-specific gene set share only three genes with the HESS_TARGETS_OF_HOXA9_AND_MEIS1_UP gene set. (C) Gene set enrichment analysis (GSEA) showed up-regulated Notch targets in NIK-stabilized LSCs compared to control LSCs.
Figure S4. Enhanced expression of RelA or knockdown of Dnmt3a is unable to restore the suppression of AML associated with stabilization of NIK, Related to Figure 3. (A) Relative expression of Rela or Relb mRNA (left and middle panels) and proteins (right panel) in sorted primary MLL-AF9 AML cells transduced with vector (Vec), RelA, or RelB (n = 3). (B) Sorting strategy used for experiments to restore stabilization of NIK-induced suppression of leukemia. The primary AML cells generated using MLL-AF9–transduced NIKERT2 BM were infected with bi-cistronic retroviruses expressing RelA or RelB or expressing Dnmt3a shRNAi. The infected cells were labeled by mCherry. The transduced cells (mCherry+) were FACs-sorted and treated with 4-OHT, then resorted for mCherry+ or mCherry+GFP+ cells for colony assays. (C) Number of colonies formed in methylcellulose using sorted vector (Vec)- or RelA-transduced primary AML cells in the presence (4-OHT) or absence of 4-OHT (n = 3). (D) Survival curves of sublethally irradiated mice given vector (Vec)- or RelA-transduced AML cells with or without tamoxifen treatment (n = 5 each). (E) Number of colonies formed in methylcellulose using sorted vector (Vec)- or pLKO-Dnmt3a shRNAi-transduced primary AML cells in the presence (4-OHT) or absence of 4-OHT (n = 3). The expression of Dnmt3a is approximately 50% of controls by qPCR analysis. EtOH, ethanol vehicle. **p < 0.01
Supplemental Experimental Procedures

Mice
NIKΔT3\textsuperscript{STOP} mice have been described previously (Sasaki et al., 2008). Vav-Cre mice (Stock Number: 008610) were obtained from the Jackson Laboratory and the ROSA-CreER\textsuperscript{T2} mice were kindly provided by Dr. Yiyi Zhang (Guo et al., 2007). Both strains are in a C57BL/6 background and were used at 8–12 weeks old. All the mice used throughout our experiments were NIKΔT3\textsuperscript{STOP} heterozygous, therefore only one allele of NIKΔT3 was activated. Transplant recipients (C57BL/Ka CD45.1) were 8–12 weeks old. The Institutional Animal Care and Use Committee of the University of Iowa approved all animal experiments.

Human Samples
De-identified human AML samples were obtained from the flow cytometry laboratory at the University of Iowa (Institutional IRB Approval #201508729). Mononuclear cells were purified by Ficoll-density gradient centrifugation and enriched for CD34\textsuperscript{+} or lineage \textsuperscript{−} leukemic stem/progenitor cells by positive or negative selection using the EasySep\textsuperscript{™} Human CD34 Positive Selection Kit or the Human Progenitor Cell Enrichment Kit, respectively (StemCell Technologies), depending on CD34 positivity of the blasts.

HSPC Isolation and LSC Frequency Analysis
We used the following antibodies from e-Bioscience and BD Biosciences: B220, CD3, CD4, CD8, CD11b, CD11c, CD16/32, CD34, CD45.1, CD45.2, CD93, CD117, Fc\textsuperscript{γ}II/III, Gr-1, Sca1, and Ter119. For isolation of lineage \textsuperscript{−}c-kit\textsuperscript{+}Sca-1\textsuperscript{+} (KLS) cells, whole bone marrow (WBM) cells were incubated with a cocktail of lineage antibodies from BD Biosciences (biotinylated anti-mouse antibodies directed against CD3\textsubscript{e}, CD11b, CD45R/B220, Gr-1, Ter119) followed by lineage depletion using BD IMag streptavidin particles Plus-DM, then stained with Sca-1 Percp-Cy5.5, c-kit BV421, and streptavidin PE-CF594. For LSC frequency, proliferation (Ki67\textsuperscript{+}), and apoptosis analyses, the following antibodies were used when appropriate: biotinylated lineage markers (CD3, CD4, CD5, CD8, B220, and Ter119) plus streptavidin PE-CF594, Gr-1, Mac-1 and Sca1-PE or PE-Cy5, c-kit-BV421, CD34-A700, CD16/32-APC or -PECy7, Annexin V-APC, and Ki67-PE. LSRII was used for all the analyses, and FACSArria was used for cell sorting. Data were analyzed using FlowJo (TreeStar).

DNA Constructs and Virus Production
The plasmids MSCV-MLL-AF9, pMSCV-IRES-mCherry, and pCMV-NIKΔ78-84 are gifts from Drs. Warren Pear, Brian Sorrentino, and Shao-cong Sun, respectively. The pMSCV-MLL-AF9-IRES-mCherry was generated by transferring MLL-AF9 from MSCV-MLL-AF9 into pMSCV-IRES-mCherry using the EcoRI site. The NIKΔ78-84, RelA, and RelB retroviral expression vectors were generated by subcloning the polymerase chain reaction (PCR) products into BamH1/EcoRI or EcoRI/XhoI sites of pMSCV-IRES-mCherry using pCMV-NIKΔ78-84, MIG-RelA (Addgene #26986), and pCDNA3-RelB (Addgene #20017) as templates, respectively. DNMT3a (Addgene #35521) and MEF2C (Addgene #32515) were subcloned into pLV-RES-mCherry (a gift from Dr. Monica Guzman) using in-fusion following the manufacturer’s instructions.
Clontech). pLKO-mCherry was generated by replacing GFP in pLKO-GFP (a gift from Dr. John Dick, University of Toronto) with mCherry. The sequences for knockdown of Relb are: TRCN0000233391 Targeted sequence: ATTGGAAATCATCGACGAATA; TRCN0000233392 Targeted Sequence: ACACATCGGAGCTGCGGATTT; TRCN0000238800 Targeted Sequence: CGGTTCCTTGGAGCCCATTT; and for knockdown of Dnmt3a they are: TRCN0000231276 Targeted Sequence: CGCTCCGCTGAAGGAATATTT; TRCN0000231277 Targeted Sequence: CTATATGTTCTGGCCTATAATT; TRCN0000039034 Targeted Sequence: CCAGATGTCTTTTGGCCTATTTA. 293T cells were transiently transfected with MSCV vectors with pCL-Eco or pLVX vectors with psPAX2 and pMD2.G using lipofectamine 3000 for retrovirus and lentivirus production, respectively.

In Vitro Colony-Forming Assays
Fluorescence-activated cell sorting (FACS)-sorted (GFP±lin–c-kit+Sca1–CD16+CD34+) or unsorted leukemia cells were plated (100–200 cells/96-well plate, 500–2000/24- or 6-well plate) in methylcellulose media (Methocult M3434, StemCell Technologies). Colonies were counted after 5–7 days and replated with the same numbers of cells. Transduced human AML cells were plated in H4034 (StemCell Technologies).

In Vivo Leukemogenesis Assays and Verteporfin Treatment
FACS-sorted lineage−c-kit+Sca-1+ or lineage−c-kit− BM cells from control, ROSAcreER<sup>T2</sup>, or NIKERT2 mice were cultured in Dulbecco Modified Eagle Medium supplemented with 10% FBS, 50 ng/ml SCF, 50 ng/ml TPO, 50 ng/ml FLT3 ligand, and 10 ng/ml IL-3 (all from Peprotech, Rocky Hill, NJ) overnight. Then the cells were plated on virus-loaded retronectin-coated plates for 24 hours and spin-infected with viral supernatant supplemented with polybrene (4 mg/ml) at 1000 g for 90 min at room temperature and transplanted retro-orbitally into lethally irradiated (950 cGy, single dose) CD45.1 recipients (45–100K/recipient) along with 2–3 × 10<sup>5</sup> rescue cells. Tamoxifen (5 mg/mouse/day, Sigma, St. Louis, MO) was administered daily by oral gavage for 4–5 days at indicated time points post–BM transplantation. For secondary BM transplantations, bulk leukemia cells (300,000/recipient) or sorted leukemia stem cells (500/recipient) from spleens or BM of primary recipient mice were transplanted into sublethally (6 Gy) irradiated CD45.1 recipients. For therapeutic treatments of the primary recipients, verteporfin (Sigma) was given at 140 mg/kg body weight by intraperitoneal injection 1 week after transplantation for 12 days and then observed daily for signs of morbidity.

Cell Proliferation Analysis
For Ki67 staining, leukemia cells from BM or spleen were first stained with surface markers to define the LSC population, followed by fixation and permeabilization with Cytofix/Cytopherm (BD Biosciences) and intracellular staining with Ki67-PE. For drug treatment, leukemia cells were cultured in RPMI with 20% fetal bovine serum (FBS) and 10 ng/ml IL-3 with dimethyl sulfoxide (DMSO) vehicle alone or different small molecules at the indicated concentrations. Cell viability was measured by CellTiter-Blue<sup>®</sup> Cell Viability Assay (Promega).
RNA-Seq and Transcriptome Analysis
Total RNA was extracted from sorted LSCs with or without stabilized NIK (GFP ± lin−c−kit−Sca1−CD16+CD34+) from tamoxifen-treated or control leukemic mice, and three biological replicates, each pooled from two control or tamoxifen-treated leukemic mice, were obtained for each genotype. cDNA synthesis and amplification were performed using the SMARTer Ultra Low Input RNA Kit (Clontech) starting with 20 ng of total RNA per sample, following the manufacturer’s instructions. cDNA was fragmented with Q800R sonicator (Qsonica) and used as input for NEBNext Ultra DNA Library Preparation Kit (NEB). Libraries were sequenced on Illumina’s HiSeq2000 in single-read mode with read length of 50 nucleotides producing 60–70 million reads per sample. Sequence data in fastq format were generated using the CASAVA 1.8.2 processing pipeline from Illumina. We considered genes differentially expressed between two groups of samples when the DESeq2 analysis resulted in an adjusted \( p \)-value of <0.01 and the fold-change in gene expression was 1.5 or higher. Heat maps were generated with normalized data of RNA-seq analyses.

Western Blotting
Whole-cell lysate or cytoplasmic or nuclear protein preparations were performed, as previously described (Xiu et al., 2014). Lysates (20–40 µg) were loaded in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and immunoblotted with antibodies (all from Cell Signaling Technologies) to RelA/p65, RelB, p50, p100/52, NIK, IkBa, A20, CyclinD1, GAPDH, or β-actin and histone H3. Membranes were scanned with the ChemiDoc Touch Imaging system (Bio-Rad).

Chromatin Immunoprecipitation (ChIP)
ChIP was performed as described (Xiu et al., 2014). In brief, NIK-stabilized and control primary MLL-AF9 leukemia cells were fixed in 1% formaldehyde. Chromatin was sheared by sonication with a Covaris E220 using the following settings: acoustic duty factor: 5%; peak incident power: 140; cycles per burst: 200; treatment time: 300 s. Sixteen rounds of 20 pulses were made with 2 min between rounds (Qsonica 125 sonicator). Then samples were incubated overnight at 4 °C with Abs to RelB or rabbit immunoglobulin G (IgG) (negative control). Precipitated DNAs were analyzed by real-time PCR. A primer set that covers the Dnmt3a proximal promoter region was used as a negative control: 5′-GGGCAAGGCTCATCAAAAACAGG-3′ and 5′-AGGAGATGAGTTGACTCC-3′. Specific primers for the NF-κB site in the Dnmt3a enhancer region are: 5′-AAGAAGCCTAGAGTTGACTGGG-3′ and 5′-CCTGACAGCAGGAGGAAGG-3′; for Nfkbia are: 5′-GCCCTATAAACGCTGGCAA-3′; and 5′-CTACCAGGTGGTGGCTAGAAC-3′; for Tifab are 5′-GGACTTTCCAGCCACTCAG-3′; for Mef2c are: 5′-CACAAAGTGAAACCCATACAAGG-3′ and 5′-CCTTCCTGCTTCTAGCTTTC-3′.

Connectivity Map Analysis
The gene expression profiles of control HSPCs and LSCs were compared to those of NIK-stabilized HSPCs and LSCs. Significantly \( p \leq 0.01 \) and log2-fold change ≥1.5 up- and down-regulated genes exclusively present in NIK-stabilized LSCs were used to query
CMAP (http://www.broadinstitute.org/cmap/) database. Connectivity scores were derived according to the published methodology (Ashton et al., 2012; Lamb et al., 2006). Based on the scores of “positive connectivity” and “enrichment”, candidate drugs were selected for further testing.

**Supplemental References**

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