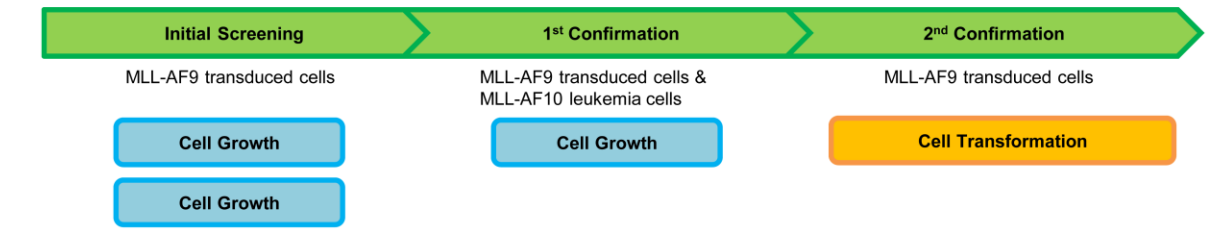
Supplemental Data

A



B

PTPN21	ZAP70	BUB1	IKKγ	GCK	CDC25C	WEE1	INSR	HR1
PLK1	IKKβ	PPP2CB	FGFR1	PPM1J	MAPK4	CDK8	GPRK4	YANK3
MAST1	IKKα	WNK2	PPP1R 14A	MET	PPP1R8	ATR	MLK2	H11
PPP4C	VRK3	MAPK13	ULK1	LATS2	IRAK3			

Figure S1, related to Figure 1. Summary of single cell-based shRNA screening and confirmation strategy.

(A) A lentiviral-based shRNA knockdown approach was applied on a gene-per-gene basis. Puromycin was added in R20/20 culture medium for selection three days after virus transduction. Two rounds of cell growth analyses (equal number of cells were plated in R20/20 medium, and cell numbers were counted after two days) were performed using mouse MLL-AF9 transduced cells in the initial screening, followed by cell growth confirmation studies in mouse MLL-AF9 transduced cells and MLL-AF10 leukemia cells. MLL-AF9 transduced cells were used in the final cell transformation analysis (equal number of cells were plated in methylcellulose-containing medium, and colony numbers were counted after five days).

(B) Candidate kinases and phosphatases identified in shRNA screen. Shading indicates candidates implicated in NF-κB signaling pathways.

Table S1, related to Figure 1. shRNA sequences in lentiviral-based kinase/phosphatase shRNA Library. Provided as an Excel file

Table S2, related to Figure 1. Gene sets used for GSEA analyses. Provided as an Excel file

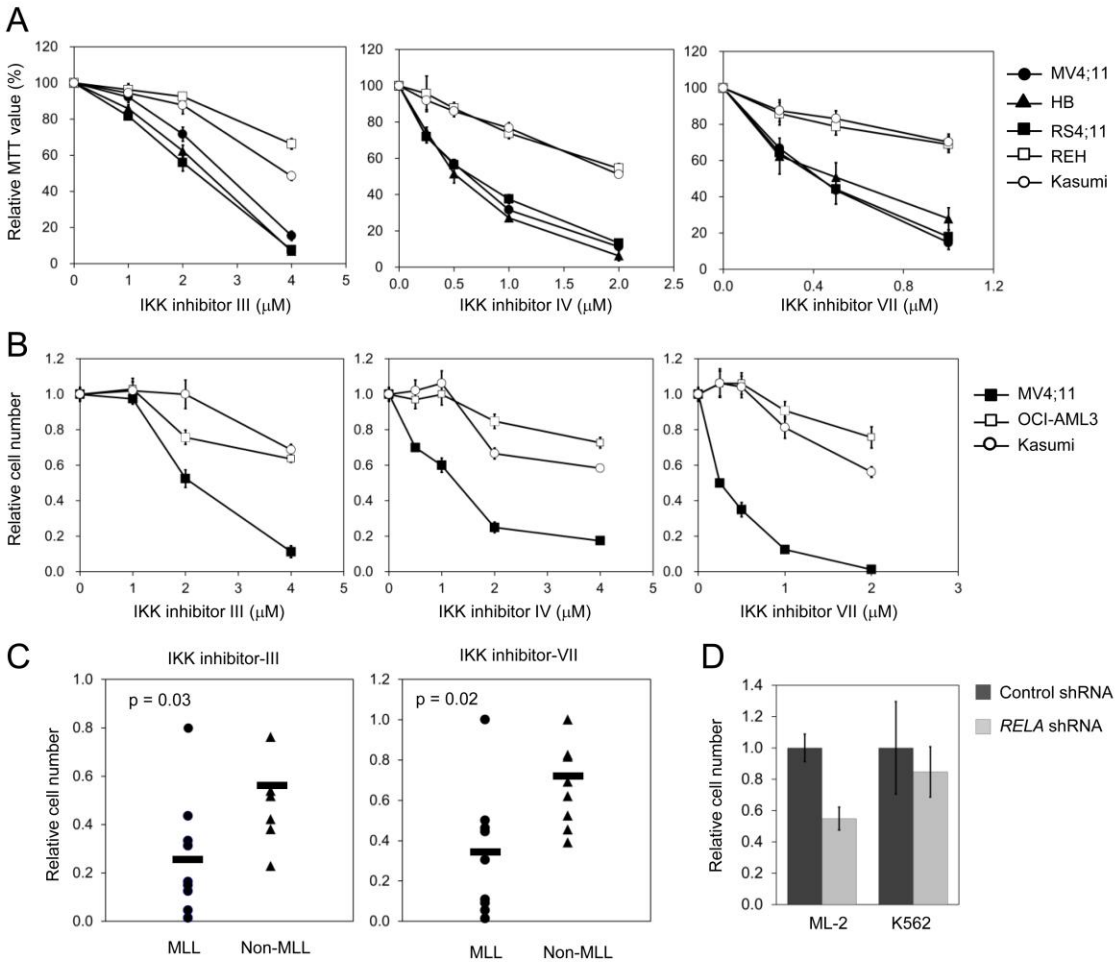


Figure S2, related to Figure 2. Human MLL cell lines show enhanced sensitivity to IKK inhibitors.

(A) Human leukemia cell lines were cultured in the absence or presence of the indicated concentrations of IKK inhibitors III, IV or VII for four days. Cell growth rate was determined by MTT assay and expressed relative to vehicle treated samples. Similar results were obtained by direct cell count (data not shown). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(B) The growth of human myeloid cell lines MV4;11 (MLL-AF4 translocation), OCI-AML3 ($NPM1c^+$) and Kasumi (AML-ETO translocation) was assessed after three days culture in the absence or presence of the indicated concentrations of IKK inhibitors III, IV or VII. The results are expressed as the relative cell number compared to vehicle treated cells.

(C) Human MLL leukemia cell lines (MV4;11, RS4;11, SEMK2, HB, ML-2, KP-L-RY, B1, Mono Mac 6, and THP1) and non-MLL leukemia cells lines (RCH-ACV, REH, Kasumi, K562, NALM-6, HAL-01, SUP-B15, and 697) were cultured in the absence or presence of IKK inhibitors III (4 μM) or VII (2 μM) for four days. The results are expressed as the relative cell number compared to vehicle treated cells.

(D) ML-2 and K562 cells were transduced with lentiviral vectors expressing the indicated shRNAs. Cell numbers were enumerated at three days, and expressed relative to cells transduced with control shRNA. All error bars represent SD of triplicate analyses.

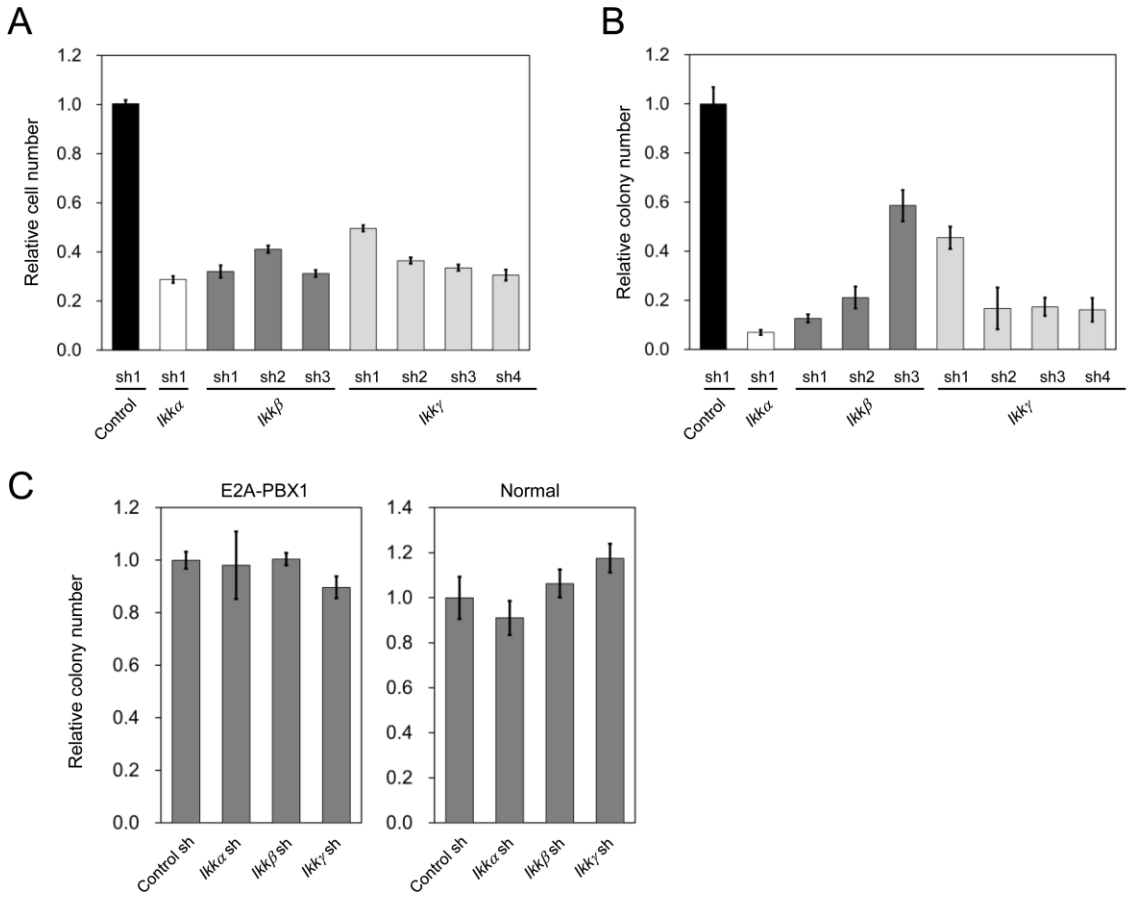


Figure S3, related to Figure 3. Depletion of components of the IKK complex reduces cell growth and colony formation of mouse MLL leukemia cells.

(A) Mouse MLL-AF10 leukemia cells were stably transduced with lentiviral vectors expressing control shRNA or shRNAs targeting *Ikk*α, *Ikk*β, or *Ikk*γ. Cells were cultured for two days and viable cell numbers are expressed relative to the number obtained with control shRNA transduced cells.

(B) Mouse MLL-AF10 leukemia cells transduced with control or *Ikk* shRNAs were plated in methylcellulose medium for five days and colonies were enumerated and expressed relative to the number obtained with control shRNA transduced cells.

(C) Mouse E2A-PBX1 transduced cells and normal hematopoietic progenitors were transduced with control or *Ikk* shRNAs and plated in methylcellulose medium for five days and colonies were enumerated and expressed relative to the number obtained with control shRNA transduced cells. All error bars represent SD of triplicate analyses.

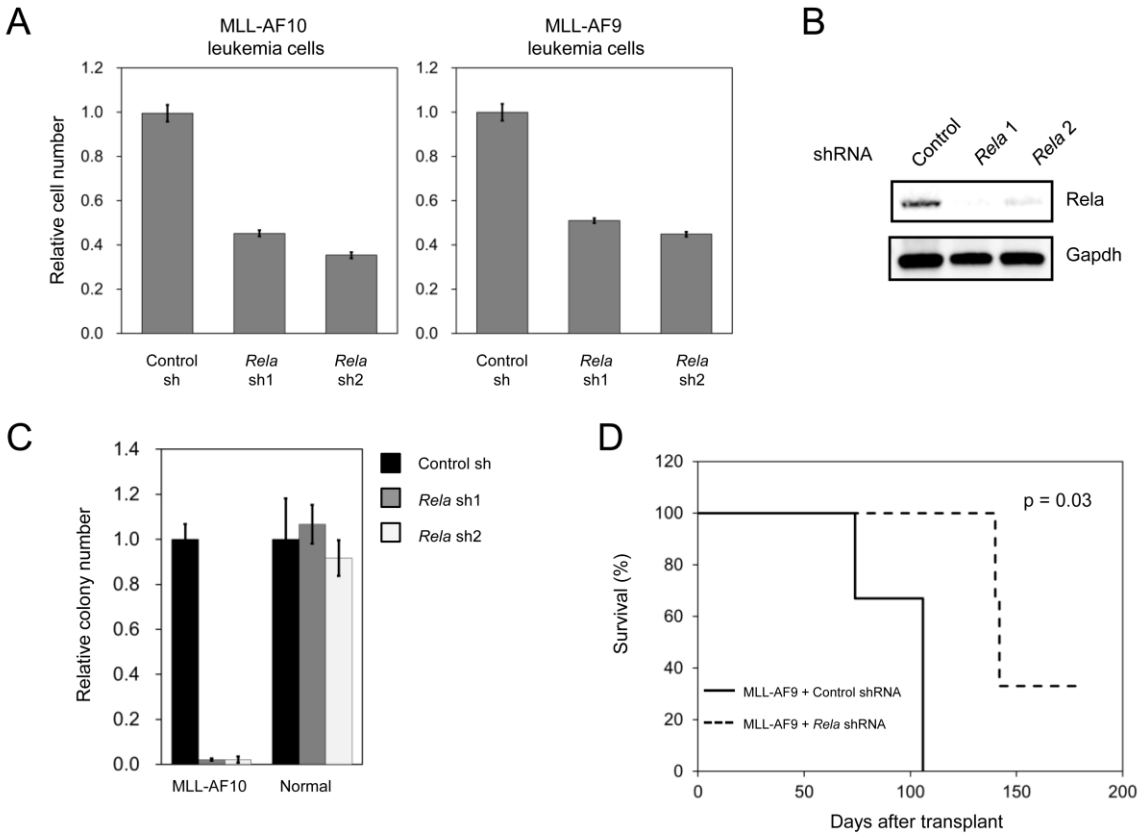


Figure S4, related to Figure 4. Depletion of Rela reduces cell growth and colony formation of mouse MLL leukemia cells.

(A) Mouse MLL-AF10 and MLL-AF9 leukemia cells transduced with lentiviral vectors expressing control or two different *Rela* shRNAs were cultured for two days. Cell numbers were counted and expressed relative to the numbers obtained with control shRNA transduced cells.

(B) Mouse MLL-AF10 leukemia cells were transduced with lentiviral vectors expressing control or *Rela* shRNAs. Protein levels of Rela were detected by western blot analysis.

(C) Mouse MLL-AF10 leukemia cells and bone marrow c-kit$^+$ cells (normal hematopoietic progenitors) were transduced with lentiviral vectors expressing control or *Rela* shRNAs. Colony numbers were enumerated after five days, and expressed relative to the numbers obtained with control shRNA transduced cells. All error bars represent SD of triplicate analyses.

(D) Survival curves are shown for cohorts of mice transplanted with mouse MLL-AF9 transformed cells (1×10^6) transduced with control or *Rela* shRNAs (n = 3 each cohort). Acute leukemia was confirmed by peripheral blood leukocyte count and necropsy. Log-rank Test was used for statistical analysis (p = 0.03).

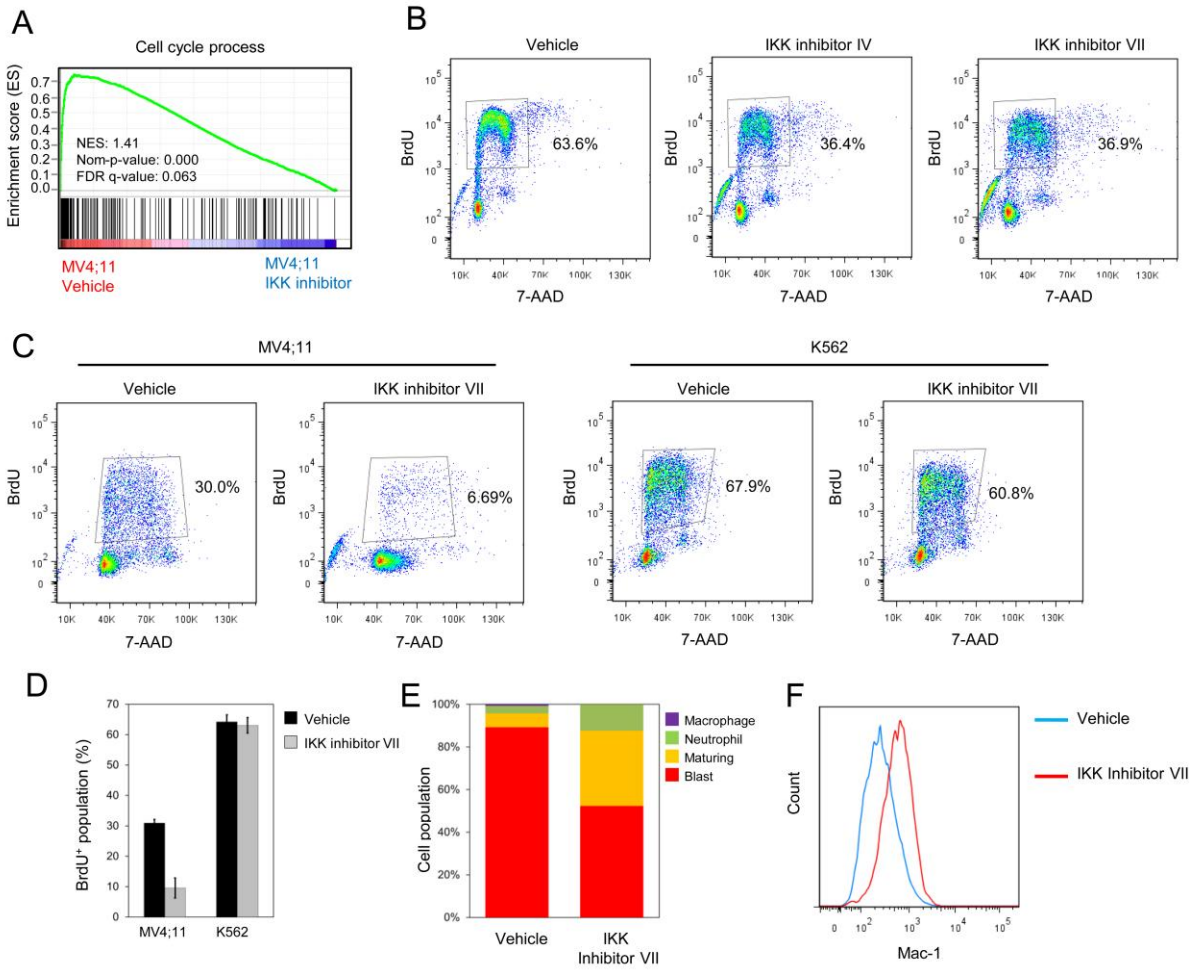


Figure S5, related to Figure 5. IKK inhibitor treatment reduces proliferation and increases differentiation of mouse and human MLL cells.

(A) GSEA plot shows downregulation of cell cycle process related genes in human MLL cell line MV4;11 treated with IKK inhibitor versus vehicle treated cells.

(B) Mouse MLL-AF9 transduced cells were cultured in the presence of 2 μM IKK inhibitor IV or 1 μM IKK inhibitor VII for two days, and BrdU incorporation was quantified by flow cytometry analysis.(C) IKK inhibitor treatment substantially reduced proliferation of human MLL cell line MV4;11, but not non-MLL cell line K562. Cells were cultured in the presence IKK inhibitor VII (1 μM) for two days, and $BrdU^+$ population was analyzed by flow cytometry. Representative results are shown for one of two independent experiments.

(D) Quantification results of (C) from two independent experiments. Error bars represent SD.

(E) Quantification of mouse MLL-AF9 cell populations with indicated morphological features after two days of IKK inhibitor VII treatment (1 μM).

(F) Flow cytometry analysis of Mac-1 surface expression by mouse MLL-AF9 transduced cells after two days of 1 μM IKK inhibitor VII treatment.

Table S3, related to Figure 5. Gene sets used for GSEA analyses. Provided as an Excel file

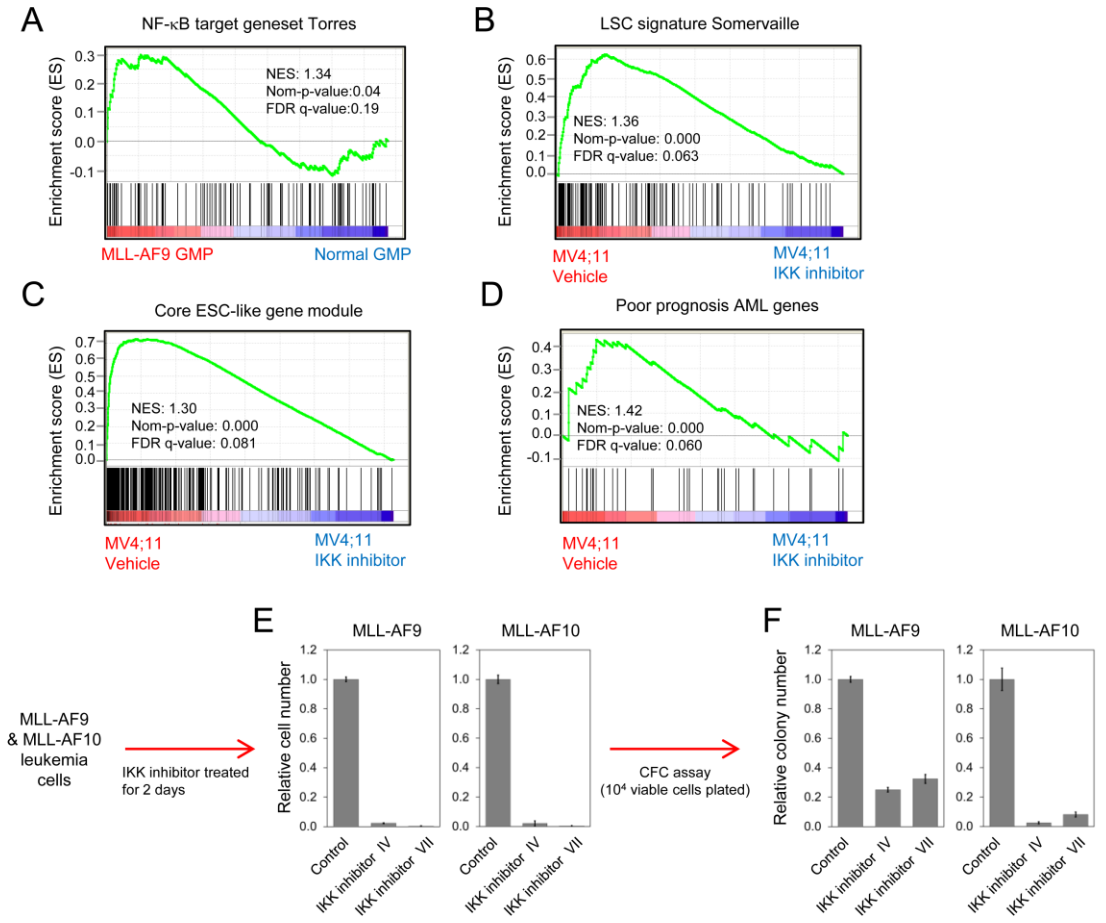


Figure S6, related to Figure 6. Treatment with IKK inhibitors reduces LSC population in mouse and human MLL cells.

(A) GSEA analysis identifies enrichment of NF-κB targets expressed in the GMP population of MLL-AF9 knockin mice. The normalized enrichment score (NES) is correlated with NF-κB target gene expression between normal GMP and MLL-AF9 GMP by using public dataset (GSE10627).

(B) GSEA plot shows downregulation of MLL LSC maintenance signature genes in human MLL cell line MV4;11 treated with IKK inhibitor versus vehicle treated cells.

(C) GSEA plot shows downregulation of core ESC-like gene module in human MLL leukemia cell line MV4;11 treated with IKK inhibitor versus vehicle treated cells.

(D) GSEA plot shows downregulation of poor prognosis AML genes in human MLL leukemia cell line MV4;11 treated with IKK inhibitor versus vehicle treated cells.

(E, F) Total cell numbers (E) are shown for mouse MLL-AF9 and MLL-AF10 leukemia cells following treatment for 2-3 days with IKK inhibitor IV or VII. IKK inhibitors were then removed and equal numbers of viable cells were plated in methylcellulose medium to analyze CFC frequencies (F), which correlated with LSCs. Error bars represent SD of triplicate analyses.

Table S4, related to Figure 6. Gene sets used for GSEA analyses. Provided as an Excel file

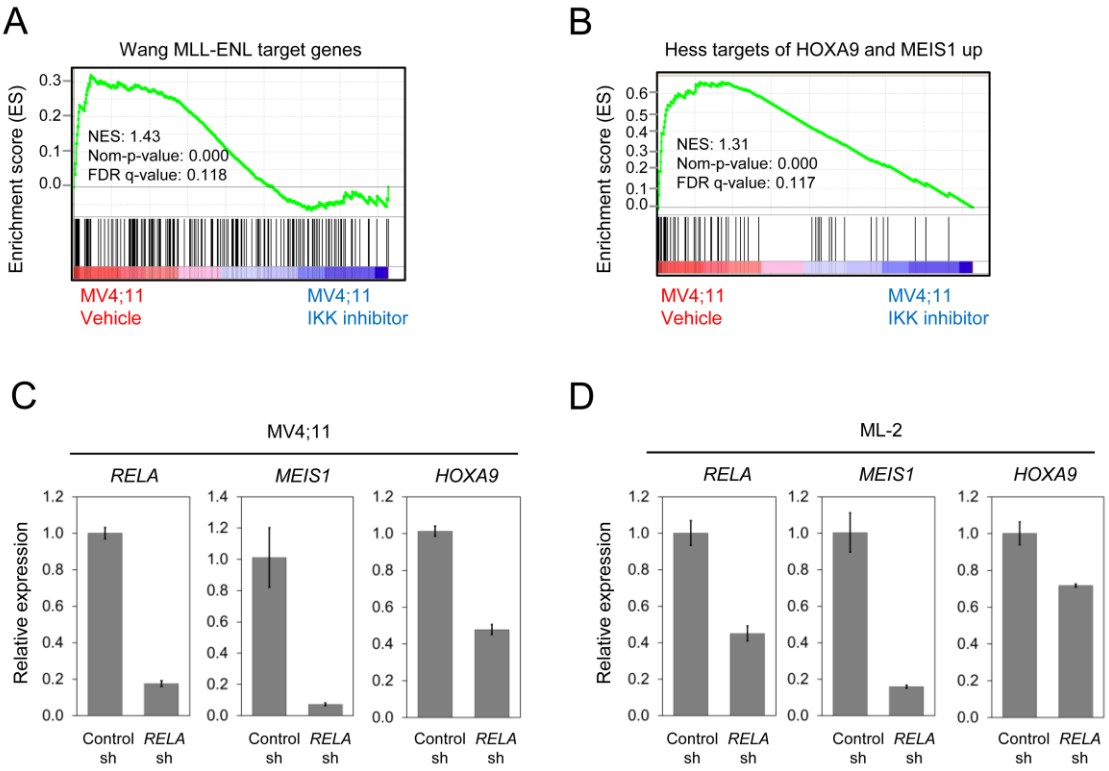


Figure S7, related to Figure 7. RELA depletion reduces *MEIS1* and *HOXA9* gene expression in human cells.

(A, B) GSEA plot shows downregulation of MLL-ENL target genes (A) and HOXA9 and MEIS1 upregulated target genes (B) in human MLL cell line MV4;11 treated with IKK inhibitor versus vehicle treated cells.

(C, D) MV4;11 (C) and ML-2 (D) cells transduced with lentiviral vectors expressing control or *RELA* shRNAs were assessed for *MEIS1* or *HOXA9* transcript levels by qRT-PCR. Results are displayed relative to control shRNA-transduced. Error bars represent SD of triplicate analyses.

Table S5, related to Figure 7. Gene sets used for GSEA analyses. Provided as an Excel file

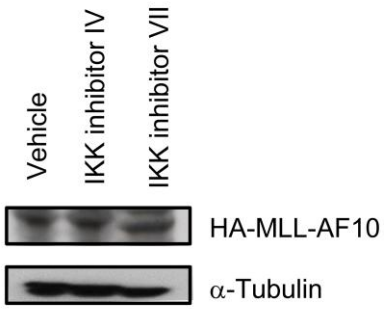


Figure S8, related to Figure 8. There is no significant change of MLL-AF10 level after IKK inhibitor treatment.

Mouse MLL-AF10 leukemia cells were cultured in the absence or presence of IKK inhibitors (IV or VII) for two days. Protein level of HA-MLL-AF10 was detected by antibodies against the HA epitope tag.

Supplemental Experimental Procedures

Cell culture

Human leukemia cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin/L-glutamine (PSG), and non-essential amino acids (NEAA). Transformed mouse myeloid cells or leukemia cells were cultured in RPMI 1640 medium supplemented with 20% FBS, 20% WEHI-conditioned medium and PSG (R20/20 medium), or in methylcellulose-containing medium (Methocult M3231, Stem Cell Technologies) with cytokines as previously described (Lavau et al., 1997). HEK-293T and Phoenix-Eco cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and PSG.

Mice

C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, Maine). *RelA*$^{+/-}$ mice were maintained on a mixed genetic background by interbreeding.

Isolation of mouse myeloid progenitors

Normal hematopoietic progenitors (c-kit^{+} cells) were isolated from mouse bone marrow using CD117 (c-kit) microbeads (130-091-224, Miltenyi Biotec) and autoMACS magnetic cell sorting.

shRNA design, virus generation, and cell transduction

The design of shRNAs (Tables S1 and below) used the RNAi consortium (TRC) shRNA library database (http://www.broadinstitute.org/rnai/public/) to predict suitable target regions in the gene of interest. Sense and antisense oligonucleotides for each shRNA duplex were annealed and cloned into lentiviral-based shRNA expression vectors pLKO.pig (pLKO.1 **P**uro^{R}-**I**RES-**G**FP)

or pLKO.pim (pLKO.1 **P**uro^{R}-**I**RES-**m**Cherry) using published protocols (Schaniel et al., 2010), and confirmed by sequencing.

Target	**shRNA sequence**
Human *RELA*-1	CGGATTGAGGAGAAACGTAAA
Human *RELA*-2	GCCTTAATAGTAGGGTAAGTT
Mouse *Rela*-1	GCGAATCCAGACCAACAATAA
Mouse *Rela*-2	CGGATTGAAGAGAAGCGCAAA
Mouse *Ikk*α-1	GCTCTCTTGAAGGTTGATGTA
Mouse *Ikk*β-1	GCTGCACATTTGAATCTGTAA
Mouse *Ikk*β-2	CCTAAGTTTGACTATCCTATA
Mouse *Ikk*β-3	GCTTGCACTTTAGCCAGAGAA
Mouse *Ikk*γ-1	GTAGCCAAACAGGAATTGATT
Mouse *Ikk*γ-2	GCTCCTGATATGGACACTCTA
Mouse *Ikk*γ-3	GCCTTAAAGGAGTTGGAGCAA
Mouse *Ikk*γ-4	CCACACTTAAGGGCTTGCTTT

Lentiviruses were generated by lipofectamine 2000-mediated co-transfection of shRNA constructs with *pCMV-dR8.2* (packaging) and *pCMV-VSVG* (envelope) into HEK-293T cells. Mouse and human cells were transduced with virus supernatants supplemented with 6 μg/ml polybrene (sc-134220, Santa Cruz Biotechnology) overnight, changed to fresh medium the following day, and cultured another 3 days under puromycin (0.8-1 μg/ml) selection. Retroviruses were generated in Phoenix-Eco cells and the resulting virus supernatants were supplemented with 4 μg/ml polybrene for transduction of mouse cells by spinoculation (2500 rpm, 32 ^{o}C for 2.5 hr).

Apoptosis and proliferation analyses

Apoptotic cells were quantified on the basis of annexin V staining (88-8007, eBioscience) by flow cytometry. BrdU incorporation assays were performed according to the manufacturer's protocol (559619, BD Pharmingen). Following a 2 hr pulse with BrdU, cells were co-stained with 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry for quantification of DNA

content. Flow cytometry data were acquired on a FACSAria using FACS Diva Software (BD Biosciences) and analyzed using FlowJo (TreeStar Software).

May–Grünwald–Giemsa cytospin staining

Following IKK inhibitor treatment, cells (5 x 10^4) were suspended in 100 μl PBS/20% FBS and cytospun onto glass slides at 600 rpm for 5 min. After fixation with methanol, cells were stained with May–Grünwald (Sigma) and Giemsa (Sigma) according to the manufacturer's protocol.

Quantitative real-time PCR

RNA was isolated using TRIzol reagent (15596-026, Invitrogen) and cDNA was synthesized using SuperScript III First-Strand Synthesis System (18080051, Life Technologies). Quantitative PCR analysis was performed using an ABI 7900HT with SYBR green and primers. All signals were quantified using the ΔCt method and were normalized to the levels of *GAPDH* (*Gapdh*) or *β-Actin.*

Primer sequences used in quantitative real-time PCR analyses	
Target	**Primer sequence**
Mouse *Hoxa9*	Forward: CCCCGACTTCAGTCCTTGC
	Reverse: GATGCACGTAGGGGTGGTG
Mouse *Meis1*	Forward: CATGATAGACCAGTCCAACCGA
	Reverse: ATTGGCTGTCCATCAGGGTTA
Mouse *Rela*	Forward: TGCGATTCCGCTATAAATGCG
	Reverse: ACAAGTTCATGTGGATGAGGC
Mouse *Ikkα*	Forward: GGTTTCGGGAACGTCAGTCTG
	Reverse: GCACCATCGCTCTCTGTTTTT
Mouse *Ikkβ*	Forward: CTGAAGATCGCCTGTAGCAAA
	Reverse: TCCATCTGTAACCAGCTCCAG
Mouse *Ikkγ*	Forward: GAGGCCCTGGTAGCCAAAC
	Reverse: ATGGCAGCCAACTTTCAGCTT
Mouse *Gapdh*	Forward: AGGTCGGTGTGAACGGATTTG
	Reverse: TGTAGACCATGTAGTTGAGGTCA
Mouse *β-Actin*	Forward: GGCTGTATTCCCCTCCATCG
	Reverse: CCAGTTGGTAACAATGCCATGT
Human *HOXA9*	Forward: TACGTGGACTCGTTCCTGCT
	Reverse: CGTCGCCTTGGACTGGAAG
Human *MEIS1*	Forward: GGGCATGGATGGAGTAGGC
	Reverse: GGTCCCCATACATCGTGGAG

Human *RELA*	Forward: GTGGGGACTACGACCTGAATG
	Reverse: GGGGCACGATTGTCAAAGATG
Human *GAPDH*	Forward: AAGGTGAAGGTCGGAGTCAAC
	Reverse: GGGGTCATTGATGGCAACAATA

Primer sequences used in ChIP assays	
Target	**Primer sequence**
H4 promoter	Forward: GACACCGCATGC AAAGAATAGCTG
	Reverse: CTTTCCCAAGGCCTTTACCACC
Ey-globin	Forward: GAGAGTTTTTGTTGAAGGAGGAGC
	Reverse: CAGGAGTGTCAGAAGCAAGTACGT
Hoxa9 (-2087)	Forward: CTGTCTCTCCGCTTCCATTC
	Reverse: CTGACCAGCTTCCCTTATGC
Hoxa9 (-1473)	Forward: CAAACTGGGGCACTGGTACT
	Reverse: TTCTGTGGTCTCAGGCAGTG
Hoxa9 (-1171)	Forward: GAGCATGAGGTGTTGTGTGG
	Reverse: CCAAAAGGGGGAAAATTCAT
Hoxa9 (-536)	Forward: TGTCAGAGCGTTGGAAAGTG
	Reverse: TGTGAATTTTGTGCCTTCCA
Hoxa9 (24)	Forward: ACCAGAGCGGTTCATACAGG
	Reverse: CAGACTGGAGATGGGGAAAA
Meis1 (-1551)	Forward: TTTGGGTGAGCTCATTTTCC
	Reverse: GGCTCTCGTGGCTTAGATTG
Meis1 (-1379)	Forward: GGGCGCAGAAACATAAAGAG
	Reverse: TCCACTTCGGAAGCTGAGAT
Meis1 (-687)	Forward: ACGTCCGGACTCAAAGTGAC
	Reverse: TGGAACTGGACTGGAAAAGG
Meis1 (-471)	Forward: CAGTCCAGTTCCAGCTCCTC
	Reverse: TCGAAATTATTGGGGTCTGC
Meis1 (153)	Forward: GCAGTTGCAAAGAGGGAGAG
	Reverse: GGTTGTCAACGTGGTGAATG
Meis1 (764)	Forward: CCATTATGGGGGTATGGATG
	Reverse: GGTACTGATGCGAGTGCAGA

Supplemental Reference

Schaniel, C., Lee, D.-F., and Lemischka, I. R. (2010). Exploration of self-renewal and pluripotency in ES cells using RNAi. Methods Enzymol. *477*, 351-365.