

Epigenetic Roles of MLL Oncoproteins Are Dependent on NF- κ B

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SUMMARY

MLL fusion proteins in leukemia induce aberrant transcriptional elongation and associated chromatin perturbations; however, the upstream signaling pathways and activators that recruit or retain MLL oncoproteins at initiated promoters are unknown. Through functional and comparative genomic studies, we identified an essential role for NF- κ B signaling in MLL leukemia. Suppression of NF- κ B led to robust antileukemia effects that phenocopied loss of functional MLL oncoprotein or associated epigenetic cofactors. The NF- κ B subunit RELA occupies promoter regions of crucial MLL target genes and sustains the MLL-dependent leukemia stem cell program. IKK/NF- κ B signaling is required for wild-type and fusion MLL protein retention and maintenance of associated histone modifications, providing a molecular rationale for enhanced efficacy in therapeutic targeting of this pathway in MLL leukemias.

INTRODUCTION

MLL is an epigenetic regulator that loses its inherent histone methyltransferase activity and acquires abnormal functionalities due to fusions with various partner proteins in leukemias. Although most MLL fusion proteins are strong transactivators that induce inappropriate expression of target genes, they do not typically display features of classical activators that recruit RNA polymerase II. Rather, substantial evidence shows that many MLL fusion proteins assemble into multiprotein complexes involved in transcriptional elongation and associated chromatin modifications. Epigenetic factors known to physically interact with MLL oncoproteins include lens epithelium-derived growth factor (LEDGF) (Yokoyama and Cleary, 2008), histone methyltransferase DOT1L (Okada et al., 2005), chromobox homolog 8 (CBX8) (Tan et al., 2011), histone demethylase KDM1A (Harris et al., 2012), and bromodomain-containing 4 (BRD4) (Zuber et al., 2011b). Chemical inhibitors that target some of these

accessory factors validate their crucial roles in leukemia pathogenesis and offer promising therapeutic strategies to block inappropriate expression of key subordinate genes such as *HOXA9* and *MEIS1* that suppress differentiation and induce aberrant self-renewal capabilities in progenitors critical for leukemogenic potential (Wong et al., 2007).

The DOT1L histone methyltransferase is implicated in playing a central role physically interacting with several MLL fusion partners including AF10, AF9, and ENL (Okada et al., 2005; Zhang et al., 2006; Bitoun et al., 2007). Aberrant levels of its H3K79 methylation mark, which denotes recently elongated genes, are a key feature of MLL primary target genes (Krivtsov et al., 2008; Bernt et al., 2011), suggesting that it might contribute a common mechanism of oncogenic activation in MLL leukemias. The elongation factor P-TEFb is also implicated to serve a central role. Fusion partners that account for most MLL leukemias assemble into higher order protein complexes that contain P-TEFb (Lin et al., 2010; Yokoyama et al., 2010) and are

Significance

MLL is a large multifunctional epigenetic regulator whose transcriptional activity is corrupted by protein fusions with various partner proteins. Extensive studies have characterized the transcriptional and epigenetic perturbations caused by MLL mutations; however, little is known about the upstream signaling pathways and factors that may cooperate with MLL oncoproteins to deregulate critical target genes in leukemia pathogenesis. In a forward genetic screen, we identified and validated a crucial role for NF- κ B in MLL-mediated transformation. IKK/NF- κ B signaling is required for MLL oncoprotein chromatin occupancy and epigenetic function within key target genes of the MLL transcriptional program that sustains leukemia stem cell potential. Therefore, targeting the NF- κ B pathway may be particularly efficacious in MLL leukemia compared with other genetic subtypes of AML.

aberrantly tethered or indirectly recruited to MLL target genes (Yokoyama et al., 2010). P-TEFb phosphorylates the C terminus of stalled RNA polymerase II as well as factors (NELF, DSIF) that otherwise pause the polymerase after promoter initiation (Fujinaga et al., 2004; Yamada et al., 2006). The role of MLL fusion associated factors such as P-TEFb in elongation of initiated transcripts strongly suggests that MLL oncoproteins must function in partnership with more conventional transcriptional activators. However, little is known about the upstream signaling pathways or activators that may recruit, retain, or cooperate with MLL oncoproteins at subordinate genes.

These studies were undertaken to identify requisite signaling pathways that sustain the transcriptional roles of MLL oncoproteins in leukemia pathogenesis.

RESULTS

Functional and Comparative Genomics Implicate the NF- κ B Signaling Pathway in MLL Leukemia Pathogenesis

A functional genomic screen was conducted to identify essential signaling pathways in MLL leukemia cells. A lentiviral-based shRNA knockdown approach was applied on a gene-per-gene basis using a library of 321 small hairpin RNAs (shRNAs) that target 211 phosphoregulators (Table S1 available online) with potential roles in stem cell biology (Lee et al., 2012). An initial screen and subsequent confirmatory rounds of analysis quantified the effects of specific shRNA knockdown on growth of myeloid progenitors transformed by *MLL* oncogenes (Figure S1A). This identified 33 candidate kinases or phosphatases whose knockdown resulted in 50% or greater impairment (Figure S1B). Among the high-scoring candidates, several have implicated roles in mediating NF- κ B signaling, including the IKK complex (IKK α , IKK β , and IKK γ) (Karin and Ben-Neriah, 2000), polo-like kinase 1 (PLK1) (Lin et al., 2011), protein phosphatase 4 catalytic subunit (PPP4C) (Yeh et al., 2004), and interleukin-1 receptor (IL-1R)-associated kinase 3 (IRAK3) (Wesche et al., 1999) (Figure 1A), suggesting that the NF- κ B pathway may contribute to MLL leukemias.

PANTHER analysis showed the enrichment of identified candidate kinases/phosphatases in the Toll-like receptor signaling pathway (data not shown). Consistent with this, expression of transcripts encoding proteins of Toll-like signaling pathways (KEGG pathway: HSA04620) that mediate p105-dependent NF- κ B activation (Table S2) are enriched in MLL-AF1p and MLL-AF10 leukemia cells compared to normal bone marrow cells (Figure 1B) (enrichment was also observed in MLL-AF9 leukemia cells but did not achieve statistical significance; results not shown), further suggesting the importance of NF- κ B signaling in mouse models of MLL leukemia.

Network analysis of global DNA methylation profiles in human MLL-associated acute myeloid leukemia (AML) also implicates the NF- κ B pathway. AMLs with *MLL* chromosomal translocations cluster in two of 16 distinct subgroups defined by DNA methylation profiles (Figuerola et al., 2010) and are distinguished by the hypomethylation of genes that organize into NF- κ B networks (Figure 1C), which is consistent with the role for NF- κ B signaling in sustaining MLL leukemia. Furthermore, gene expression analysis in the same AML cohort identified 121

genes whose expression correlated with both *HOXA9* and *NF- κ B* expression in MLL-associated AML, whereas no overlapping genes were observed in *NPM1c* mutant AML, another genetic subtype associated with aberrant *HOXA9* expression (Figure 1D). Taken together, these findings suggest a potential pathogenic role of the NF- κ B signaling pathway in MLL-associated leukemia.

MLL Leukemia Cells Are Preferentially Dependent on IKK/NF- κ B Signaling

Pharmacologic inhibitors of the IKK complex, a major upstream regulator of NF- κ B signaling, were assessed for their effects on the growth of human cell lines representative of different genetic subtypes of AML. Cell lines with *MLL* aberrations displayed enhanced sensitivity to three different IKK inhibitors compared to non-*MLL* leukemia cells (Figure 2A; Figures S2A–S2C). Furthermore, knockdown of the NF- κ B subunit *RELA* (p65), a transcriptional effector of the pathway, showed marked impairment of cell growth that correlated with the extent of knockdown in the MLL leukemia cell lines MV4;11 (Figures 2B and 2C) and ML-2 but not in non-*MLL* cells K562 (Figure S2D). Consistent with observations that phosphorylation of *RELA* modulates NF- κ B transcriptional activity, lipopolysaccharide (LPS) stimulation induced substantial *RELA* phosphorylation (Figure 2D) and nuclear localization (Figure 2E) in MLL leukemia cell lines compared to non-*MLL* cell lines and was blocked by IKK inhibition. These results demonstrate that human MLL leukemia cells are highly responsive to NF- κ B upstream activation and are dependent on pathway signaling for sustained in vitro growth.

RELA Is Required for Initiation and Establishment of MLL-Mediated Oncogenesis

A murine transformation model was used to further investigate the role of NF- κ B in MLL leukemogenesis. Primary mouse myeloid progenitors transduced with *MLL* oncogenes display enhanced self-renewal in vitro and develop AML in vivo that accurately models the features of human MLL leukemia. A selective requirement for NF- κ B signaling in this model was evidenced by sensitivity to IKK inhibitors, which substantially reduced clonogenic potentials of myeloid progenitors transduced by *MLL* oncogenes (*MLL-AF6* and *MLL-AF9*), as compared to progenitors transduced by other fusion oncogenes (*E2A-PBX1* or *E2A-HLF*) (Figures 3A and 3B). Similarly, genetic reduction of *Ikk α* , *Ikk β* , or *Ikk γ* levels by shRNA-mediated knockdown (Figures 3C and 3D) resulted in decreased cell growth and clonogenicity in *MLL* oncogene-transduced cells (Figures 3E and 3F) and AML cells (Figures S3A and S3B) but not in *E2A-PBX1*-transduced cells and normal hematopoietic progenitors (Figure S3C). Growth and clonogenicity suppression correlated with *Ikk β* expression knockdown level and with *Ikk α* and *Ikk γ* inhibition to a lesser extent. Differential effects of IKK inhibition on colony formation and cell growth were also observed in MLL leukemia cells compared with normal hematopoietic progenitors (Figures 3G and 3H).

Rela knockdown also induced a pronounced reduction of cell growth and clonogenicity in MLL-transformed cells and MLL leukemia cells but not normal hematopoietic progenitors (Figures 4A–4C; Figures S4A–S4C). Conversely, forced *Rela* expression increased cell growth and clonogenicity in MLL

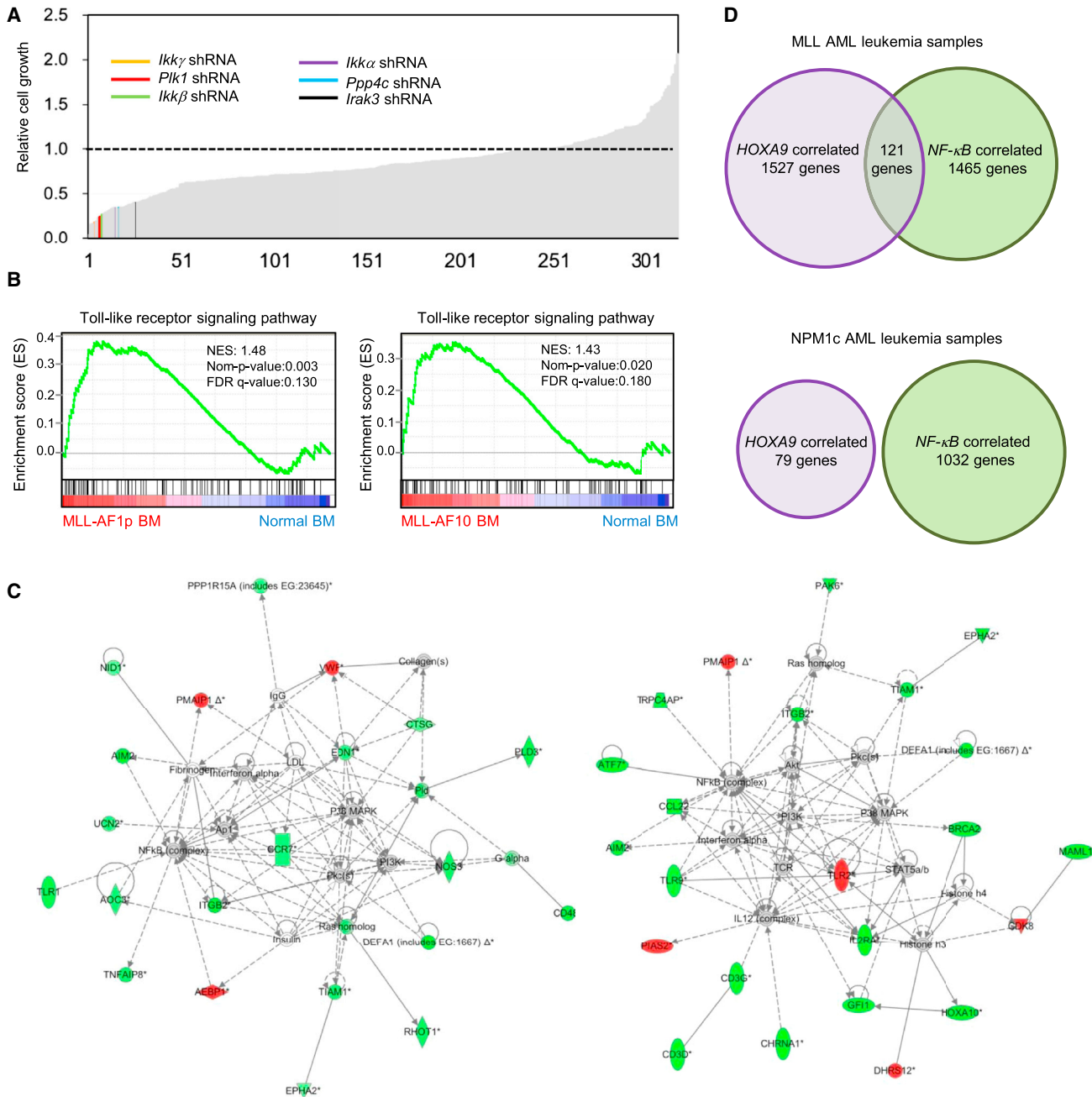


Figure 1. Enrichment of NF-κB Signaling in MLL Leukemia Cells

(A) Single-cell-based shRNA screening was used to identify the effects of kinase and phosphatase knockdowns on the growth of mouse myeloid cells transduced by MLL-AF9. Combined results from two independent replicates are expressed as the relative cell number compared to cells transduced with control shRNA. Potential candidates associated with NF-κB signaling are indicated.

(B) GSEAs demonstrate that expression of genes associated with the Toll-like receptor signaling pathway is enriched in leukemic bone marrow (BM) cells from mice with AML induced by MLL-AF1p (left) and MLL-AF10 (right) compared to normal BM. The normalized enrichment scores (NES) are based on analysis of a public data set (GSE13796).

(C) Aberrantly methylated NF-κB gene networks in two MLL-associated epigenetically defined human AML clusters. Genes with DNA hypomethylation compared with normal CD34⁺ cells are shown in green, and hypermethylated genes appear in red.

(D) Comparison of *HOXA9*-correlated and *NF-κB*-correlated (correlation, >0.5) gene expression in human MLL acute myeloid leukemias showed 121 unique genes whose expression overlapped ($p = 10^{-16}$, calculated using R package: SAGx_1.32.0 under R version 2.15.3). A similar analysis in NPM1c acute myeloid leukemias showed no overlap.

See also [Figure S1](#) and [Tables S1](#) and [S2](#).

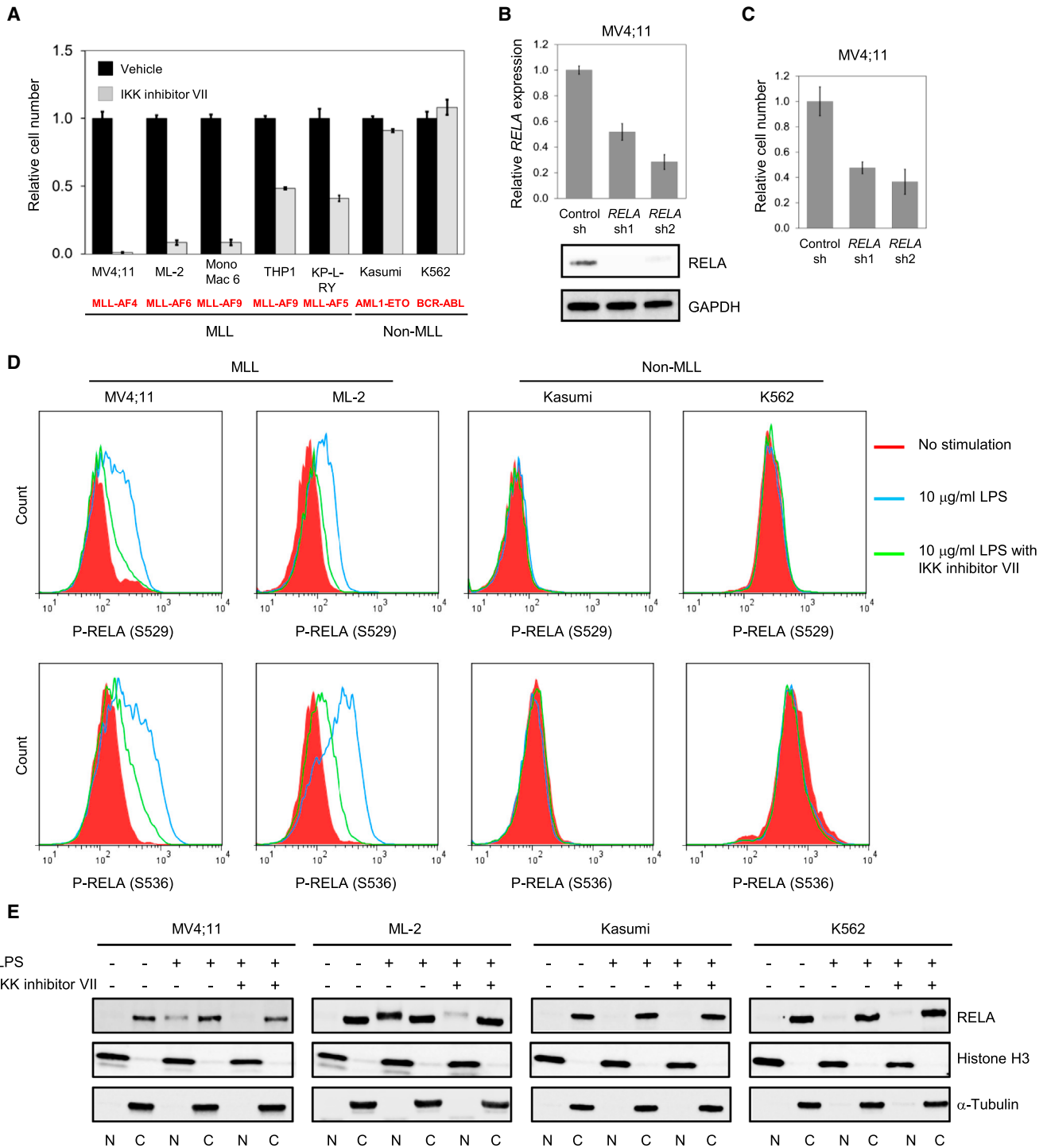


Figure 2. Differential Sensitivity of Human MLL Leukemia Cells to IKK/NF- κ B Signaling

(A) The growth of human myeloid leukemia cell lines was assessed after 4 days' culture in the absence or presence of 2 μ M IKK inhibitor VII. Results are expressed as the relative cell number compared to vehicle-treated cells.

(B and C) MV4;11 human leukemia cells were transduced with lentiviral vectors expressing the indicated shRNAs. Relative *RELA* transcript and protein levels were measured by qRT-PCR and western blot analysis, respectively (B). Cell numbers (C) were enumerated at 3 days and expressed relative to cells transduced with control shRNA.

(D) Human myeloid leukemia cell lines were serum starved (0.1% FBS) overnight, then stimulated with 10 μ g/ml LPS for 30 min with or without IKK inhibitor VII pretreatment, and analyzed by phospho-flow cytometry with antibodies specific to p65 *RELA* phosphorylated at S529 or S536. Representative results are shown from three independent experiments.

(legend continued on next page)

oncogene-transduced cells (Figures 4D and 4E). For further study of the *Rela* requirement, myeloid progenitors were isolated from fetal livers of *Rela*^{+/+} or *Rela*^{-/-} mouse embryos at embryonic day 13.5 (E13.5), transduced with *MLL* oncogenes (*MLL-AF9* or *MLL-ENL*) or oncogenes involved in other genetic subtypes of acute leukemia (*E2A-PBX1*, *NUP98-HOXA9*, or *E2A-HLF*), and serially plated in methylcellulose medium to assess self-renewal. *Rela*^{-/-} cells were unable to sustain colonies induced by *MLL* oncogenes beyond the second plating yet were fully capable of continuous replating induced by non-*MLL* oncogenes (Figure 4F), demonstrating a specific requirement for NF- κ B at early stages of *MLL*-mediated oncogenic transformation.

To assess if *Rela* is required to establish *MLL* leukemia in vivo, *MLL-AF10* AML and *MLL-AF9*-transformed cells were transduced with lentiviral constructs expressing control or *Rela* shRNAs and transplanted into syngeneic recipients. *Rela* knockdown resulted in reduced leukemia penetrance and increased survival (Figures 4G and S4D). Conversely, hyperexpression of *Rela* in *MLL-AF6*-transduced cells substantially shortened leukemia latency and survival of transplanted mice (Figure 4H), suggesting that *Rela* serves a critical role in leukemia development. Our results using several *MLL* fusions further demonstrate a requirement for NF- κ B in the broad context of *MLL* leukemia.

The NF- κ B Pathway Maintains Proliferation, Survival, and Differentiation Arrest of *MLL* Leukemia Cells

Gene expression profiling indicated that genes with decreased expression in *MLL* leukemia cells treated with IKK inhibitors for 24 hr were significantly enriched for cell cycle genes (Gene Ontology [GO] ID number 0022402) (Subramanian et al., 2005), whereas upregulated genes were enriched for apoptosis (GO ID number 0006915) and monocyte/granulocyte signature genes (Chambers et al., 2007) (Figures 5A, 5C, and 5E; Figure S5A; Table S3).

Consistent with gene expression profiles, flow cytometry analysis demonstrated a marked reduction of proliferation following 2-day IKK inhibitor treatment of mouse (Figures 5B and S5B) and human (Figures S5C and S5D) *MLL* leukemia cells, whereas non-*MLL* leukemia cells (K562) were minimally affected (Figures S5C and S5D). Annexin-V staining confirmed the presence of apoptosis at 3 days (Figure 5D). Notably, the differentiation block of *MLL* leukemia cells was reversed by IKK inhibitor IV treatment (2 μ M), which induced morphological maturation (Figures 5F and 5G) associated with increased expression of myeloid differentiation antigens Mac-1 and Gr-1 (Figure 5H) within 2 days. Similar results were found by treatment with IKK inhibitor VII (data not shown), different concentration of IKK inhibitor IV (data not shown), or in *MLL-AF9*-transduced cells (Figures S5E and S5F). These results phenocopy the loss of functional *MLL* fusion protein complex and indicate that IKK/NF- κ B signaling serves a crucial role in supporting proliferation, sustaining survival, and arresting differentiation of *MLL*-transformed and leukemia cells.

IKK/NF- κ B Signaling Sustains LSC Potential

In murine AML induced by *MLL-AF9*, expression of NF- κ B target genes is enriched in leukemic granulocyte/macrophage progenitors (L-GMPs), which have features of leukemia stem cells (LSCs), compared with normal GMPs (Figure S6A; Table S4) (Chen et al., 2008), consistent with a possible role of the NF- κ B pathway in LSCs. In IKK inhibitor-treated mouse and human leukemia cells, genes showing decreased expression were significantly enriched for a transcriptional signature previously shown to distinguish LSCs from non-self-renewing leukemia cells and involved in LSC maintenance (Somerville et al., 2009) (Figure 6A; Figure S6B; Table S4). A transcriptional program that *MLL* LSCs share with embryonic stem cells (ESCs) (Somerville et al., 2009) was also downregulated in treated cells (Figures 6B and S6C; Table S4). The ESC-like program overlaps with a transcriptional program controlled by the MYC oncoprotein (Kim et al., 2010), and MYC core module genes (the set of genes bound by the combination of MYC, MAX, MYCN, DMAP1, E2F1, E2F4, and ZFX in murine ESCs) (Kim et al., 2010) were also downregulated in IKK inhibitor-treated cells (Figure 6C; Table S4). Since poor prognosis in a diverse set of human malignancies is associated with expression of an ESC-like program (Wong et al., 2007; Ben-Porath et al., 2008; Yagi et al., 2003), its downregulation by IKK inhibition has potential therapeutic implications. Indeed, a gene set associated with poor prognosis in pediatric AML was downregulated in IKK inhibitor-treated cells concomitant with upregulation of a good prognosis gene set (Figures 6D and S6D; Table S4).

Functional studies confirmed that IKK inhibition negatively impacts LSCs. Pretreatment of *MLL-AF10* and *MLL-AF9* AML cells with IKK inhibitor for 2 days not only significantly reduced cell growth (Figure S6E) but resulted in a substantial reduction in colony-forming cells (CFCs) in the remaining viable cell population (Figure S6F), which correlate with LSCs (Somerville and Cleary, 2006). Limit-dilution secondary transplantation of inhibitor-pretreated AML cells showed at least a 10-fold reduction in LSC frequency (Figure 6E). Thus, the NF- κ B pathway is critically required for maintenance of LSCs in *MLL* leukemia.

RELA Controls Essential Gene Expression Programs in *MLL* Leukemia by Direct Regulation of *MEIS1* and *HOXA9*

Genes showing decreased expression in *MLL* leukemia cells treated with IKK inhibitors were significantly enriched for genes previously shown to be regulated by the *MLL* oncoprotein (gene sets downregulated upon *MLL-AF9* withdrawal in murine *MLL-AF9*;Nras^{G12D} AML cells) (Zuber et al., 2011a) or for *MLL-ENL* direct targets (Wang et al., 2011) (Figures 7A and S7A; Table S5) (other published direct target gene sets were also downregulated in IKK inhibitor-treated cells but the enrichment p value did not achieve statistical significance). Transcripts for primary *MLL* target genes *Hoxa9* and *Meis1* were significantly reduced in IKK inhibitor-treated leukemia cells (Figure 7C), which were enriched for *HOXA9* and *MEIS1* downregulated gene sets (Hess et al., 2006) (Figures 7B and S7B; Table S5). *Rela* (*RELA*) knockdown

(E) Human leukemia cells were treated as in (D). Nuclear (N) and cytoplasmic (C) fractions were collected and detected by antibodies specific to *RELA*, histone H3 (nuclear control), and α -tubulin (cytoplasmic control).

Error bars represent SD of triplicate analyses.

See also Figure S2.

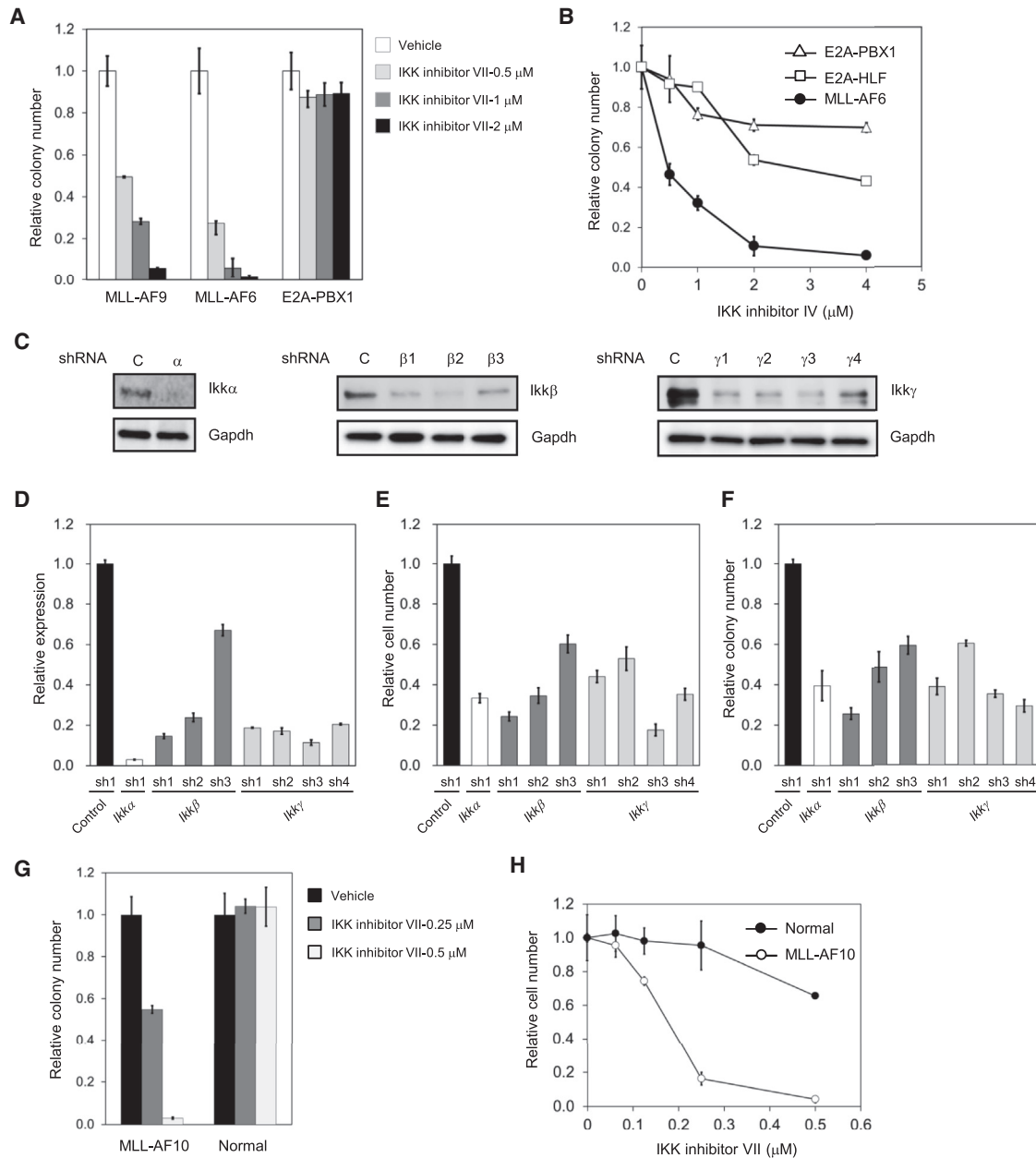


Figure 3. Inhibition of IKK Signaling Suppresses Cell Growth and Colony Formation of Mouse MLL Leukemia Cells

(A and B) Mouse myeloid progenitors transformed by the indicated oncogenes (bone marrow c-kit⁺ cells transduced with *MLL* oncogene) were plated in methylcellulose medium with different concentrations of IKK inhibitor VII (A) or IV (B). Colonies were enumerated after 5 days and expressed relative to cells treated with vehicle (DMSO) alone.

(C and D) Mouse myeloid progenitors transformed by MLL-AF9 were stably transduced with lentiviral vectors expressing control shRNA or shRNAs targeting *Ikkα*, *Ikkβ*, or *Ikkγ*. Protein (C) and relative messenger RNA (D) levels were determined by western blot analysis and qRT-PCR, respectively.

(E and F) Mouse MLL-AF9 transduced cells were treated as in (C) and (D). Viable cell numbers at day 2 (E) and colony numbers at day 5 (F) were enumerated and expressed relative to the number obtained with control shRNA-transduced cells.

(G) Mouse MLL-AF10 leukemia cells and bone marrow c-kit⁺ cells (normal hematopoietic progenitors) were plated in methylcellulose medium with different concentrations of IKK inhibitor VII. Colonies were enumerated after 5 days and expressed relative to cells treated with vehicle (DMSO) alone.

(H) The growth of mouse MLL-AF10 leukemia cells and c-kit⁺ cells was assessed after 2 days culture in the absence or presence of the indicated concentrations of IKK inhibitor VII. Results are expressed as relative cell number compared to vehicle-treated cells.

Error bars represent SD of triplicate analyses.

See also Figure S3.

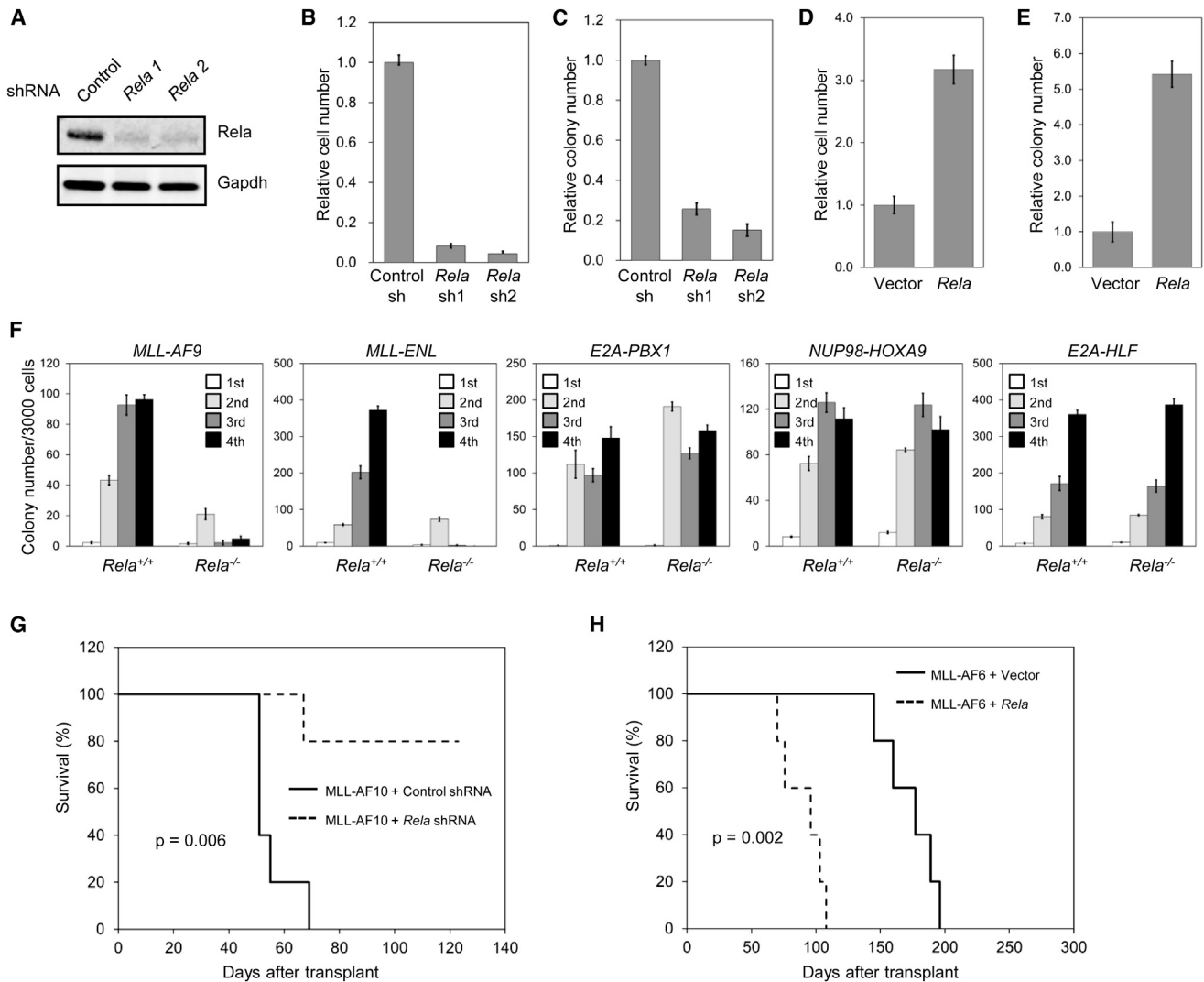


Figure 4. RELA Is Required for MLL Leukemia Development

(A–C) Mouse MLL-AF9 cells were transduced with lentiviral vectors expressing control or *Rela* shRNAs. Protein levels of RelA were detected by western blot analysis (A). Cell numbers (B) and colony numbers (C) were enumerated after 2 and 5 days, respectively, and expressed relative to the numbers obtained with control shRNA-transduced cells.

(D and E) Mouse MLL-AF9 cells were transduced with *Rela* overexpression or control vectors. Cell numbers (D) and colony numbers (E) were enumerated after 3 and 5 days, respectively, and expressed relative to the numbers obtained with control vector-transduced cells.

(F) Hematopoietic progenitors obtained from mouse fetal livers (E13.5) of wild-type (*Rela*^{+/+}) or *Rela* knockout (*Rela*^{-/-}) embryos were transduced with the indicated oncogenes and used for serial myeloid replating assays. Representative results from two independent replicates through four rounds of serial methylcellulose culture are shown as colony number per 3,000 cells.

(G) Survival curves are shown for cohorts of mice transplanted with mouse MLL-AF10 leukemia cells (5×10^5) transduced with control or *Rela* shRNAs ($n = 5$ in each cohort). Acute leukemia was confirmed by peripheral blood leukocyte count and necropsy. Log-rank test was used for statistical analysis ($p = 0.006$).

(H) Survival curves are shown for mice transplanted with mouse MLL-AF6-transformed cells (1×10^6) cotransduced with *Rela* or control vectors ($n = 5$ in each cohort). Log-rank test was used for statistical analysis ($p = 0.002$).

Error bars represent SD of triplicate analyses.

See also Figure S4.

in MLL-transduced mouse progenitors or human leukemia cell lines also markedly reduced *Hoxa9* (*HOXA9*) and *Meis1* (*MEIS1*) transcript levels (Figures 7D, S7C, and S7D), whereas forced *Rela* expression substantially increased their levels (Figure 7E). These results indicated that NF- κ B upregulates *HOXA9/MEIS1* expression. Consistent with this suggestion, myeloid progenitors transformed by forced expression of

Hoxa9 and *Meis1* to bypass the MLL oncoprotein were relatively resistant to IKK inhibitor VII (Figures 3G and 7F).

Promoter Occupancy and Epigenetic Roles of MLL Proteins Are Dependent on IKK/NF- κ B Signaling

In support of a direct role for NF- κ B in *Hoxa9* and *Meis1* transcriptional regulation, chromatin immunoprecipitation (ChIP)

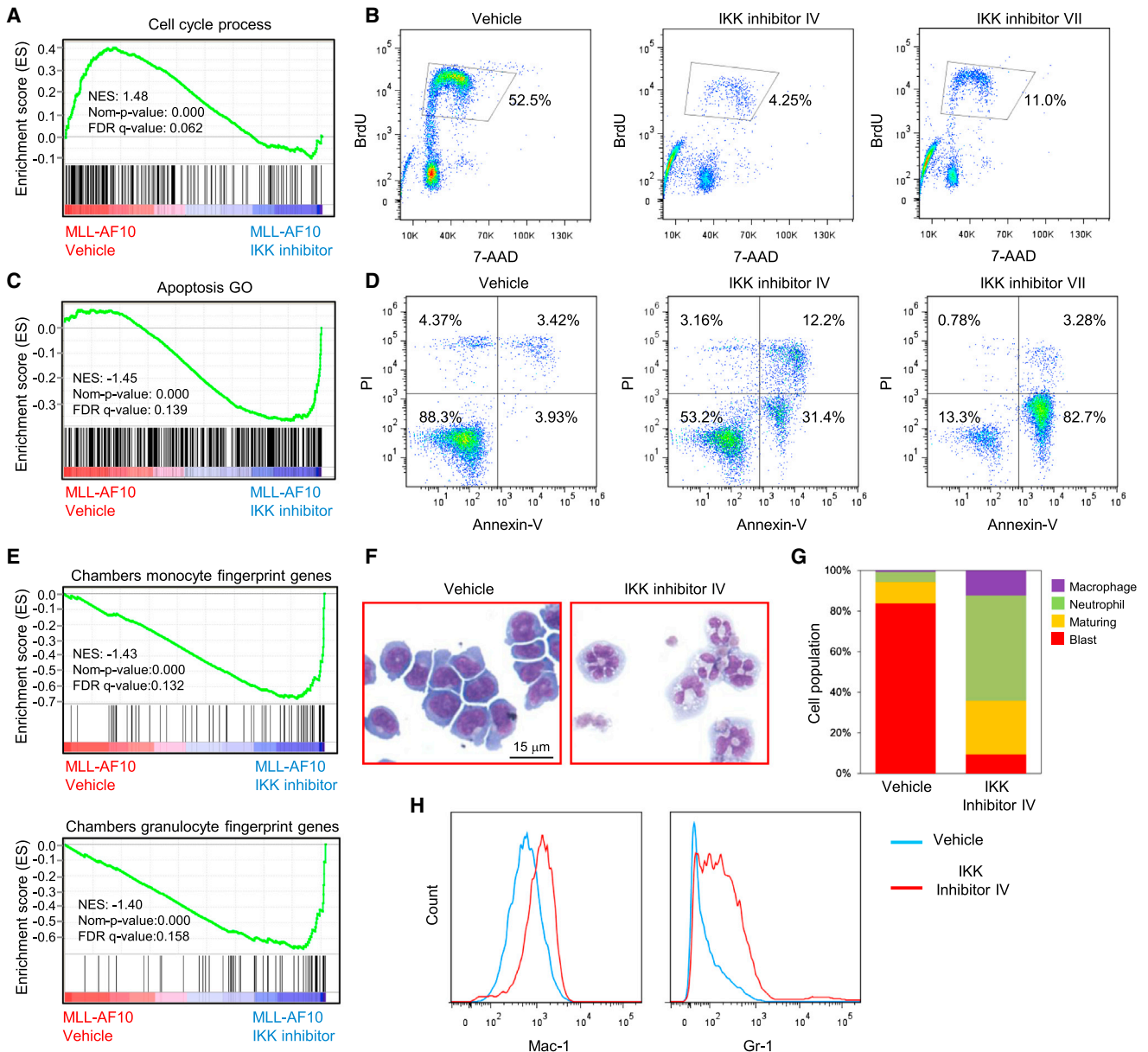


Figure 5. IKK Inhibition Decreases Proliferation, Increases Apoptosis, and Induces Differentiation of MLL Leukemia Cells

(A) GSEA plot shows downregulation of cell cycle process-related-genes in mouse MLL-AF10 leukemia cells treated for 24 hr with IKK inhibitor versus vehicle-treated cells.

(B) Mouse MLL-AF10 leukemia cells were cultured in the presence of 2 μ M IKK inhibitor IV or 0.5 μ M IKK inhibitor VII for 2 days, and BrdU incorporation was quantified by flow cytometry analysis.

(C) GSEA plot shows upregulation of apoptosis-related genes in mouse MLL-AF10 leukemia cells treated for 24 hr with IKK inhibitor versus vehicle-treated cells.

(D) Mouse MLL-AF10 leukemia cells were cultured in the presence of 1 μ M IKK inhibitor IV or 0.5 μ M IKK inhibitor VII for 3 days. The annexin-V positive and propidium iodide (PI)-negative populations constitute early apoptotic cells.

(E) GSEA plots show upregulation of monocyte or granulocyte fingerprint genes in mouse MLL-AF10 leukemia cells treated for 24 hr with IKK inhibitor versus vehicle-treated cells.

(F) Light microscopy of May-Grünwald/Giemsa-stained mouse MLL-AF10 leukemia cells after 2 days of IKK inhibitor IV treatment (2 μ M).

(G) Quantification of leukemia cell populations with indicated morphological features after 2 days of IKK inhibitor IV treatment (2 μ M).

(H) Flow cytometry analysis of Mac-1 and Gr-1 surface expression by mouse MLL-AF10 leukemia cells after 2 days of 2 μ M IKK inhibitor IV treatment.

See also [Figure S5](#) and [Table S3](#).

demonstrated Rel α occupancy that peaked within specific regions of the *Hoxa9* and *Meis1* promoters in proximity of consensus NF- κ B binding sites (Figures 8A and 8B) in mouse

AML cells. The Rel α occupancy profile was similar to that of MLL-AF10 detected with anti-hemagglutinin (anti-HA) antibody specific for HA-tagged MLL-AF10 in AML cells (Figure 8A). The

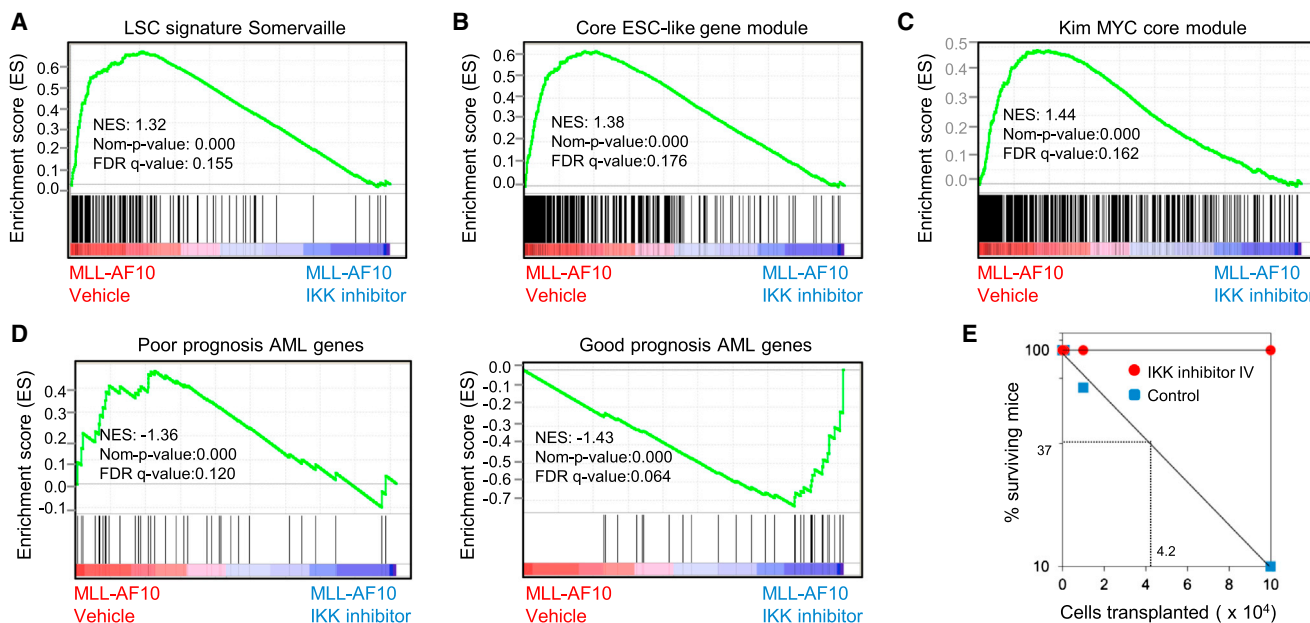


Figure 6. IKK Inhibition Reduces the LSC Population

(A–C) GSEA plots show downregulation of MLL LSC maintenance signature genes (A), core ESC-like gene module (B), and MYC core module genes (C) in mouse MLL-AF10 leukemia cells treated for 24 hr with IKK inhibitor versus vehicle-treated cells.

(D) GSEA plots show downregulation of poor prognosis AML genes and upregulation of good prognosis AML genes in mouse MLL-AF10 leukemia cells treated with IKK inhibitor versus vehicle-treated cells.

(E) Limit-dilution analyses show the estimated cell number of transplanted mouse MLL-AF10 leukemia cells required to initiate AML in sublethally irradiated recipient mice ($n =$ three for each cell dose). MLL-AF10 leukemia cells were pretreated with IKK inhibitor IV ($2 \mu\text{M}$) or control vehicle for 2 days. Viable cells used for transplantation were confirmed by trypan blue staining. Mice were followed for 190 days, with the longest disease latencies being 84 days.

See also [Figure S6](#) and [Table S4](#).

observed RelA occupancy was reduced by treatment with IKK inhibitors and, therefore, dependent on IKK/NF- κ B signaling ([Figure 8C](#)). Notably, IKK inhibition also substantially reduced promoter occupancy of MLL-AF10 ([Figure 8C](#)), indicating that chromatin association of the MLL oncoprotein was highly dependent on IKK/NF- κ B signaling. The marked reduction of promoter-associated MLL-AF10 occurred despite stable cellular MLL-AF10 protein levels in treated cells ([Figure S8](#)). IKK inhibition also reduced histone H3K79 dimethylation ([Figure 8D](#)) consistent with reduced occupancy of MLL-AF10, which interacts with the DOT1L H3K79 histone methyltransferase. Aberrant recruitment of the latter promotes broad spreading of the H3K79 dimethyl mark in MLL target genes characteristic of “epigenetic lesions” ([Guenther et al., 2008](#)). IKK inhibitor treatment also substantially reduced promoter occupancy of wild-type MLL ([Figure 8E](#)) and the level of H3K4 trimethylation ([Figure 8F](#)) on the *Meis1* and *Hoxa9* genes. Thus, NF- κ B signaling is specifically required for promoter association of MLL proteins and maintenance of epigenetic marks necessary for transcription of key MLL target genes in leukemia cells.

DISCUSSION

Using pharmacologic, biochemical, genetic, and genomic approaches, we demonstrate the requirement of IKK/NF- κ B signaling to maintain MLL-mediated transformation in vitro and in vivo and its important role in promoting leukemia cell prolifer-

ation, survival, differentiation arrest, and LSC potential. This strong phenocopy with functions of the MLL oncoprotein and associated cofactors reflects that IKK/NF- κ B signaling sustains the MLL-dependent LSC gene expression program and is required for promoter occupancy of the MLL oncoprotein at key target genes *HOXA9* and *MEIS1*. Thus, NF- κ B constitutes an upstream signaling pathway to converge on key MLL subordinate genes, in support of a model whereby NF- κ B and MLL oncoproteins cooperate to, respectively, initiate and aberrantly elongate transcription of essential target genes in MLL leukemia pathogenesis.

By using a nonbiased genomic screening approach targeting phosphoregulators, we identified the crucial roles of multiple kinases/phosphatases involved in NF- κ B signaling in MLL leukemia cells, including IKK α , IKK β , IKK γ , PPP4C, IRAK3, and PLK1. In the canonical NF- κ B signaling pathway, the IKK $\alpha/\beta/\gamma$ complex phosphorylates I κ B α and triggers its degradation, thereby liberating the NF- κ B heterodimer and inducing its nuclear translocation ([Karin and Ben-Neriah, 2000](#)). PPP4C and IRAK3 also function upstream on the pathway, as their overexpression has been shown to activate NF- κ B mediated transcription ([Yeh et al., 2004](#); [Wesche et al., 1999](#)). Conversely, PLK1 is downstream since the NF- κ B subunit RELA transcriptionally activates its promoter through direct binding ([Lin et al., 2011](#)). Inactivation or knockdown of PLK1 showed reduced cell growth in human and mouse MLL cells (data not shown). Notably, *PLK1* is also an MLL-AF9-regulated gene

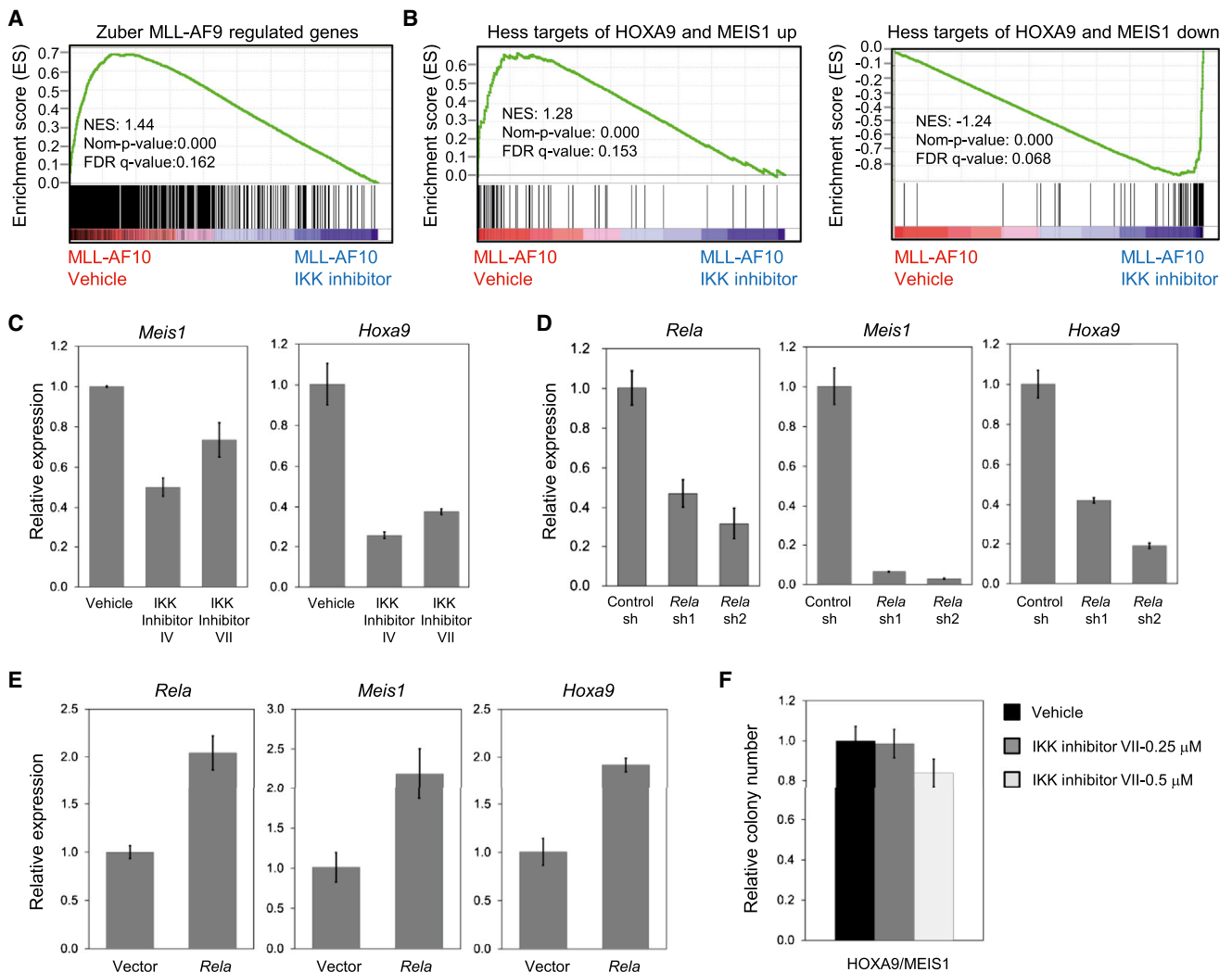


Figure 7. IKK/NF- κ B Signaling Regulates *Meis1* and *Hoxa9* Gene Expression

(A) GSEA plot shows downregulation of MLL-AF9 regulated genes in mouse MLL-AF10 leukemia cells treated with IKK inhibitor versus vehicle-treated cells. (B) GSEA plots show downregulation of HOXA9 and MEIS1 upregulated target genes (left) and upregulation of HOXA9 and MEIS1 downregulated target genes (right) in mouse MLL-AF10 leukemia cells treated with IKK inhibitor versus vehicle-treated cells. (C) Mouse MLL-AF10 leukemia cells were cultured in the absence or presence of IKK inhibitors (IV or VII) for 2 days. *Meis1* or *Hoxa9* transcripts were quantified by qRT-PCR and expressed relative to vehicle-treated cells. (D) Mouse MLL-AF9-transformed cells transduced with lentiviral vectors expressing control or *Rela* shRNAs were assessed for *Rela*, *Meis1*, or *Hoxa9* transcript levels by qRT-PCR. Results are displayed relative to control shRNA-transduced cells. (E) Mouse MLL-AF9-transformed cells transduced with retroviral vector expressing *Rela* or control vector were assessed for *Rela*, *Meis1*, or *Hoxa9* transcript levels by qRT-PCR. Results are displayed relative to control vector-transduced cells. (F) Mouse HOXA9/MEIS1 leukemia cells were plated in methylcellulose medium with different concentrations of IKK inhibitor VII. Colonies were enumerated after 5 days and expressed relative to cells treated with vehicle (DMSO) alone. Error bars represent SD of triplicate analyses. See also Figure S7 and Table S5.

(Zuber et al., 2011a), further demonstrating the potential for integrated transcriptional roles of MLL oncoproteins with NF- κ B. The identification of multiple direct hits on the NF- κ B pathway strongly reinforces its role in MLL leukemia pathogenesis and suggests several alternative strategies to target NF- κ B activity in MLL leukemia therapy.

Previous studies have implicated NF- κ B in a subset of AML, particularly in chemoresistance and regulation of cell survival (Jiang et al., 2012). FLT3 overexpression and PI3-K signaling

contribute to activation of the NF- κ B pathway in AML (Takahashi et al., 2005; Birkenkamp et al., 2004), and NF- κ B participates in deregulation of the Sp1/NF- κ B/HDAC/*miR-25b* signaling network that drives *KIT* overexpression in some AMLs (Liu et al., 2010). Furthermore, the AML1-ETO fusion protein lacks the ability of wild-type AML1 to attenuate NF- κ B, resulting in activated NF- κ B signaling compared with MLL leukemia cells (Nakagawa et al., 2011). In contrast to the latter, our broader studies using several different experimental systems compared

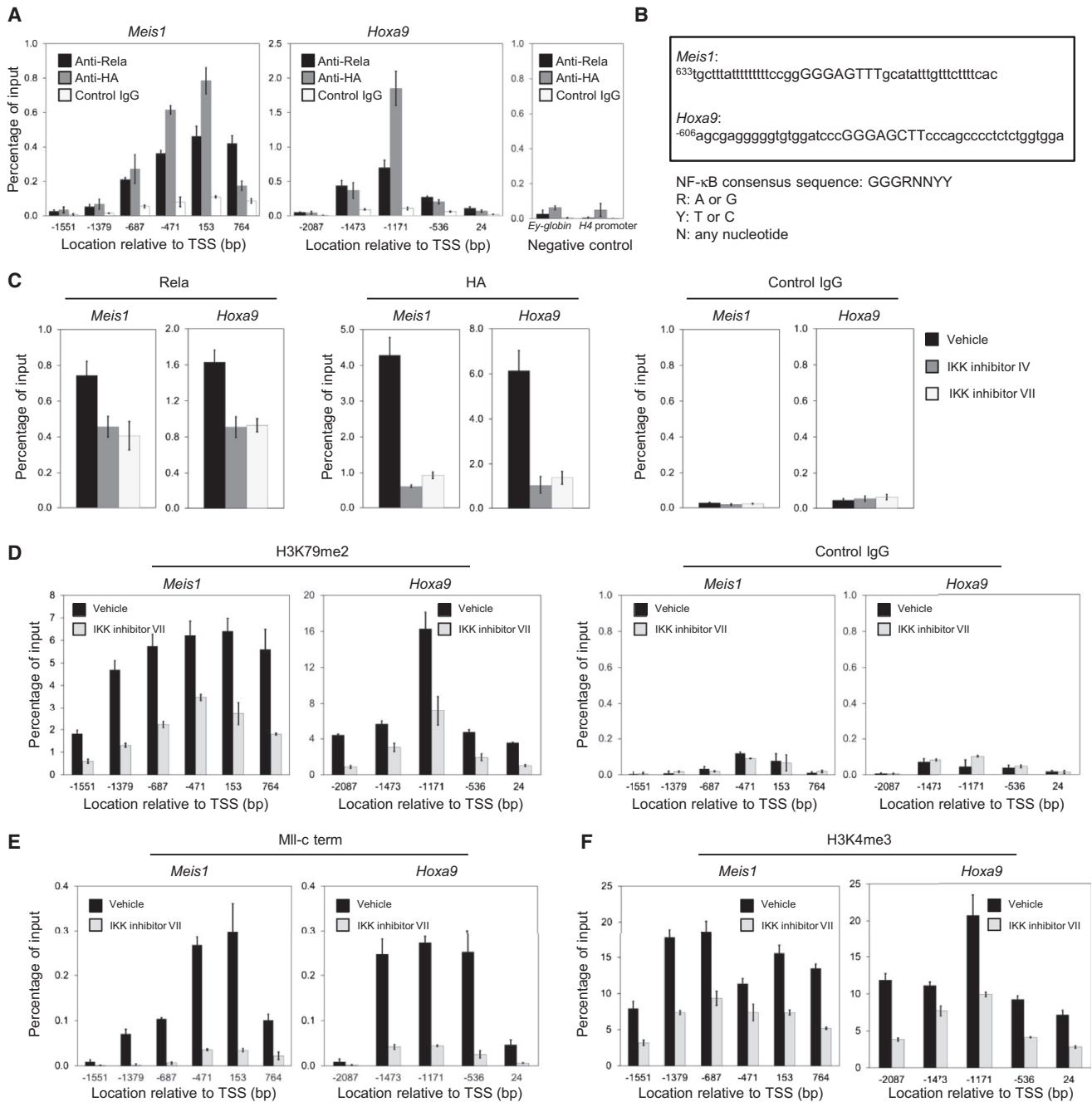


Figure 8. Promoter Occupancies of MLL Wild-Type and Oncoprotein Are Dependent on IKK/NF- κ B Signaling

(A) ChIP was performed on mouse MLL-AF10 leukemia cells with antibodies against Rela, HA (MLL-AF10), and control IgG. Genomic regions amplified by qRT-PCR are indicated relative to the transcription start site (TSS) on *Meis1* and *Hoxa9* genes. *Ey-globin* and *H4* promoter primers were used for negative controls.

(B) Promoter sequence of *Meis1* and *Hoxa9*. NF- κ B consensus sequence is indicated in capital letters.

(C) Mouse MLL-AF10 leukemia cells were cultured in the absence or presence of IKK inhibitors (IV or VII) for 2 days. ChIP was performed using antibodies against Rela, HA (MLL-AF10), and control IgG.

(D-F) Mouse MLL-AF10 leukemia cells were cultured in the absence or presence of IKK inhibitor VII for 2 days. ChIP was performed using antibodies against H3K79me2 and control IgG (D), MII-c term (E), and H3K4me3 (F) and qRT-PCR primers amplifying the indicated genomic regions.

Error bars represent SD of triplicate analyses.

See also Figure S8.

to Nakagawa et al. (2011) clearly demonstrate and provide a mechanistic basis for the strong dependence of MLL leukemia cells on NF- κ B signaling.

Various signaling pathways and kinases have been implicated in MLL leukemia pathogenesis, including FLT3, which is highly expressed in a subset of MLL-rearranged acute lymphoblastic

leukemia (ALL) (Stam et al., 2005), and the AMPK pathway, which directly contributes to the survival of ALL cells with MLL translocations (Accordi et al., 2013). Glycogen synthase kinase 3 (GSK3) supports leukemia cell proliferation and transformation by facilitating the transcriptional activity of HOX proteins (Wang et al., 2008, 2010). However, these studies did not illuminate the upstream signaling pathways or activators that may recruit, retain, or cooperate with MLL oncoproteins at subordinate genes.

Our studies demonstrate that IKK/NF- κ B signaling impinges on the MLL-dependent transcriptional program and serves a major role in its maintenance and deregulation. MLL target genes previously shown to be regulated by MLL-AF9 are significantly downregulated in IKK inhibitor-treated MLL leukemia cells. This includes *Hoxa9* and *Meis1*, which are essential for leukemia pathogenesis, as well as the transcriptional program subordinate to these key MLL target genes. Thus, in addition to the actions of MLL oncoproteins, NF- κ B signaling is also necessary to sustain key MLL target gene expression. Identification of RelA occupancy on *Hoxa9* and *Meis1* promoters in proximity of NF- κ B binding sites (Cartharius et al., 2005) suggests a direct function of NF- κ B in their transcriptional regulation.

MLL oncoproteins facilitate aberrant transcription of their target genes by recruitment of elongation factors in conjunction with epigenetic cofactors, as opposed to functioning as classical activators to recruit RNA polymerase II. P-TEFb, in particular, phosphorylates substrates that otherwise keep RNA polymerase II paused on primed promoters. Despite extensive implication of MLL oncoproteins in aberrant elongation, the conventional transcriptional activators that may functionally cooperate to recruit or retain MLL fusions at primed promoters have not been defined. Our studies demonstrate that IKK/NF- κ B signaling is necessary for promoter occupancy of the MLL oncoprotein and maintenance of histone marks on MLL target genes in leukemia cells. This suggests that NF- κ B may initiate the promoter as a prerequisite for recruitment and/or retention of MLL and associated factors that affect aberrant elongation of the stalled polymerase.

A potential role for NF- κ B in the recruitment of wild-type MLL to chromatin has recently been reported in other promoter contexts (Wang et al., 2012). Consistent with this, our results also demonstrate the importance of IKK/NF- κ B signaling in sustaining H3K4 trimethylation of *Hoxa9* and *Meis1* genes in AML cells and recruitment of wild-type MLL, whose cooperation with MLL fusions is essential for leukemogenesis (Thiel et al., 2010). Although wild-type MLL has been reported to associate with NF- κ B, physical association of RELA or p50 with MLL-fusion proteins was not detected in AML cells by immunoprecipitation (IP) western blot analysis (data not shown) suggesting that NF- κ B is not a component of the MLL fusion protein complex and does not directly tether the MLL oncoprotein to chromatin. Although the mechanism underlying their codependent functions in MLL leukemia requires further study, one possibility is that establishment of an appropriate chromatin context by NF- κ B allows for subsequent binding of the MLL oncoprotein complex given that several of its integral components contain motifs that bind epigenetically modified chromatin or DNA. Alternatively, functional interactions may occur through shared cofactors such as BRD4, a bromodomain protein that positively regulates P-TEFb (Jang et al., 2005) and is present in the MLL on-

coprotein higher order complex. BRD4 not only associates with acetylated histones but also binds to acetylated NF- κ B and coactivates its transcriptional function (Huang et al., 2009). This functional overlap may contribute to the substantial efficacy of therapeutically targeting BRD4 in preclinical models of MLL leukemia (Zuber et al., 2011b).

Small molecule inhibitors targeting the NF- κ B pathway are in development, and some are in preclinical testing. CHS828, which impairs LPS-induced NF- κ B nuclear translocation and transcriptional activation, is currently in its phase I/II clinical trial (Hjarnaa et al., 1999). Also in clinical testing is bortezomib, a potent 26S proteasome inhibitor that indirectly inhibits NF- κ B activity by preventing I κ B α proteasomal degradation (Dai et al., 2011). Recent studies, however, indicate that bortezomib can induce NF- κ B, rather than inhibit it, through calpain (Li et al., 2010) or caspase-independent (Hideshima et al., 2009) mechanisms. Activation of NF- κ B by bortezomib has been observed in various tumor types (Hideshima et al., 2009; Li et al., 2010) and normal PBMCs (Hideshima et al., 2009). Other studies suggest that NF- κ B inhibition may not be a key mechanism of bortezomib's anticancer activity (Chen et al., 2011).

Our studies demonstrate that NF- κ B serves a critical role in sustaining MLL LSC potential. The expression level of RelA quantitatively correlates with LSC activity and leukemia latency, likely reflecting its molecular function as a transcriptional regulator of MEIS1, which serves as a rate-limiting regulator of LSC potential and leukemia latency (Wong et al., 2007). LSCs constitute a subpopulation of leukemia cells with unlimited self-renewal and whose acquired drug-resistant properties are responsible for relapse and, therefore, represent a crucial target for therapeutic intervention. However, targeting LSCs while sparing hematopoietic stem cells (HSCs) is challenging due to similarities in their biological and molecular properties. Constitutive activation of NF- κ B signaling has been observed in primitive AML cells but not in normal primitive hematopoietic cells (Guzman et al., 2001), suggesting the possibility that dependence on NF- κ B may distinguish LSCs from HSCs. Consistent with this notion, indirect inhibition of NF- κ B by proteasome blockade induces LSC apoptosis while leaving normal HSCs viable (Guzman et al., 2002). Our studies are consistent with these earlier observations and demonstrate the differential sensitivity of normal progenitors versus MLL LSCs. The LSC transcriptional program is regulated by NF- κ B, which is required for promoter occupancy of MLL proteins and maintenance of subordinate histone marks in chromatin of crucial target genes (*Hoxa9* and *Meis1*). This provides a mechanistic basis for enhanced dependence of MLL leukemia on the NF- κ B pathway compared with other genetic subtypes of AML and suggests that targeting the pathway may be particularly efficacious in MLL leukemia.

EXPERIMENTAL PROCEDURES

Inhibitors

IKK inhibitors III, IV, and VII (product ID numbers 401480, 401481, and 401486, respectively; EMD Chemicals) were dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations.

Cellular Fractionation and Western Blot Analysis

Cells were washed with PBS and then lysed in hypotonic buffer (10 mM HEPES, pH 7.5, 25 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol,

1 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors). Nuclei were separated from cytoplasmic proteins by centrifugation (1,000 \times g) and resuspended in cell extraction buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, and protease inhibitors) for 30 min on ice. Nuclear fraction was separated from insoluble chromatin fraction by centrifugation (17,000 \times g). Western blot analysis was performed as described elsewhere (Yokoyama et al., 2004). The blots were reacted with antibodies specific to RELA (ab7970, Abcam), GAPDH (G9545, Sigma), α -tubulin (A01410, GenScript), HA (ab9110, Abcam), histone H3 (ab1791, Abcam), Ikk α (2682, Cell Signaling), Ikk β (MAB7155, R&D Systems), and Ikk γ (ab137363, Abcam), followed by IRDye secondary antibodies (LI-COR Biosciences) or peroxidase-conjugated secondary antibodies. Images were detected by an Odyssey imaging system (Odyssey Fc, LI-COR Biosciences) or an ECL system (GE Healthcare).

In Vivo Leukemogenesis Assays

Mouse MLL-AF10 and MLL-AF9 leukemia cells were transduced with control or *Rela* shRNAs. After puromycin selection, cells (5×10^5) were transplanted intravenously into sublethally irradiated (450 rad) C57BL/6 mice.

Mouse myeloid cells transformed by MLL-AF6 were transduced with empty vector or *Rela* expression vector. After puromycin selection, cells (10^6) were transplanted intravenously into sublethally irradiated (450 rad) C57BL/6 mice.

All experiments on mice in this study were performed with the approval of, and in accordance with, the Stanford University Administrative Panel on Laboratory Animal Care.

ChIP

ChIP assays were performed as described elsewhere (Yokoyama et al., 2005), using primary antibodies specific for HA tag (ab9110, Abcam), *Rela* (ab7970, Abcam), Mll-c term (05-765, Millipore), H3K4me3 (ab8580, Abcam), H3K79me2 (ab3594, Abcam), or control immunoglobulin G (IgG) antibodies (ab46540, Abcam). Immunocomplexes were precipitated using Protein A Dynabeads (10001D, Invitrogen) or Protein G Dynabeads (10003D, Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed on the precipitated DNA using primers flanking the *Hoxa9*, *Meis1*, *Ey-globin*, and *H4* promoter sites. The relative values to input were determined using SYBR Green.

Phosphoflow Analysis

Leukemia cell lines were serum starved overnight with 0.1% fetal bovine serum (FBS) contained in RPMI 1640 medium, and stimulated with 10 μ g/ml LPS (L3024-5MG, Sigma) for 30 min with or without IKK inhibitor VII (1 μ M) pretreatment for 15 min. Cells were subsequently fixed with 1.5% paraformaldehyde and permeabilized with 100% ice cold methanol as described elsewhere (Krutzik and Nolan, 2003). Conjugated antibodies to intracellular proteins P-RELA (S529)-Alexa 488 (558421, BD Biosciences) and P-RELA (S536)-PE (5733, Cell Signaling Technology) were used for staining. Flow cytometry data were acquired on a LSR Fortessa using FACS Diva Software (BD Biosciences) and analyzed using FlowJo (TreeStar Software).

Microarray and GSEA Analyses

RNA used for microarray analysis was prepared using an RNeasy Mini kit (QIAGEN). Gene 1.0 ST arrays (Affymetrix) were used according to the manufacturer's instructions. Normalizations of CEL file data were performed using dChip 2010 (DNA-Chip Analyzer) software (Li and Wong, 2001). Gene set enrichment analyses (GSEAs) were performed using GSEA v2.07 software (<http://www.broad.mit.edu/gsea>) with 1,000 data permutations. Enriched gene sets were selected on the basis of statistical significance (false discovery rate q value < 0.25 , and normalized p value < 0.05).

ACCESSION NUMBERS

The Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo>) accession number for the microarray raw data reported in this article is GSE46252.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.08.019>.

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