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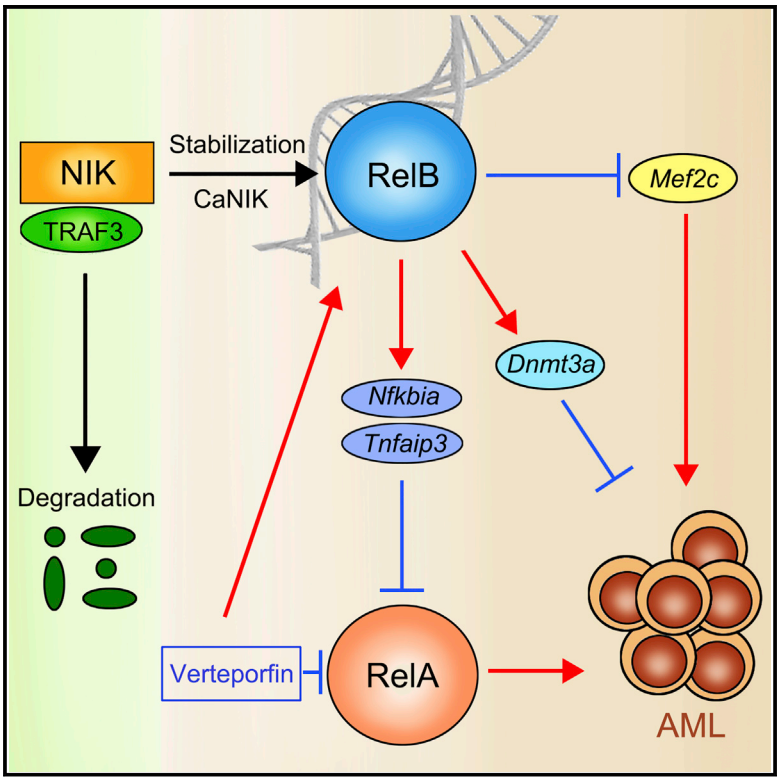
Stabilization of NF-κB-Inducing Kinase Suppresses MLL-AF9-Induced Acute Myeloid Leukemia

Graphical Abstract



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In Brief

Xiu et al. identify a myeloid-leukemia-suppressive role of NIK, which is different from NIK's tumor-promoting role in lymphoid neoplasms. They show that stabilization of NIK activates non-canonical but represses canonical NF-κB signaling, indicating that NF-κB non-canonical signaling has a role opposite to that of canonical signaling in AML.

Highlights

- Stabilization of NIK suppresses MLL-AF9-induced AML
- NIK-induced leukemic suppression acts through NF-κB non-canonical signaling
- NF-κB non-canonical signaling upregulates *Dnmt3a* and downregulates *Mef2c*
- NF-κB non-canonical and canonical signaling have opposite roles in AML stem cells

Data and Software Availability

GSE97389

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

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SUMMARY

Canonical NF-κB signaling is constitutively activated in acute myeloid leukemia (AML) stem cells and is required for maintenance of the self-renewal of leukemia stem cells (LSCs). However, any potential role for NF-κB non-canonical signaling in AML has been largely overlooked. Here, we report that stabilization of NF-κB-inducing kinase (NIK) suppresses AML. Mechanistically, stabilization of NIK activates NF-κB non-canonical signaling and represses NF-κB canonical signaling. In addition, stabilization of NIK-induced activation of NF-κB non-canonical signaling upregulates *Dnmt3a* and downregulates *Mef2c*, which suppresses and promotes AML development, respectively. Importantly, by querying the connectivity MAP using up- and downregulated genes that are present exclusively in NIK-stabilized LSCs, we discovered that verteporfin has anti-AML effects, suggesting that repurposing verteporfin to target myeloid leukemia is worth testing clinically. Our data provide a scientific rationale for developing small molecules to stabilize NIK specifically in myeloid leukemias as an attractive therapeutic option.

INTRODUCTION

Acute myeloid leukemia (AML) is the most common acute leukemia in adults; it occurs increasingly with age, with devastating outcomes (Dombret and Gardin, 2016). AML is initiated and maintained by a small minority of self-renewing leukemia stem cells (LSCs) (Kreso and Dick, 2014). Defining and targeting the key molecules specific to LSCs hold clinical promise for the eradication of AML (Pollyea and Jordan, 2017). Despite advances in understanding of the pathophysiology of AML, current treatment still largely relies on standard "7+3" chemotherapy and allogeneic stem cell transplantation (Dombret and Gardin, 2016).

Canonical nuclear factor κB (NF-κB) signaling is constitutively activated in AML stem cells required for maintenance of LSC self-renewal and positively correlates with resistance to therapy (Bosman et al., 2016; Gasparini et al., 2014; Guzman et al., 2007). Suppression of canonical signaling by ectopic expression of the so-called super-repressor form of IκBα attenuates AML development, validating targeting the pathway as potential therapy in AML in combination with current treatment (Cilloni et al., 2007; Hsieh and Van Etten, 2014). However, the existing studies have focused almost entirely on the canonical NF-κB pathway, and any potential role for NF-κB non-canonical signaling in AML has been largely overlooked. A limited number of papers suggest that expression of the non-canonical signaling components may be beneficial in AML; for example, the upregulation of non-canonical NF-κB components (NF-κB-inducing kinase [NIK] and p52) induces AML cell differentiation (Olsnes et al., 2009). In contrast, using a biphenotypic B-myelomonocytic leukemia cell line with lymphoblastic morphology, expression of RelB was shown to repress a tumor suppressor, death-associated protein kinase 1 (Shanmugam et al., 2012). While these studies suggest that non-canonical signaling components can suppress or accelerate AML, no functional studies have been reported.

NIK activates NF-κB non-canonical signaling by directly phosphorylating IKKα, which, in turn, phosphorylates p100 and induces its processing to p52, facilitating the formation and nuclear translocation of RelB/p52 complexes (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009). In normal, unstimulated cells, NIK protein is continually degraded to prevent unnecessary NF-κB activation (Sun, 2012). NIKΔT3 is a non-degradable NIK mutant lacking the TRAF3-binding domain (Liao et al., 2004). To constitutively or conditionally stabilize NIK in the hematopoietic system, mice carrying a NIKΔT3^{flSTOP} allele were crossed with either Vav-Cre mice (hereinafter termed caNIK) or Rosa-CreER^{T2} mice (hereinafter termed NIKERT2) (Sasaki et al., 2008; Xiu et al., 2017). We used these mutant mice in combination with the well-characterized MLL-AF9-induced AML mouse model to investigate the role of NIK-induced non-canonical signaling in AML (Krivtsov et al., 2006). Unexpectedly, we found that stabilization of NIK suppressed MLL-AF9-induced AML, which is different from the tumor-promoting role of NIK in B cell neoplasms.

RESULTS

Stabilization of NIK Impairs MLL-AF9-Induced Leukemic Transformation

We first tested the impact of NIK-induced signaling on the colony-forming ability of MLL-AF9-transduced hematopoietic

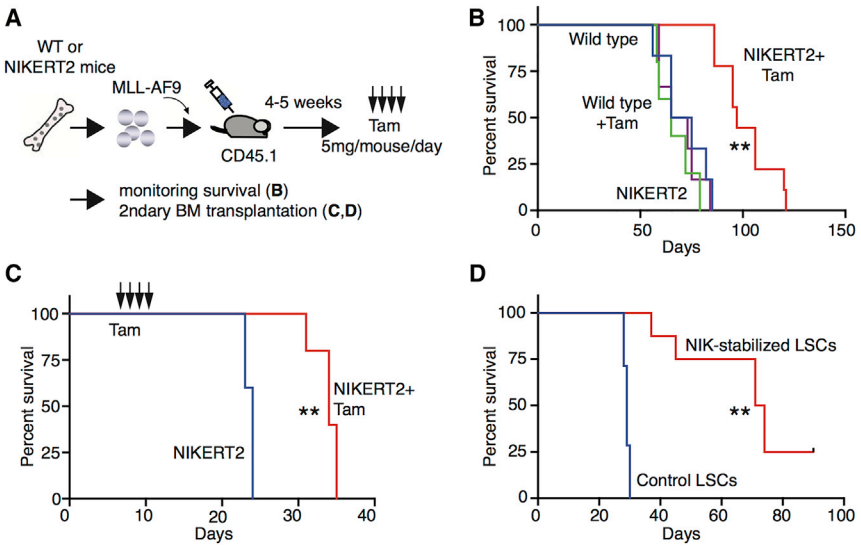

Figure 1. Stabilization of NIK Suppresses MLL-AF9-Induced AML

(A) Scheme of MLL-AF9-induced AML and the timing of tamoxifen delivery.

(B) Kaplan-Meier survival curve of lethally irradiated recipients receiving MLL-AF9-transduced wild-type or NIKERT2 Lin$^-$cKit$^+$ BM with or without tamoxifen treatment (n = 5–9 mice in each group).

(C) Survival curve of sublethally irradiated recipient mice receiving full-blown NIKERT2 AML cells with or without tamoxifen treatment (n = 5 each). Tamoxifen was started 8 days post-transplantation.

(D) Survival curve of sublethally irradiated mice receiving sorted LSCs (500 GFP ± Lin$^-$Kit$^+$Sca1$^-$CD16$^+$CD34$^+$ cells) from control or tamoxifen-treated fully developed NIKERT2 AML mice (control, n = 7; NIK, n = 8). **$p < 0.01$.

stem/progenitor cells (HSPCs; Lin$^-$cKit$^+$Sca1$^+$). MLL-AF9-transduced HSPCs from caNIK mice formed fewer colonies in the first plating and exhausted during the second plating (Figure S1A). We then performed a similar assay using HSPCs isolated from NIKERT2 mice to exclude the possibility that the impaired HSPC self-renewal upon NIK stabilization prevents subsequent oncogenic transformation (Xiu et al., 2017). Accordingly, all the following experiments were performed using cells from NIKERT2 mice, unless otherwise specified. After retroviral MLL-AF9 transduction and four rounds of serial plating, MLL-AF9-transformed cells grew in liquid medium supplemented with interleukin (IL)-3 (Figure S1B). Compared to vehicle treatment, stabilization of NIK by 4-hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen, reduced colony and cell numbers in both methylcellulose and liquid cultures and reduced colony size (Figures S1C–S1E).

Since NIK promotes cell proliferation in various solid tumors and B cell neoplasms (Cildir et al., 2016; Gasparini et al., 2014), these unexpected *in vitro* findings prompted us to test whether NIK suppresses AML *in vivo*. We transplanted MLL-AF9-transduced Lin$^-$cKit$^+$ bone marrow (BM) cells from NIKERT2 or wild-type (WT) mice into lethally irradiated recipients, along with radioprotective cells, and treated recipients with daily doses of tamoxifen for 4 days, 4–5 weeks post-transplantation (Figure 1A). Mice that received MLL-AF9-transduced WT or NIKERT2 cells developed AML within 3 months. However, the development of AML in recipients of NIKERT2 cells treated with tamoxifen was significantly delayed. In contrast, tamoxifen treatment had no effect on mice transplanted with MLL-AF9-transduced WT cells or HSPCs from CreER^{T2} (ERT2) mice, indicating that the delayed AML onset is not due to Cre toxicity (Figures 1B, S1F, and S1G). These results suggest that NIK impairs the initiation of MLL-AF9-induced AML, which encouraged us to further investigate whether NIK has a similar effect on fully developed AML. The LSC immunophenotype in this model is well defined as lin$^-$c-kit$^+$Sca1$^-$CD16$^+$CD34$^+$ leukemia cells (Krivtsov et al., 2006). We prospectively isolated LSCs from mice with fully developed AML, transplanted them into sublethally irradiated recipients, and gave tamoxifen 1 week later, daily for 4 days. Strikingly, tamoxifen treatment also significantly delayed full-blown LSC repopulation (Figure 1C). In our system, stabilization of NIK by tamoxifen can be monitored by the expression of GFP. To directly test the effect of NIK on LSC self-renewal, NIK-stabilized LSCs (GFP$^+$lin$^-$c-kit$^+$Sca1$^-$CD16$^+$CD34$^+$) and control LSCs were isolated and re-transplanted into sublethally irradiated recipients. Stabilization of NIK significantly inhibited LSC repopulation capacity and extended the latency of AML development (Figure 1D). No homing defects of NIK-activated LSCs compared with control LSCs were detected (0.099 ± 0.032 versus 0.121 ± 0.037; n = 4, p = 0.403). The aforementioned results differ from those produced from the tumor-promoting NIK in B cell neoplasms, but they are consistent with NIK's inhibitory role in anaplastic large-cell lymphoma and also are in agreement with the notion that, in certain circumstances, NIK inhibits cell proliferation (Cildir et al., 2016; Lu et al., 2005; Muro et al., 2014). Most importantly, stabilization of NIK has a broad anti-human myeloid leukemia role (Figure S2).

Stabilization of NIK Inhibits Leukemic Cell Proliferation and Dysregulates Multifaceted Pathways that Regulate LSC Self-Renewal

To understand the cellular mechanisms underlying the ability for NIK to inhibit AML, we compared the proliferation and apoptosis of NIK-stabilized LSCs to control LSCs. Colony-forming assays showed that NIK-stabilized LSCs formed fewer and smaller colonies and exhausted after the second plating, compared to controls (Figure 2A). The frequency and the proliferation (Ki67$^+$) index of NIK-stabilized LSCs were also significantly decreased compared to those of controls (Figures 2B and 2C). However, annexin V staining showed that apoptosis was only modestly increased in NIK-stabilized leukemic cells isolated from tamoxifen-treated mice with fully developed leukemia (Figure 2D). In addition, stabilization of NIK-associated impaired

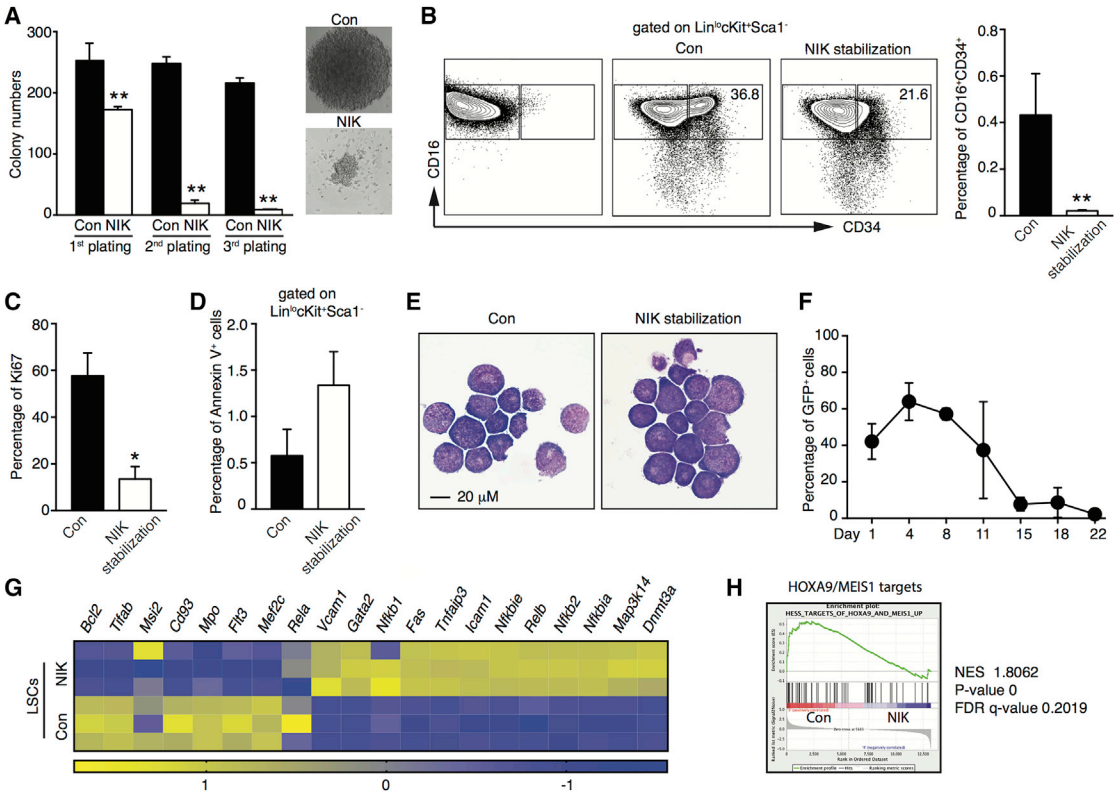

Figure 2. Stabilization of NIK Impairs Leukemic Cell Proliferation, Induces Apoptosis, and Dysregulates Multifaceted Pathways that Control LSC Self-Renewal

(A) Number of colonies formed in methylcellulose using sorted LSCs from tamoxifen-treated (NIK, GFP$^+$) or untreated NIKERT2 leukemic mice (n = 3). Representative colony pictures from the first plating are shown on the right.

(B) Representative fluorescence-activated cell sorting (FACS) plots and frequencies of LSCs in control or tamoxifen-treated NIKERT2 leukemic mice (n = 7). Left: no CD34 antibody was added, for gating control.

(C) Percentage of Ki67$^+$ LSCs in control or tamoxifen-treated NIKERT2 leukemic mice (n = 4).

(D) Percentage of apoptotic (annexin V$^+$) leukemic stem/progenitor cells in control or tamoxifen-treated NIKERT2 leukemic mice (n = 4, p = 0.175).

(E) Wright Giemsa stains of control and NIK-stabilized leukemia cells.

(F) Dynamic changes of NIK-stabilized (GFP$^+$) leukemia cells cultivated *in vitro* (n = 3).

(G) RNA-seq analyses of sorted LSCs from control or tamoxifen-treated leukemic mice. Heatmaps were generated from selected genes of interest.

(H) Downregulated HOXA9/MEIS signature in NIK-stabilized LSCs. FDR, false discovery rate. NES, normalized enrichment score.

Values are mean ± SEM. *p < 0.05; **p < 0.01.

leukemogenesis was not due to enhanced leukemic cell differentiation (Figure 2E). When cultured *in vitro*, these cells lost their proliferative capacity and were eventually outcompeted by unrecombinated control leukemic cells (Figure 2F).

To elucidate the molecular mechanisms, we performed RNA sequencing (RNA-seq) using sorted LSCs. This analysis demonstrated that stabilization of NIK in LSCs significantly upregulated the expression of NF-κB non-canonical genes, *Nfκb2* and *Relb*, without significantly altering canonical pathway components (*Nfkb1*, *Rela*, and *Rel*), suggesting that NIK mainly activated non-canonical signaling in LSCs (Figure 2G). In addition, RNA-seq results provided additional potential mechanistic insights underlying NIK-associated AML suppression. For example, stabilization of NIK induced upregulation of *Gata2*, which suppresses MLL-AF9-induced AML (Danis et al., 2015). Furthermore, downregulation of *Bcl2*, *Flt3*, *Mef2c*, and *Cd93* promotes apoptosis, inhibits the proliferation of LSCs, and contributes to AML suppression (Canté-Barrett et al., 2014; Iwasaki et al., 2015) (Figures 2G and S3A). Consistent with these gene expression data, gene set enrichment analysis (GSEA) further revealed that NIK suppressed the expression of leukemia-maintaining genes controlled by the MLL-AF9 targets, *Hoxa9* and *Meis1* (Faber et al., 2009) (Figure 2H). This is likely due to non-canonical signaling directly regulating the expression of Hoxa9/Meis1 target genes in NIK-stabilized LSCs, given that the expression of *Hoxa9* and *Meis1* themselves was not significantly altered. In addition, stabilization of NIK upregulated Notch signaling, which has been show to inhibit MLL-AF9-induced AML (Lobry et al., 2013) (Figures S3B and S3C).

NIK Exerts Its Leukemic Inhibitory Role Mainly through Non-canonical Signaling

To further understand the underlying molecular mechanisms, we assessed the expression of NF-κB proteins. NIK protein

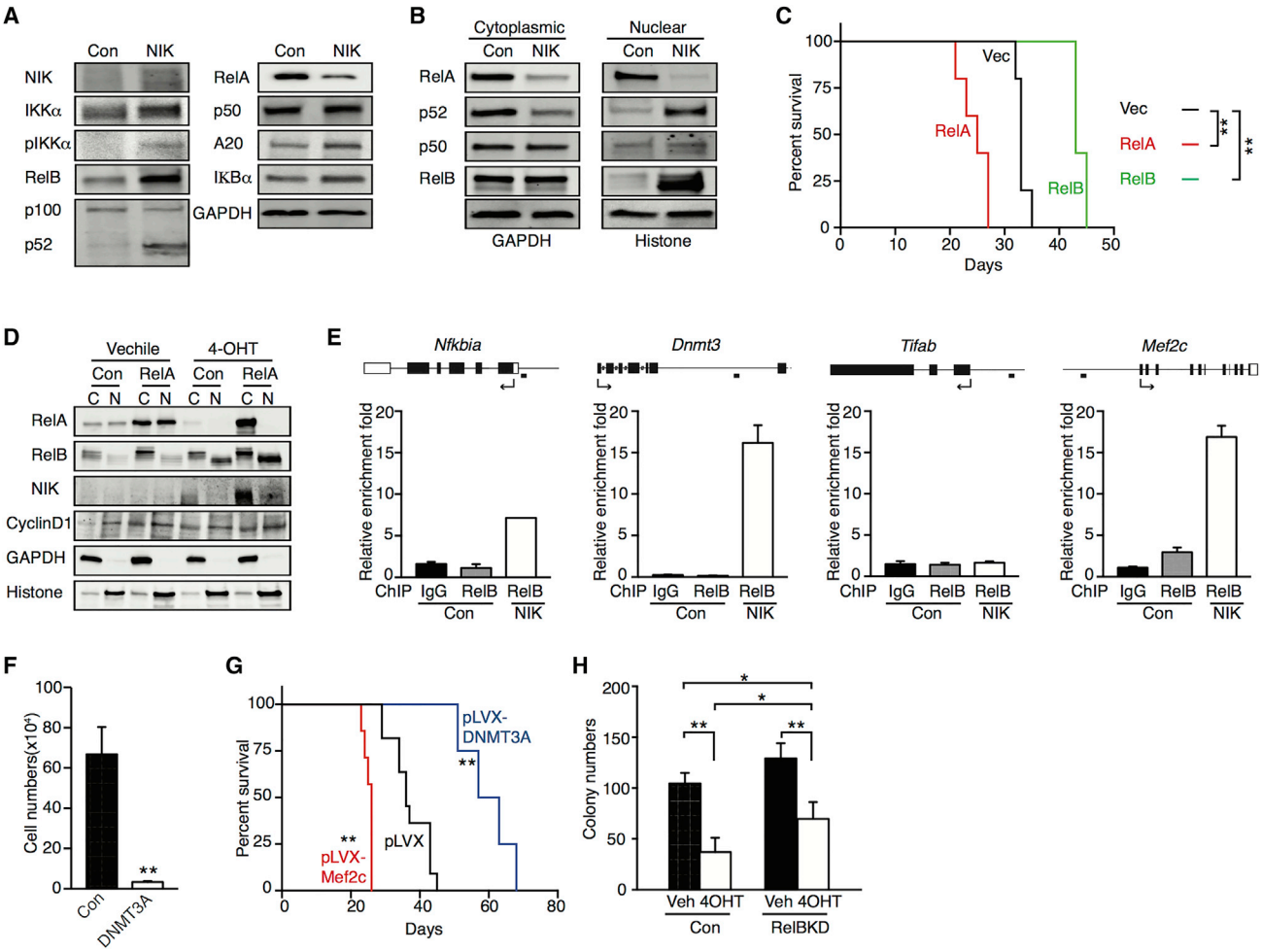

Figure 3. Stabilization of NIK Suppresses AML Mainly through Activation of NF-κB Non-canonical Signaling

(A and B) The expression and cellular localization of different NF-κB pathway components in control or tamoxifen-treated leukemia cells. Whole cellular proteins (A) or fractionated cytoplasmic or nuclear proteins (B) were used for immunoblotting. GAPDH was used as whole-cell and cytoplasmic extract loading control, and histone H3 was used as nuclear protein loading control.

(C) Survival curve of sublethally irradiated mice given RelA-, RelB-, or empty-vector-transduced MLL-AF9 leukemia cells (n = 5 each).

(D) The expression of NIK, RelA, RelB, and their target cyclin D1 in control and RelA-overexpressing leukemia cells in the presence or absence of 4-OHT.

(E) Sheared chromatin from control or tamoxifen-treated leukemia cells were precipitated with RelB or immunoglobulin G (IgG). RelB binding to the indicated regulatory regions were quantified by real-time PCR. Arrows mark transcriptional start site, and black boxes indicate putative NF-κB sites. The binding of *Nfkbia* is used as a positive control.

(F) Cell growth of vector (Con) or WT DNMT3A (DNMT3A)-transduced leukemic cells cultivated in RPMI media (n = 4).

(G) Survival curve of sublethally irradiated recipient mice given vector (pLVX)-, MEF2C- or WT DNMT3A (pLVX-MEF2C or DNMT3A)-transduced leukemic cells (ns = 10, 7, and 8, respectively).

(H) Number of colonies formed in methylcellulose using FACS-sorted pLKO-scramble-mCherry (Con) or pLKO-RelB-mCherry (RelBKD) knocked-down cells in the presence or absence of 4-OHT (n = 3 each). The expression of *Relb* is approximately 40% of controls by qPCR analysis.

Data are representative of at least three independent experiments. Values are mean ± SEM. *$p < 0.05$; **$p < 0.01$.

was ~2-fold increased in NIK-stabilized AML cells and was associated with increased IKKα phosphorylation, upregulation of RelB, and activation of p100 processing to p52 (Figure 3A). Consistent with activation of non-canonical signaling, nuclear localization of RelB and p52 was increased (Figure 3B). Interestingly, the expression and nuclear localization of RelA protein were markedly reduced (Figures 3A and 3B). The decrease in nuclear RelA was at least partially due to NIK-induced up-regulation of the canonical pathway inhibitors *Nfkbia*/IκBα and *Tnfaip3*/A20, which form a negative-feedback control of canonical NF-κB signaling (Figures 2G and 3A) (O'Dea and Hoffmann, 2010). These results are consistent with a previous report that NIK can antagonize canonical signaling in a context-dependent manner (Mao et al., 2016). It has been suggested that inhibition of canonical NF-κB signaling leads to robust anti-leukemia effects, phenocopying loss of functional MLL oncoprotein (Kuo et al., 2013). However, overexpression of RelA, confirmed by real-time PCR and western blot, failed to restore the reduced

colony formation and the delay in leukemogenesis caused by NIK stabilization (Figures S4A–S4D). To test whether activation of non-canonical signaling suppresses AML directly, we overexpressed RelB or RelA in MLL-AF9 leukemic cells. Strikingly, overexpression of RelB significantly repressed—while overexpression of RelA significantly accelerated—AML (Figure 3C). The latter is consistent with RelA overexpression, which accelerates AML with MLL mutations (Kuo et al., 2013). Consistent with activation of NIK-reduced RelA protein, overexpression of RelB also repressed RelA protein expression (Figure S4A). We further investigated the expression of NIK, RelA, RelB, and their target, cyclin D1, in control and RelA-overexpressing leukemia cells in the presence or absence of 4-OHT. The expression of cytoplasmic and nuclear RelA was increased in RelA-overexpressing cells, correlated with the expression of its target cyclin D1. However, when NIK was stabilized by 4-OHT, the nuclear translocation of RelA was blocked with only slightly decreased expression of cyclin D1 (Figure 3D), possibly due to increased expression of RelB (Demicco et al., 2005). Collectively, these data indicate that non-canonical and canonical signaling have opposite roles in AML.

Dnmt3a and *Mef2c* Are Direct Targets of RelB

The inactivating DNMT3A mutation enhances HSPC self-renewal and is a driver mutation in about 30% of AML (Yang et al., 2015). It has recently been shown that overexpression of DNMT3A inhibits AML (Lu et al., 2016). In addition, *Mef2c* is a known MLL-AF9 downstream target, and deletion of *Mef2c* impairs MLL-AF9-induced leukemogenesis (Canté-Barrett et al., 2014). To determine the direct downstream targets of non-canonical signaling in NIK-stabilized leukemic cells, we focused and tested *Dnmt3a*, *Mef2c*, and *Tifab* using chromatin immunoprecipitation. We found that RelB directly binds to Mef2c and one of the *Dnmt3a* promoters in front of exon 7, which functionally regulates the expression of an active DNMT3a variant (Chen et al., 2002), but not Tifab promoter (Figure 3E). Consistent with previous reports (Canté-Barrett et al., 2014; Lu et al., 2016), overexpression of MEF2C and DNMT3A significantly enhanced and suppressed AML development, respectively (Figures 3F and 3G). However, neither knockdown of *Dnmt3a* (Figure S4E) or overexpression of MEF2C (data not shown) in AML cells restored NIK-induced delayed leukemogenesis. In contrast, knockdown of *Relb* partially restored NIK's anti-leukemic effect, suggesting that upregulation of DNMT3a and downregulation of MEF2C, along with other RelB-regulated molecules, contribute to AML suppression (Figure 3H).

Identification of Verteporfin as a Potential Anti-AML Drug through the Connectivity Map

As NIK also impairs the self-renewal of normal HSPCs, a key challenge is the need to stabilize NIK, specifically in leukemia cells (Xiu et al., 2017). One potential, but currently unavailable, method would be to develop specific NIK activators/stabilizers and deliver them via LSC-specific surface receptors (e.g., Tim3 and CD93) (Iwasaki et al., 2015; Jan et al., 2011). We noted that NIK impaired the function of HSPCs and LSCs through different sets of genes. One prevailing concept for identifying therapeutic targets for the treatment of cancer is that a transcriptional program can be used to identify druggable targets (Ashton et al., 2012; Lamb et al., 2006; Li et al., 2017). The Connectivity Map (CMAP) database comprises a large reference catalog of gene expression profiles from cultured human cells stimulated with various chemicals. The database can be queried with a gene signature of interest to identify those compounds that induce desired gene expression changes. We queried CMAP using genes that are exclusively and significantly up- and downregulated in LSCs and identified verteporfin as a candidate for the treatment of AML (Figures 4A and 4B). Verteporfin is a Food and Drug Administration (FDA)-approved photosensitizer for eliminating abnormal blood vessels in macular degeneration by increasing cellular reactive oxygen species levels. It inhibits tumor growth in various models, including acute lymphoblastic leukemia, with minimal effects on normal hematopoiesis (Gibault et al., 2016; Morishita et al., 2016). We found that verteporfin dose-dependently suppressed leukemic cell growth in liquid culture and colony formation and delayed AML development *in vivo* (Figures 4B–4F). Verteporfin treatment transiently upregulated the expression of non-canonical NF-κB signaling components at the mRNA and protein levels (Figures 4G and 4H). Interestingly, verteporfin downregulated RelA protein in primary MLL-AF9 leukemia cells, mimicking the effects of NIK stabilization (Figures 3A and 4H). Importantly, the inhibitory effect of verteporfin was largely attenuated by knockdown of RelB and has no additive effects on NIK-stabilized AML cells (Figure 4I). Similarly, verteporfin had a moderate additive inhibitory effect on RelB-overexpressing cells only at a higher (0.5-μM) dose, not at a lower (0.2-μM) dose, and overexpression of RelA minimally antagonized low-dose verteporfin treatment (Figure 4J). Collectively, these results indicate that the effect of verteporfin is initially mainly through non-canonical signaling.

DISCUSSION

In this study, we have shown that stabilization of NIK suppresses myeloid leukemia through the activation of NF-κB non-canonical signaling and simultaneous repression of NF-κB canonical signaling. In addition, we provide evidence that NF-κB non-canonical signaling has an opposite role to that of NF-κB canonical signaling. Mechanistically, our data showed that the upregulation of DNMT3A and Notch signaling and downregulation of RelA and MEF2C are, at least in part, responsible for NIK-associated AML suppression. Other possibilities, for example, through RelB-independent, NIK/p52-dependent, non-canonical signaling in cooperation with other transcription factors, such as ETS1, may negatively control the genes maintaining AML development (Li and Tergaonkar, 2016; Li et al., 2015). As RUNX1 can interact with MLL1 and NF-κB at its N-terminal and C-terminal domains, respectively, the non-canonical signaling component may inhibit the function of MLL1 through interfering with the RUNX1-MLL1 complex (Koh et al., 2013). Future studies are required to determine the genes differentially regulated by RelA and RelB in AML and whether deletion of RelB will enhance AML development and block stabilization of NIK-induced impaired leukemogenesis using genetic RelB-deficient or NIK-stabilized/RelB-deficient compound mutant

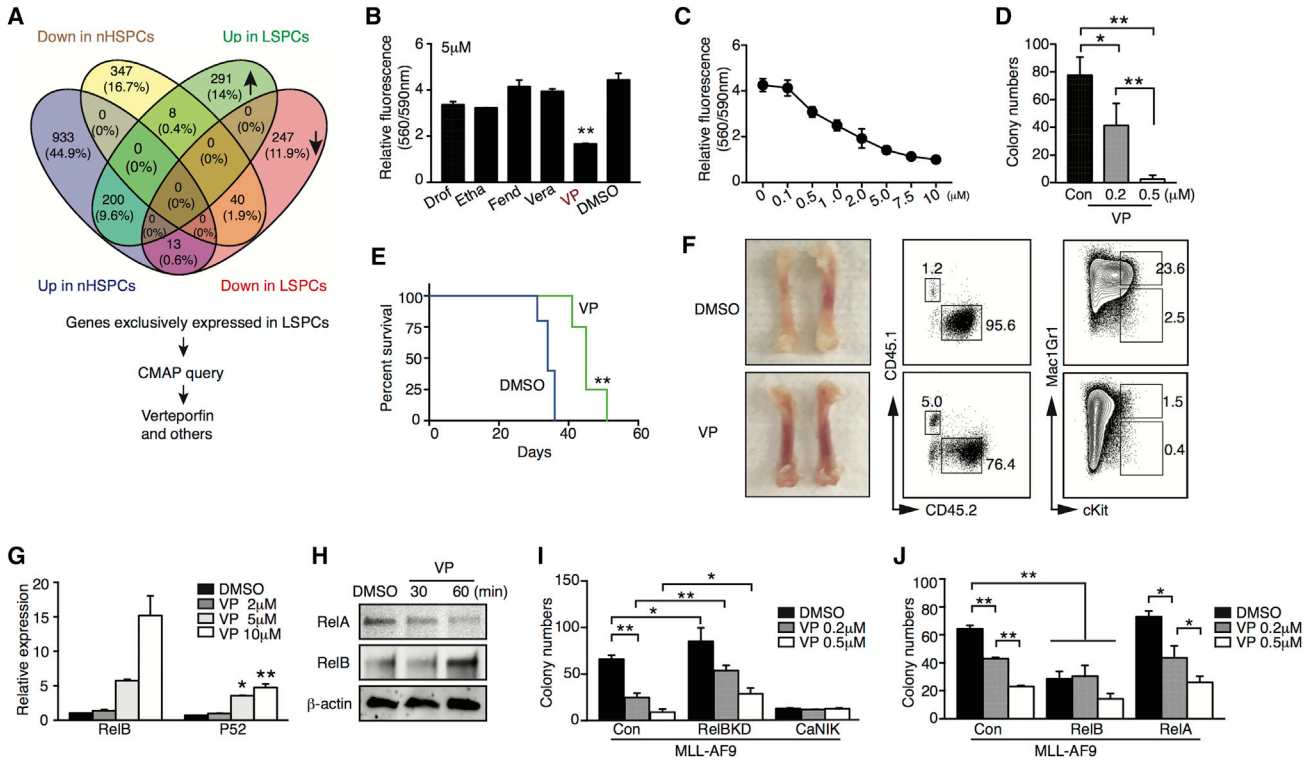

Figure 4. Verteporfin Represses AML Development by Mimicking Stabilization of NIK-Induced Gene Expression Changes

(A) Venn diagrams show the genes that were significantly up- and downregulated in HSPCs and LSCs. The genes that were exclusively and significantly up- and downregulated in LSCs were used to query the CMAP. nHSPC, normal hematopoietic stem/progenitor cell; LSPC, Leukemia stem/progenitor cell.

(B) Cell viability of MLL-AF9-transformed cells treated with different candidate chemicals. Drof, drofenine; Etha, ethaverin; Fend, fendiline; Vera, verapamil; Vert, verteporfin; VP, verteporfin; each at 5 μM.

(C) Cell viability of MLL-AF9-transformed cells treated with verteporfin at the indicated concentrations.

(D) Number of colonies formed in methylcellulose using primary AML cells in the presence or absence of verteporfin.

(E) Survival curve of sublethally irradiated mice given MLL-AF9-induced AML cells with or without verteporfin treatment (n = 5 each).

(F) Representative gross pictures and FACS profiles with or without VP treatment. Note that the VP-treated BMs were pinker, compared to DMSO-treated BMs (paler, indicating advanced disease). Also, the cKit$^+$ immature leukemia cells were substantially reduced.

(G) qPCR analysis of the expression of non-canonical (Relb/p52) pathway components in primary AML cells treated with verteporfin at the indicated concentrations.

(H) Western blots of whole-cell lysates extracted from verteporfin-untreated (DMSO) or -treated primary MLL-AF9 leukemia cells using indicated antibodies.

(I) Colony numbers of control knockdown (Con), RelB-knockdown (RelBKD), or NIK-stabilized (CaNIK) leukemic cells treated with different doses of VP (n = 4).

(J) Colony numbers of control, RelB-overexpressing (RelB), or RelA-overexpressing (RelA) leukemic cells treated with different doses of VP (n = 4).

Data are representative of at least three independent experiments in (B)–(D) and (G)–(J). Values are mean ± SEM. *p < 0.05; **p < 0.01.

mice. Nevertheless, the key finding of the present study is that AML is significantly suppressed by stabilization of NIK protein.

Although activating mutations in components of both canonical and non-canonical NF-κB signaling are frequently detected in B cell neoplasms, no gain- or loss-of-function mutations in canonical and non-canonical signaling have been reported in AML (Cildir et al., 2016; Gasparini et al., 2014). This is not unprecedented: β-catenin has been shown to be critical for chronic myeloid leukemia (CML) transformation and MLL-AF9-induced AML, although no mutations have been reported thus far (Jamieson et al., 2004; Wang et al., 2010). Even without mutations, the critical roles of canonical signaling and β-catenin in AML are undisputed. We have shown that either blocking or activating non-canonical signaling impairs HSPC self-renewal, and other research has shown that deletion of NIK also impairs HSPC function through non-canonical signaling (González-Murillo et al., 2015; Xiu et al., 2017; Zhao et al., 2012). AML is a stem cell disease; thus, the majority, if not all, of the genetic mutations or translocations that drive AML need to target stem cells. However, stem cells whose ability to self-renew is impaired are not "good targets" for leukemic transformation, and we speculate that this is why no loss-of-function mutations in non-canonical signaling components have been detected. The same reasoning can explain why no mutations of genes involved in canonical signaling have been discovered, as both activating and blocking canonical signaling also interfere with HSPC self-renewal (Nagamachi et al., 2014; Nakagawa et al., 2015; Stein and Baldwin, 2013). This is the opposite of what happens in mature B cell neoplasms, which are non-stem-cell diseases. Furthermore, we want to emphasize that NIK protein is constantly degraded, its expression in normal BM cells and AML blasts was very low to undetectable, and it even cannot be detected after WT NIK is overexpressed, generating a "null, naturally

mutated condition" even without mutations (Figures 3A, S2D, and S2E) (Liao et al., 2004; Sasaki et al., 2008). Our data also suggest that there are no activating mutations of NIK in AML, because of NIK's leukemia inhibitory effects (Figure S1A). Importantly, stabilized NIK suppressed not only the growth of MLL-AF9-induced AML but also BCR-ABL-induced CML, BCR−ABL+NUP98−HOXA9-induced blast crisis CML (an AML equivalent), and different subtypes of human myeloid leukemias (Figures S2A–S2C), indicating that NIK has broad anti-myeloid leukemic effects and implying the clinical significance of targeting NIK degradation pathways in myeloid leukemias. Lastly, the identification of a potential anti-AML drug, verteporfin, by querying CMAP suggests that repurposing verteporfin to target myeloid leukemia is worth further testing clinically.

In summary, we have identified an unexpected anti-myeloid leukemia role of NIK and provide a scientific rationale for developing drugs to stabilize NIK specifically in myeloid leukemia cells. Focusing on the continually degraded protein NIK will be an attractive therapeutic option to target a broad spectrum of myeloid leukemias.

EXPERIMENTAL PROCEDURES

Mice Experiments

The experiments were conducted under Institutional Animal Care and Use Committee (IACUC)-approved protocol 7061109. All the mouse strains are in a C57BL6 background and were used at 8–12 weeks old with a mix of male and female mice.

In Vivo Leukemogenesis Assays and Verteporfin Treatment

FACS-sorted lineage$^-$c-kit$^+$Sca-1$^+$ or lineage$^-$c-kit$^+$ BM cells from control, ROSA-CreER^{T2}, or NIKERT2 mice were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 ng/mL stem cell factor (SCF), 50 ng/mL thrombopoietin (TPO), 50 ng/mL FLT3 ligand, and 10 ng/mL IL-3 (all from Peprotech, Rocky Hill, NJ, USA) overnight. Then the cells were plated on virus-loaded retronectin-coated plates for 24 hr, spin-infected with viral supernatant supplemented with polybrene (4 mg/mL) at 1,000 × *g* for 90 min at room temperature, and transplanted retro-orbitally into lethally irradiated (950 cGy, single dose) CD45.1 recipients (45–100K per recipient) along with 2–3 × 10^5 rescue cells. Tamoxifen (5 mg per mouse per day; Sigma, St. Louis, MO, USA) was administered daily by oral gavage for 4 days at indicated time points post-BM transplantation. For secondary BM transplantations, bulk leukemia cells (300,000 per recipient) or sorted leukemia stem cells (500 per 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 100–140 mg/kg body weight by intraperitoneal injection 1 week after transplantation for 12 days and then observed daily for signs of morbidity.

Detailed information on mouse strains, human sample collection, HSPC isolation, LSC analysis, plasmid constructs, virus production, *in vitro* colony-forming assays, RNA-seq, western blotting, chromatin immunoprecipitation, and querying the CMAP are available in the Supplemental Experimental Procedures.

Statistical Analysis

Student's t test was used for all but survival curve statistical analyses, and significance was set at $p < 0.05$. Values are mean ± SEM. For the Kaplan-Meier survival curve, the log-rank test was used.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE97389.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.055.

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AUTHOR CONTRIBUTIONS

C.Z. and H.X. conceived and supervised the overall study. Y.X., Q.D., Q.L., and F.L. performed the experiments and analyzed the data. W.Z. provided reagents. N.B. and B.B. assisted with data analysis, interpretation, and writing. H.X. and C.Z. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Ashton, J.M., Balys, M., Neering, S.J., Hassane, D.C., Cowley, G., Root, D.E., Miller, P.G., Ebert, B.L., McMurray, H.R., Land, H., and Jordan, C.T. (2012). Gene sets identified with oncogene cooperativity analysis regulate in vivo growth and survival of leukemia stem cells. Cell Stem Cell *11*, 359–372.

Bosman, M.C., Schuringa, J.J., and Vellenga, E. (2016). Constitutive NF-κB activation in AML: causes and treatment strategies. Crit. Rev. Oncol. Hematol. *98*, 35–44.

Canté-Barrett, K., Pieters, R., and Meijerink, J.P. (2014). Myocyte enhancer factor 2C in hematopoiesis and leukemia. Oncogene *33*, 403–410.

Chen, T., Ueda, Y., Xie, S., and Li, E. (2002). A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J. Biol. Chem. *277*, 38746–38754.

Cildir, G., Low, K.C., and Tergaonkar, V. (2016). Noncanonical NF-κB signaling in health and disease. Trends Mol. Med. *22*, 414–429.

Cilloni, D., Martinelli, G., Messa, F., Baccarani, M., and Saglio, G. (2007). Nuclear factor kB as a target for new drug development in myeloid malignancies. Haematologica *92*, 1224–1229.

Danis, E., Yamauchi, T., Echanique, K., Haladyna, J., Kalkur, R., Riedel, S., Zhu, N., Xie, H., Bernt, K.M., Orkin, S.H., et al. (2015). Inactivation of Eed impedes MLL-AF9-mediated leukemogenesis through Cdkn2a-dependent and Cdkn2a-independent mechanisms in a murine model. Exp. Hematol. *43*, 930–935.e6.

Demicco, E.G., Kavanagh, K.T., Romieu-Mourez, R., Wang, X., Shin, S.R., Landesman-Bollag, E., Seldin, D.C., and Sonenshein, G.E. (2005). RelB/p52 NF-kappaB complexes rescue an early delay in mammary gland development in transgenic mice with targeted superrepressor IkappaB-alpha expression and promote carcinogenesis of the mammary gland. Mol. Cell. Biol. *25*, 10136–10147.

Dombret, H., and Gardin, C. (2016). An update of current treatments for adult acute myeloid leukemia. Blood *127*, 53–61.

Faber, J., Krivtsov, A.V., Stubbs, M.C., Wright, R., Davis, T.N., van den Heuvel-Eibrink, M., Zwaan, C.M., Kung, A.L., and Armstrong, S.A. (2009). HOXA9 is required for survival in human MLL-rearranged acute leukemias. Blood *113*, 2375–2385.

Gasparini, C., Celeghini, C., Monasta, L., and Zauli, G. (2014). NF-kappaB pathways in hematological malignancies. Cell. Mol. Life Sci. *71*, 2083–2102.

Ghosh, S., and Hayden, M.S. (2008). New regulators of NF-kappaB in inflammation. Nat. Rev. Immunol. *8*, 837–848.

Gibault, F., Corvaisier, M., Bailly, F., Huet, G., Melnyk, P., and Cotelle, P. (2016). Non-photoinduced biological properties of verteporfin. Curr. Med. Chem. *23*, 1171–1184.

González-Murillo, Á., Fernández, L., Baena, S., Melen, G.J., Sánchez, R., Sánchez-Valdepeñas, C., Segovia, J.C., Liou, H.C., Schmid, R., Madero, L., et al. (2015). The NFKB inducing kinase modulates hematopoiesis during stress. Stem Cells *33*, 2825–2837.

Guzman, M.L., Rossi, R.M., Neelakantan, S., Li, X., Corbett, C.A., Hassane, D.C., Becker, M.W., Bennett, J.M., Sullivan, E., Lachowicz, J.L., et al. (2007). An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells. Blood *110*, 4427–4435.

Hsieh, M.Y., and Van Etten, R.A. (2014). IKK-dependent activation of NF-κB contributes to myeloid and lymphoid leukemogenesis by BCR-ABL1. Blood *123*, 2401–2411.

Iwasaki, M., Liedtke, M., Gentles, A.J., and Cleary, M.L. (2015). CD93 marks a non-quiescent human leukemia stem cell population and is required for development of MLL-rearranged acute myeloid leukemia. Cell Stem Cell *17*, 412–421.

Jamieson, C.H., Ailles, L.E., Dylla, S.J., Muijtjens, M., Jones, C., Zehnder, J.L., Gotlib, J., Li, K., Manz, M.G., Keating, A., et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N. Engl. J. Med. *351*, 657–667.

Jan, M., Chao, M.P., Cha, A.C., Alizadeh, A.A., Gentles, A.J., Weissman, I.L., and Majeti, R. (2011). Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. Proc. Natl. Acad. Sci. USA *108*, 5009–5014.

Koh, C.P., Wang, C.Q., Ng, C.E., Ito, Y., Araki, M., Tergaonkar, V., Huang, G., and Osato, M. (2013). RUNX1 meets MLL: epigenetic regulation of hematopoiesis by two leukemia genes. Leukemia *27*, 1793–1802.

Kreso, A., and Dick, J.E. (2014). Evolution of the cancer stem cell model. Cell Stem Cell *14*, 275–291.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature *442*, 818–822.

Kuo, H.P., Wang, Z., Lee, D.F., Iwasaki, M., Duque-Afonso, J., Wong, S.H., Lin, C.H., Figueroa, M.E., Su, J., Lemischka, I.R., and Cleary, M.L. (2013). Epigenetic roles of MLL oncoproteins are dependent on NF-κB. Cancer Cell *24*, 423–437.

Lamb, J., Crawford, E.D., Peck, D., Modell, J.W., Blat, I.C., Wrobel, M.J., Lerner, J., Brunet, J.P., Subramanian, A., Ross, K.N., et al. (2006). The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science *313*, 1929–1935.

Li, Y., and Tergaonkar, V. (2016). Telomerase reactivation in cancers: Mechanisms that govern transcriptional activation of the wild-type vs. mutant TERT promoters. Transcription *7*, 44–49.

Li, Y., Zhou, Q.L., Sun, W., Chandrasekharan, P., Cheng, H.S., Ying, Z., Lakshmanan, M., Raju, A., Tenen, D.G., Cheng, S.Y., et al. (2015). Non-canonical NF-κB signalling and ETS1/2 cooperatively drive C250T mutant TERT promoter activation. Nat. Cell Biol. *17*, 1327–1338.

Li, F., He, B., Ma, X., Yu, S., Bhave, R.R., Lentz, S.R., Tan, K., Guzman, M.L., Zhao, C., and Xue, H.H. (2017). Prostaglandin E1 and its analog misoprostol inhibit human CML stem cell self-renewal via EP4 receptor activation and repression of AP-1. Cell Stem Cell *21*, 359–373.e5.

Liao, G., Zhang, M., Harhaj, E.W., and Sun, S.C. (2004). Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. J. Biol. Chem. *279*, 26243–26250.

Lobry, C., Ntziachristos, P., Ndiaye-Lobry, D., Oh, P., Cimmino, L., Zhu, N., Araldi, E., Hu, W., Freund, J., Abdel-Wahab, O., et al. (2013). Notch pathway activation targets AML-initiating cell homeostasis and differentiation. J. Exp. Med. *210*, 301–319.

Lu, L.F., Gondek, D.C., Scott, Z.A., and Noelle, R.J. (2005). NF kappa B-inducing kinase deficiency results in the development of a subset of regulatory T cells, which shows a hyperproliferative activity upon glucocorticoid-induced TNF receptor family-related gene stimulation. J. Immunol. *175*, 1651–1657.

Lu, R., Wang, P., Parton, T., Zhou, Y., Chrysovergis, K., Rockowitz, S., Chen, W.Y., Abdel-Wahab, O., Wade, P.A., Zheng, D., and Wang, G.G. (2016). Epigenetic perturbations by Arg882-mutated DNMT3A potentiate aberrant stem cell gene-expression program and acute leukemia development. Cancer Cell *30*, 92–107.

Mao, X., Phanavanh, B., Hamdan, H., Moerman-Herzog, A.M., and Barger, S.W. (2016). NFκB-inducing kinase inhibits NFκB activity specifically in neurons of the CNS. J. Neurochem. *137*, 154–163.

Morishita, T., Hayakawa, F., Sugimoto, K., Iwase, M., Yamamoto, H., Hirano, D., Kojima, Y., Imoto, N., Naoe, T., and Kiyoi, H. (2016). The photosensitizer verteporfin has light-independent anti-leukemic activity for Ph-positive acute lymphoblastic leukemia and synergistically works with dasatinib. Oncotarget *7*, 56241–56252.

Muro, I., Fang, G., Gardella, K.A., Mahajan, I.M., and Wright, C.W. (2014). The TRAF3 adaptor protein drives proliferation of anaplastic large cell lymphoma cells by regulating multiple signaling pathways. Cell Cycle *13*, 1918–1927.

Nagamachi, A., Nakata, Y., Ueda, T., Yamasaki, N., Ebihara, Y., Tsuji, K., Honda, Z., Takubo, K., Suda, T., Oda, H., et al. (2014). Acquired deficiency of A20 results in rapid apoptosis, systemic inflammation, and abnormal hematopoietic stem cell function. PLoS ONE *9*, e87425.

Nakagawa, M.M., Thummar, K., Mandelbaum, J., Pasqualucci, L., and Rathinam, C.V. (2015). Lack of the ubiquitin-editing enzyme A20 results in loss of hematopoietic stem cell quiescence. J. Exp. Med. *212*, 203–216.

O'Dea, E., and Hoffmann, A. (2010). The regulatory logic of the NF-kappaB signaling system. Cold Spring Harb. Perspect. Biol. *2*, a000216.

Olsnes, A.M., Ersvaer, E., Ryningen, A., Paulsen, K., Hampson, P., Lord, J.M., Gjertsen, B.T., Kristoffersen, E.K., and Bruserud, Ø. (2009). The protein kinase C agonist PEP005 increases NF-kappaB expression, induces differentiation and increases constitutive chemokine release by primary acute myeloid leukaemia cells. Br. J. Haematol. *145*, 761–774.

Pollyea, D.A., and Jordan, C.T. (2017). Therapeutic targeting of acute myeloid leukemia stem cells. Blood *129*, 1627–1635.

Sasaki, Y., Calado, D.P., Derudder, E., Zhang, B., Shimizu, Y., Mackay, F., Nishikawa, S., Rajewsky, K., and Schmidt-Supprian, M. (2008). NIK overexpression amplifies, whereas ablation of its TRAF3-binding domain replaces BAFF:BAFF-R-mediated survival signals in B cells. Proc. Natl. Acad. Sci. USA *105*, 10883–10888.

Shanmugam, R., Gade, P., Wilson-Weekes, A., Sayar, H., Suvannasankha, A., Goswami, C., Li, L., Gupta, S., Cardoso, A.A., Baghdadi, T.A., et al. (2012). A noncanonical Flt3ITD/NF-κB signaling pathway represses DAPK1 in acute myeloid leukemia. Clin. Cancer Res. *18*, 360–369.

Stein, S.J., and Baldwin, A.S. (2013). Deletion of the NF-κB subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function. Blood *121*, 5015–5024.

Sun, S.C. (2012). The noncanonical NF-κB pathway. Immunol. Rev. *246*, 125–140.

Vallabhapurapu, S., and Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annu. Rev. Immunol. *27*, 693–733.

Wang, Y., Krivtsov, A.V., Sinha, A.U., North, T.E., Goessling, W., Feng, Z., Zon, L.I., and Armstrong, S.A. (2010). The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science *327*, 1650–1653.

Xiu, Y., Xue, W.Y., Lambertz, A., Leidinger, M., Gibson-Corley, K., and Zhao, C. (2017). Constitutive Activation of NIK impairs the self-renewal of hematopoietic stem/progenitor cells and induces bone marrow failure. Stem Cells *35*, 777–786.

Yang, L., Rau, R., and Goodell, M.A. (2015). DNMT3A in haematological malignancies. Nat. Rev. Cancer *15*, 152–165.

Zhao, C., Xiu, Y., Ashton, J., Xing, L., Morita, Y., Jordan, C.T., and Boyce, B.F. (2012). Noncanonical NF-κB signaling regulates hematopoietic stem cell self-renewal and microenvironment interactions. Stem Cells *30*, 709–718.