

NFKB1 Is a Direct Target of the TAL1 Oncoprotein in Human T Leukemia Cells

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Abstract

We recently showed that a subset of human T acute lymphoblastic leukemia (T-ALL) cell lines expresses low basal levels of p50, a nuclear factor- κ B (NF- κ B)/Rel family member, resulting in their capacity to activate the atypical p65:cRel complex rather than the classic p50:p65 dimer. Here, we show that the transcription factor TAL1 (also known as SCL) binds to the promoter of the NFKB1 gene that encodes p50 and represses its transcription to set up this unique response in T-ALL cells. When TAL1 expression is reduced in CEM T leukemia cells, basal NFKB1 expression is increased, and the levels of p65:cRel complex and transcription of its target gene, such as intercellular adhesion molecule-1 (ICAM-1), are reduced in response to etoposide treatment. Moreover, a significant negative correlation between NFKB1 and TAL1 or LMO1 was found in primary human TAL1/LMO1 double-positive T-ALL samples previously described by Ferrando et al. Thus, TAL1 modulates NFKB1 expression and an NF- κ B-dependent transcriptional program in a subset of human T-cell leukemia cells. (Cancer Res 2006; 66(12): 6008-13)

Introduction

T-cell acute lymphocytic leukemia 1 (TAL1, also known as the stem cell factor SCL) is a member of the basic helix-loop-helix family of transcription factors and is normally involved in regulation of hematopoiesis (1). In the T-cell lineage, TAL1 is active only at early thymocyte stages and regulates expression of pre-T α , Rag2, and cyclin D1 (2–4). Aberrant expression of TAL1 in later stages of T-cell development is associated with T-ALL (5). We have recently described that certain T-ALL cell lines express low basal levels of p50, a member of the nuclear factor- κ B (NF- κ B)/Rel family of transcription factors (6). This is associated with the formation of an atypical p65:cRel heterodimer, instead of the canonical p50:p65 heterodimer, and activation of p65:cRel target genes, such as the intercellular adhesion molecule-1 (ICAM-1) gene (7). When these cells are exposed to repetitive NF- κ B activation stimuli, an augmented NF- κ B response can be observed, leading to an enhanced cell survival response against an anticancer agent etoposide (6). Below, we describe evidence that NFKB1, which encodes the p50 protein, is a novel target of TAL1 to set up this unique response in T-ALL cells.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Antibodies and chemicals. Immunoglobulin G (IgG) antibodies against actin (C-11), p65 (C-20), and RelB (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cRel antibody (SA-172) was obtained from Biomol (Plymouth Meeting, PA). Anti-p52 (06-413) and anti-p50 (06-886) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated protein A and horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Etoposide (VP16) was purchased from Sigma-Aldrich (St. Louis, MO).

General protocols. Electrophoretic mobility shift assays (EMSA); including the Ig κ - κ B and p21- κ B probe oligonucleotide probe) were done as previously described (6). The ICAM1- κ B probe is 5'-TTAGCTTG-GAAATCCCGAGCGAGAGGCCAT-3'. Immunoprecipitation and Western blotting were done as described previously (6). All experiments were repeated at least thrice unless otherwise specified, and the results were quantified by exposing dried EMSA gels on a phosphorimager screen and analyzed by the IQMac1 program.

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation solutions were purchased from Upstate Cell Signaling (Charlottesville, VA), and the assays were done according to the manufacturer's protocol with the minor modifications (as described in ref. 6). In all chromatin immunoprecipitation experiments, quantitative real-time data are presented by setting the untreated serum precipitated samples as unity. The average and SDs were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software. Forward-NFKB1-promoter primer 5'-GAATTCATGGATGGCAGAAGATGATCCATAT-3' and reverse-NFKB1-promoter primer 5'-GAATTCCTAGCTCATCAATGCTTCATCCC-3'.

Quantitative reverse transcription-PCR analysis. Total RNA from various cell types was extracted with the Qiagen RNeasy kit. cDNA was synthesized by as previously described (6). Quantitative real-time reverse transcription-PCR (RT-PCR) reactions (25 μ L) contained 2 μ L of cDNA, 12.5 μ L of SYBR Green (Applied Biosystems, Foster City, CA), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence with ABI Prism 7000 Sequence Detection Systems. The relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Forward and reverse primers for real-time RT-PCR are previously described (5, 6). The average and SDs were calculated by the Microsoft Excel program and plotted by the Kaleida-Graph software.

Generation of stable AS-TAL1 CEM cells. The human TAL1 cDNA was cloned in the antisense orientation by digesting the plasmid pMSCL (8) with *Eco*RI and religation into the pcDNA3 vector. The *Eco*RI sites come from the plasmid polylinker. The integrity of the expression vector was confirmed by direct sequencing. These constructs were then electroporated into CEM cells and selected with G418 (1 mg/mL).

Isolation of thymocytes and thymomas. Generation of transgenic animals were described previously (9). Tumors were isolated from leukemic transgenic mice and converted into cell culture. Thymi were isolated from 4-week-old mice. For protein extraction, cells were lysed in NP40 lysis buffer [150 mmol/L NaCl, 1% NP40, 50 mmol/L Tris (pH 8)]. Whole RNA extracts were prepared using TRIzol reagent (Invitrogen, San Diego, CA).

Statistical analysis. The statistical analysis was done by Graph Pad Prism program (*t* test, polynomial, or logarithmic regression) and plotted with Microsoft Excel program.

Results

When different human T-ALL cell lines were exposed to a certain stimulus, such as VP16, and activation of NF- κ B was analyzed by EMSA, different DNA-binding complexes (denoted complexes I and II) were observed. Complex I was supershifted by antibodies against p65 and p50, whereas complex II was supershifted by anti-p65 and anti-cRel antibodies but not by anti-p50 antibody (Fig. 1B; others not shown). Antibodies against p52 and RelB had little or no effect on both of these complexes (data not shown). Thus, complex I seems to be composed primarily of the classic p50:p65 NF- κ B heterodimer, whereas complex II is primarily a p65:cRel heterodimer.

We recently showed that low basal expression of p50 correlated with the reduced capacity of T-ALL cell lines to activate p50:p65 complex (6). Figure 1C shows that whereas the expression of cRel and p65 was variable between different T-cell lines, a consistent reduction of p50 expression was seen in MOLT-4 and RPMI8402 cells that display reduced p50:p65 binding. Although p50 protein levels seemed similar in CEM and ALL-SIL cells, p65 and cRel levels were higher in CEM cells. These results, combined with our previous finding (6), suggested that lower p50 expression levels relative to p65 and cRel contributed to reduced formation of the classic p50:p65 complexes in certain T-ALL cell lines.

Low basal expression of p105/p50 has also been observed in diffuse large B-cell lymphoma cells (10), and the proto-oncoprotein BCL-6 is implicated in direct transcriptional repression of the NFKB1 gene in this tumor type (11). However, BCL-6 is not expressed in T cells (12).³ Thus, we examined a potential relationship between expression levels of NFKB1 and oncogenes implicated in T leukemia (5). Among the genes, the expression of TAL1 was generally associated with low NFKB1 expression, except for MOLT-16 and PF382 (Fig. 1D; Supplementary Fig. S1). TAL1 was undetectable in MOLT-16 cells by Western blot analysis, whereas it was readily detectable in PF382 cells (data not shown).

TAL1 can mediate both transcriptional activation and repression (3). To determine if TAL1 was involved in reduced expression of the NFKB1 gene in T-ALL cells, we generated CEM cells that stably expressed an antisense-TAL1 construct (AS-TAL1 cells) with markedly reduced TAL1 expression (Fig. 2A). TAL1 reduction was associated with an increase in basal NFKB1 gene and p105/p50 protein expression (Fig. 2A; others not shown). TAL1 can associate with E box binding proteins, such as transcription factor E2A products (E12/E47) and HEB, and represses expression of genes regulated by these transcription activators (3). The NFKB1 promoter contains κ B and ETS sites (Fig. 2B), which are regulated by NF- κ B (p65) and ELF1 upon exposure of CEM cells to a phorbol ester (6). An E box (CAGTGG) is also present in this promoter sequence. We did a supershift analysis with antibodies against TAL1 using the NFKB1 promoter probe and total extracts isolated from unstimulated CEM cells. The protein complex formed on the NFKB1 promoter probe could be supershifted with anti-TAL1 antibody (Fig. 2C, lane 4). An antibody against HEB (lane 3) but not E12/E47 (lane 2) reduced the binding of the complex, suggesting that HEB

is also associating with the E-box along with TAL1. Both probes containing E-box mutations failed to form the protein complex (lanes 6 and 7). These results suggested that TAL1 and HEB could assemble on the NFKB1 promoter sequence in the E-box-dependent manner.

To determine whether TAL1 could directly associate with the NFKB1 promoter *in vivo*, chromatin immunoprecipitation was done. This analysis showed that 7-fold more NFKB1 promoter DNA was recovered by anti-TAL1 antibody when compared with the IgG control in CEM cells (Fig. 2D). Further specificity of the chromatin immunoprecipitation analysis was shown by the reduction of the NFKB1 promoter DNA recovery from AS-TAL1 cells under the similar conditions. Moreover, chromatin immunoprecipitation analysis also showed that histone deacetylase 1 (HDAC1), which is associated with transcriptional repression by TAL1 (13), was present on the NFKB1 promoter (Fig. 2D). HDAC1 recruitment depended on the presence of TAL1 because reduction of TAL1 also rendered reduction of the HDAC1 occupancy (Fig. 2D). Thus, TAL1 could bind to the NFKB1 gene promoter along with HDAC1, which was associated with basal repression of NFKB1 expression in CEM T leukemia cells.

To probe a functional significance of TAL1-mediated NFKB1 repression in T-ALL cells, we stimulated CEM and AS-TAL1 cells with VP16 and analyzed NF- κ B complexes formed on distinct κ B elements. Using the classic Ig κ - κ B probe, increased p50:p65 and reduced p65:cRel binding could be observed in AS-TAL1 cell extracts when compared with CEM cell extracts (Fig. 3A). When we examined the κ B element that selectively binds to a p65:cRel heterodimer in the ICAM-1 promoter (7), diminished binding of complex II could be seen in AS-TAL1 cell extracts (Fig. 3A, lane 8). In contrast, increased complex I binding was observed with the p21^{cip1/waf1}- κ B probe in AS-TAL1 cells (Fig. 3A, lane 12). When we analyzed VP16-inducible expression of ICAM-1 and p21^{cip1/waf1} in CEM and AS-TAL1 cells, we found that inducible ICAM-1 expression was reduced in AS-TAL1 cells (Fig. 3B; $P < 0.05$). However, there was no significant reduction of VP16-inducible p21^{cip1/waf1} expression in these cells. Together, these results suggest that repression of NFKB1 expression by TAL1 promotes not only the activation of a p65:cRel heterodimer but also the expression of target genes selectively regulated by this atypical NF- κ B complex.

To evaluate the relationship between TAL1 and NFKB1 expression in primary T-ALL samples, we obtained microarray and quantitative RT-PCR data sets from Ferrando et al. (5). Of these patient samples, 39 had both NFKB1 (microarray) and TAL1 (both microarray and quantitative RT-PCR) expression data. A linear or polynomial regression analysis displayed no significant correlation between their expression levels of NFKB1 expression and TAL1 expression (RT-PCR data; $R^2 = 0.1$; Supplementary Fig. S2A). TAL1 mediates transcriptional modulation through collaborating factors, such as LMO1 and LMO2 (14). Although samples harboring LMO2 expression showed no correlation between TAL1 and NFKB1 expression ($R^2 = 0.1$; data not shown), those with LMO1 expression showed an inverse trend ($R^2 = 0.48$; Supplementary Fig. S2B). When we eliminated three samples that had very low TAL1 and high LYL1 expression, which were previously categorized as LYL1⁺ T-ALL by Ferrando et al. (5), the reverse correlation became much more apparent ($R^2 = 0.96$; Fig. 4A). A reverse correlation ($R^2 = 0.99$) was also observed between LMO1 and NFKB1 expression in these samples (Fig. 4B).

³ P.-Y. Chang, unpublished observations.

The above analysis suggested the possibility that both TAL1 and LMO1 could be involved in repression of NFKB1 gene expression. All T-ALL cell lines expressed varying levels of LMO1 (Supplementary Fig. S1). To test whether reduction in LMO1 expression affects

NFKB1 expression, we introduced small interfering RNA (siRNA) against LMO1 in CEM cells. Although knockdown of LMO1 expression alone had a minimal effect, it augmented NFKB1 expression induced by TAL1 knockdown (Fig. 4C). siRNA designed

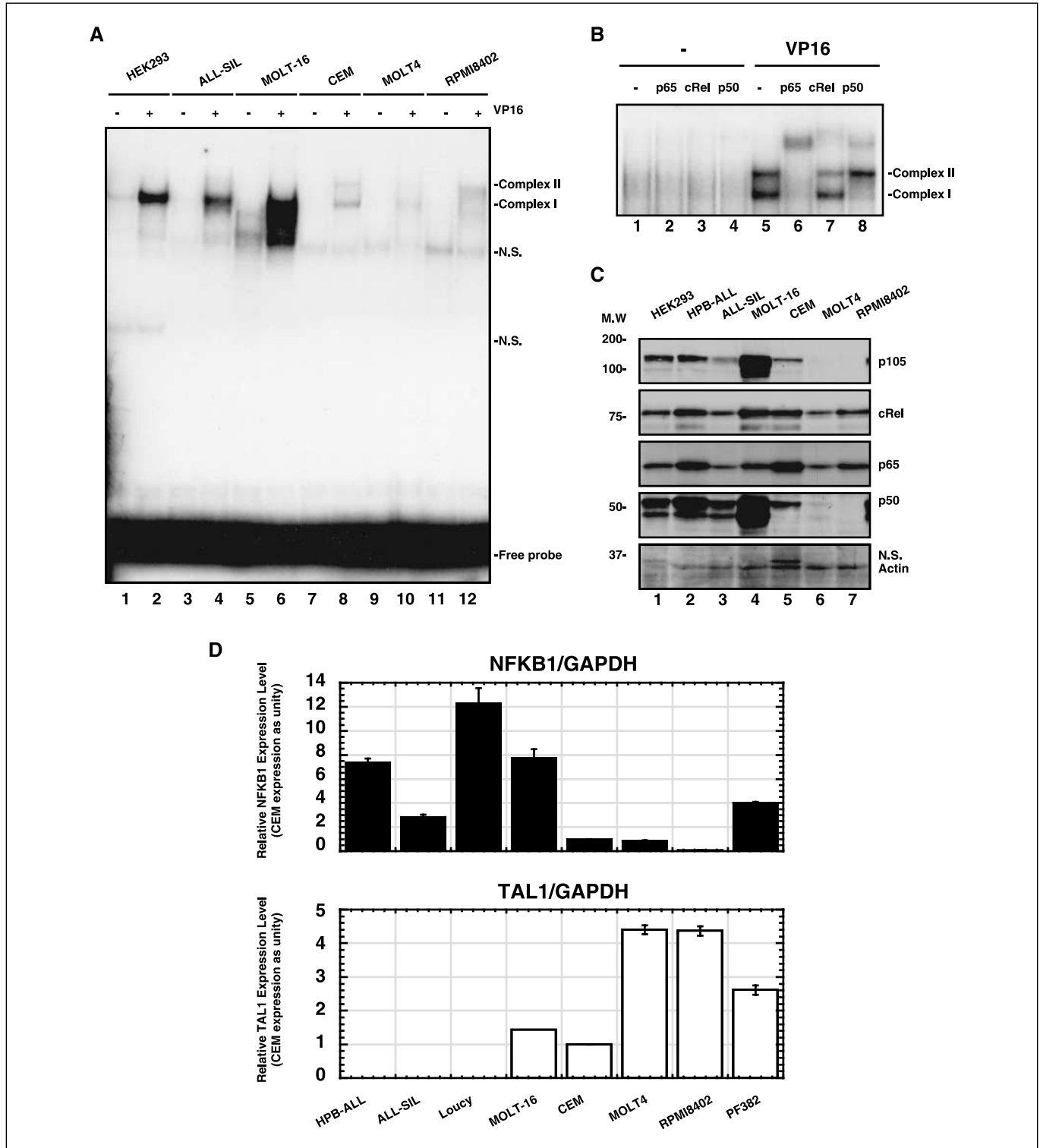


Figure 1. Reduced basal NFKB1 expression and p105/p50 synthesis correlates with the presence of TAL1. *A*, cells were either left untreated or treated with VP16 (10 μ mol/L) for 3 hours. Total protein extracts were used for EMSA with an I κ B site. *B*, extracts from CEM cells were used for supershift analyses with anti-p65, anti-p50, and anti-cRel antibodies as indicated. *C*, untreated cellular extracts were used for Western blot analysis using anti-p105/p50, anti-cRel, anti-p65 and anti-actin antibodies. *D*, total RNA was extracted from T-ALL cell lines and analyzed by quantitative RT-PCR for the expression of NFKB1 and TAL1 using GAPDH as a normalization control.

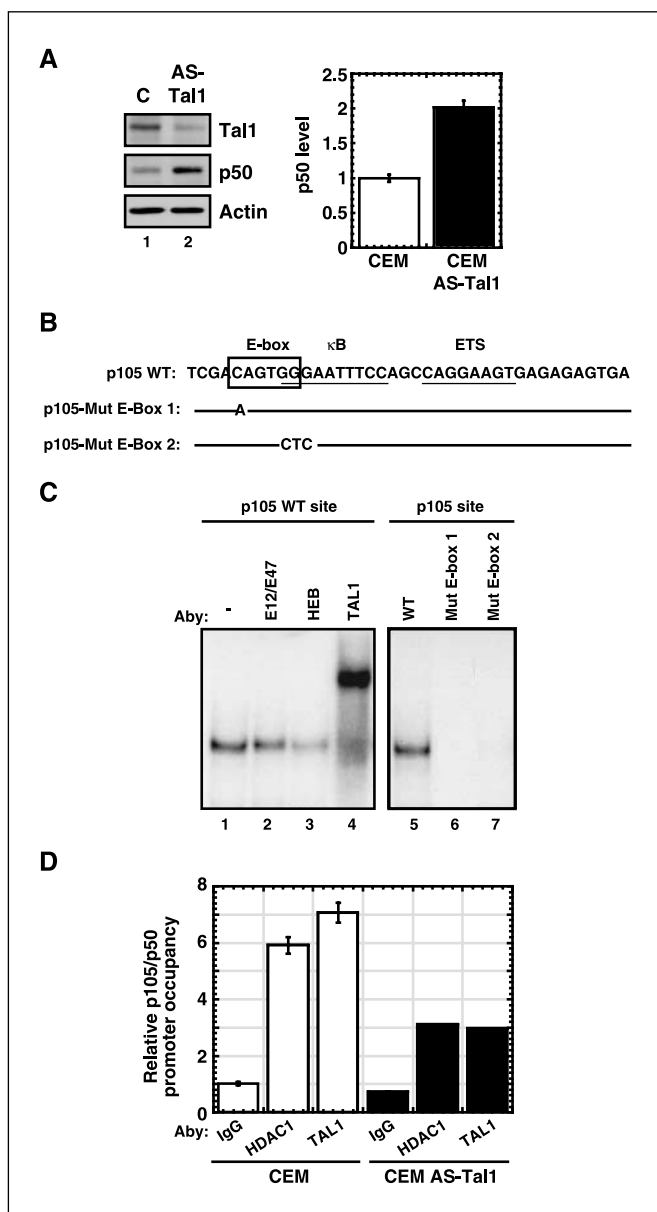


Figure 2. TAL1 associates with the NFKB1 promoter. *A*, expression of p50 and TAL1 in CEM and CEM cells stably expressing an AS-TAL1 construct was analyzed by Western blotting. *B*, sequences used for EMSA in (*C*). *C*, left, extracts from CEM cells were used for supershift analyses using the p105- κ B element with anti-E12/E47, HEB, and TAL1 antibodies as indicated. Right, extracts from CEM cells were used for EMSA analyses using either the wild-type p105 promoter element or ones with E box mutations. *D*, chromatin immunoprecipitation analysis was done using anti-TAL1 and anti-HDAC1 antibodies in parental CEM and AS-TAL1 cells. Results from two independent experiments were plotted.

for TAL1 resulted in reduced LMO1 expression (Supplementary Fig. S3B). Although an off-target effect of the TAL1 siRNA cannot be ruled out, it is possible that the expression of LMO1 may be regulated by TAL1 as a strong positive correlation between TAL1 and LMO1 expression levels was observed between TAL1 and LMO1 in primary patient T-ALL samples (Supplementary Fig. S3A). A similar trend was observed in RPMI8402 cells (data not shown). These results suggested that LMO1 participates in TAL1-dependent NFKB1 gene repression in certain human T-ALL cells.

Discussion

Human T-cell leukemogenesis is often associated with the aberrant expression of TAL1 (3, 5). However, the repertoire of TAL1 direct target genes in T-ALL cells is not well understood. Our current study identified NFKB1 as a novel TAL1-repressed gene. This repression is likely mediated via the E-box present on the NFKB1 promoter and may involve HEB, LMO1, and HDAC1. Furthermore, we found a negative correlation between expression levels of NFKB1 and TAL1 or LMO1 in TAL1⁺/LMO1⁺ primary

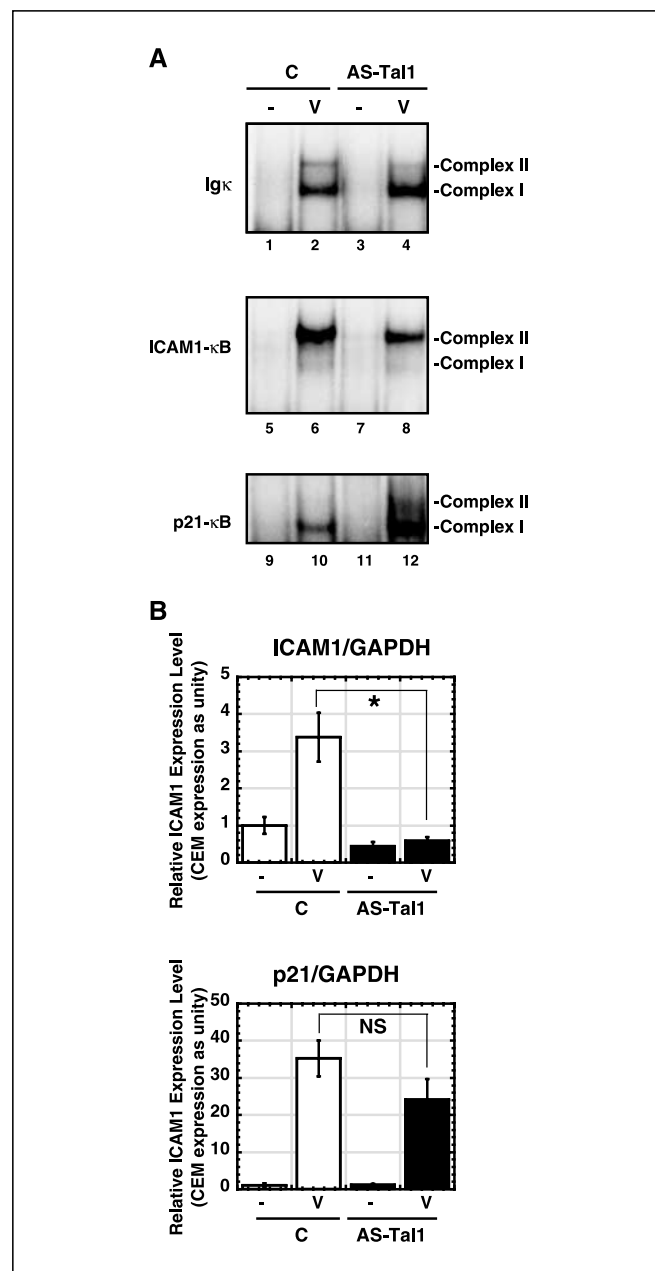


Figure 3. TAL1-mediated NFKB1 repression is associated with enhanced p65:cRel activation. *A*, CEM parental and AS-TAL1 cells were left untreated or treated with VP16 (3 hours) and analyzed by EMSA with the classical Ig κ - κ B, ICAM1- κ B or p21- κ B element. *B*, CEM parental and AS-TAL1 cells were left untreated or treated with VP16 (6 hours), and ICAM1 and p21 expression were analyzed by quantitative real-time PCR. GAPDH expression was analyzed as the control.

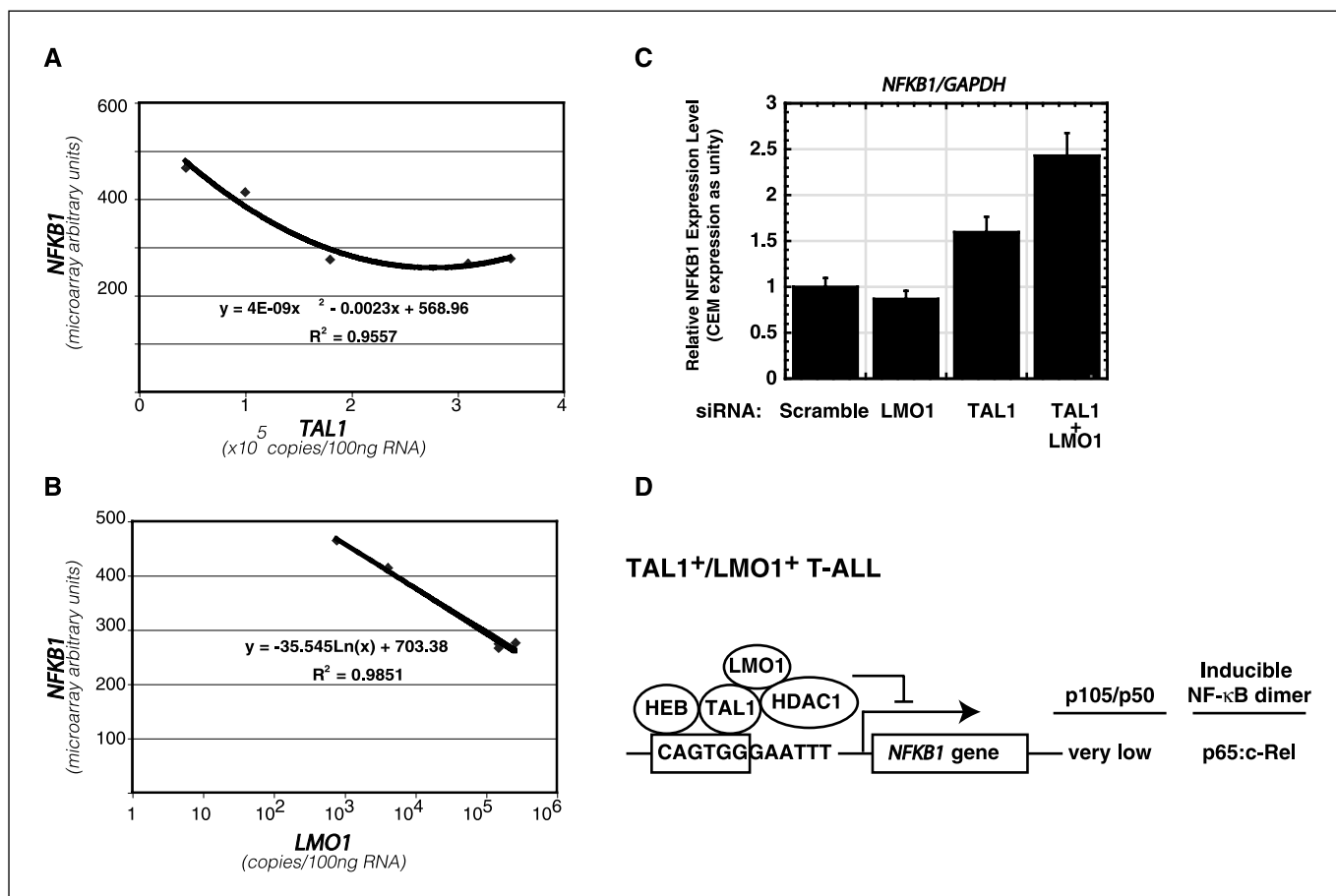


Figure 4. NFKB1 expression inversely correlates with TAL1 and LMO1 expression in TAL1⁺/LMO1⁺ primary T-ALLs. *A*, polynomial regression analysis was done to examine the relationship between TAL1 and NFKB1 expression in five LMO1⁺/TAL1⁺ patient samples. *B*, logarithmic regression analysis was done to examine the relationship between LMO1 and NFKB1 expression in five LMO1⁺/TAL1⁺ patient samples. *C*, transient knockdown of LMO1, TAL1, and TAL1 + LMO1 was done in CEM cells. NFKB1 expression was analyzed by quantitative RT-PCR. *D*, model for NFKB1 transcriptional regulation in TAL1⁺/LMO1⁺ primary T-ALLs and T-ALL cells.

T-ALL samples. In addition, we also observed a general reduction in the levels of p50 expression in thymomas induced by different oncogenes in transgenic mouse models when compared with those in thymocytes of corresponding animals (Supplementary Fig. S3C). Reduction of p50 expression results in the increased capacity to activate the atypical p65:cRel heterodimer at the expense of a p50:p65 dimer. Thus, the capacity of T-ALL cells to induce p65:cRel-regulated genes, such as ICAM-1, is increased when these cells are exposed to anticancer genotoxic agents, such as VP16. Thus, one consequence of TAL1-dependent repression of NFKB1 gene is the selective alteration in the repertoire of NF-κB-regulated genes in T-ALL cells. In addition, when these T-ALL cells are repetitively stimulated with NF-κB-activating agents, an augmented p50:p65-dependent response can be seen after the second stimulus due to augmented p50 expression after the first stimulus (6). Thus, basal repression of NFKB1 gene expression by TAL1 results in both short-term and long-term alterations of the NF-κB-dependent transcrip-

tional program in T-ALL cells. Because NF-κB-regulated genes are implicated in cancer cell resistance, proliferation, adhesion, and migration (15), it is conceivable that TAL1-dependent alterations in NF-κB functions likely contribute to T-ALL leukemogenesis and drug resistance development.

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