Transcriptional regulation of the human ALDH1A1 promoter by the oncogenic homeoprotein TLX1/HOX11

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Abstract

The homeoprotein TLX1, which is essential to spleen organogenesis and oncogenic when aberrantly expressed in immature T cells, functions as a bifunctional transcriptional regulator, being capable of activation or repression depending on cell type and/or promoter context. However, the detailed mechanisms by which it regulates the transcription of target genes such as ALDH1A1 remains to be elucidated. We therefore functionally assessed the ability of TLX1 to regulate ALDH1A1 expression in two hematopoietic cell lines, PER-117 T-leukemic cells and human erythroleukemic (HEL) cells, by use of luciferase reporter and mobility shift assays. We showed that TLX1 physically interacts with the general transcription factor TFIIB via its homeodomain, and identified two activators with the general transcription factor TFIIB via its homeodomain, and identified two activators

Introduction

TLX1 (HOX11), TLX2 and TLX3 belong to the ancient NKI family of homeobox genes that includes HEX, LBX1/2, MSX1/2, NKX3-2/BAPX1 and NKX2-5/Csx.1 TLX1 encodes a transcription factor that, although required during normal embryogenesis,2 was actually discovered as a consequence of its aberrant expression in T-cell acute lymphoblastic leukemia (TALL).3,4 Two distinct TLX1-expression categories have been identified in T-ALL.5,6 High level TLX1 expression (13%) is typically associated with 10q24 chromosomal abnormalities and confers a favorable prognosis whereas TLX1-low T-ALLs (22%) have an intact 10q24 locus and expression does not impact prognosis.7 Aberrant expression of related TLX3 is additionally found in another 22% of T-ALLs following 5q35 chromosomal rearrangements,8 which highlights the significant role of TLX family members in T-cell oncogenesis.

Confirmation of the gene product of TLX1 is an oncogene has come from mouse models, which have shown that enforced expression of TLX1 impairs cell differentiation and leads to malignancy.9,10 Current models for the mechanism by which TLX1 promotes leukemia are based on its ability to act indirectly, either by enhancing chromosome instability11,12 or by regulating gene expression through specific protein-protein interactions with key cellular regulatory molecules such as the protein serine/threonine phosphatases PP1 and PP2A, and the transcriptional coactivator/acyetyltransferase, CREB-binding protein (CBP).18-20 Thus, TLX1 may mediate its transforming function by simultaneously inhibiting the phosphatase activity of PP1/PP2A to promote cell cycle progression via upregulation of pathways such as those downstream of EZF and MYC,13 and sequestering CBP at heterochromatin to accomplish a differentiation block.20 TLX1 has also long been suspected to act as a sequence-specific transcription factor21-23 that preferentially binds to the core sequence TAA/GTG in vitro.23,24 However, with the exception of its associations with heterochromatic satellite 2 DNA2 and its own promoter,25 no direct target genes for TLX1 have been convincingly identified. Individual genes suspected to be regulated by TLX1 have been described in various settings, including spleen development (Aldh1a1, Wt1),26,27 erythroid differentiation (Aldh1a1, c-Kit, Vegf),28,29 and T-cell leukemia (Aldh1a1, Fhl1, Nrag3).23,22,26 Nevertheless, the regulatory role that TLX1 plays in such cases still remains to be determined.

The best characterized TLX1 target gene, ALDH1A1 (aldehyde dehydrogenase 1A1) belongs to a subfamily (class 1A) of ALDH genes whose main biological role is the conversion of the aldehyde form of vitamin A (retinal) to its biologically active form, retinoic acid.30 ALDH1A1 apparently has normal roles in embryonic development,31 and in the renewal/differentiation of hematopoietic stem cells (HSCs),32 where it is known to be highly expressed.33 ALDH1A1 is further implicated in regulating the polarity of HSC differentiation by favoring the development of a myeloid rather than a lymphoid cell fate.33,34 In agreement with this, ALDH1A1 expression can discriminate between acute myeloid (AML) and acute lymphoid leukemia,35 and while we have demonstrated aberrant ALDH1A1 expression in T-ALL,36 ALDH1A1 is reportedly down-regulated in AML.37 Thus, ALDH1A1, which is regulated by TLX1 in its normal chromosomal context38-40 is of interest due to its associations...
with both normal development and leukemogenesis. Here, we explored the molecular mechanism(s) by which TLX1 regulates the ALDH1A1 gene and find that it occurs in a non-DNA binding fashion through protein-protein interactions. We further show that TLX1 interacts directly with the general factor TFIIH via its homeodomain, indicating a role for TLX1 in gene regulation via the basal transcriptional machinery.

Design and Methods

Cell culture and expression plasmids

The PER-117 and ALL-SIL T-cell lines, and erythroleukemic cell line HEL, were cultured as previously described.6 The coding regions of human TLX1 and TFIIH were amplified by RT-PCR from ALL-SIL cDNA generated by Thermoscript RT (Invitrogen, Carlsbad, CA, USA) using PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA, USA) and primers containing an Nhe I restriction site. The resulting products were cloned into the Nhe I site of the pCINeo mammalian expression vector (Promega, Madison, WI, USA) and sequence verified.

 Luciferase reporter constructs

The human ALDH1A1 proximal promoter region was amplified by high-fidelity PCR from genomic DNA as previously described.10 For the construction of the -978/+42 construct, a 1020 bp fragment of the ALDH1A1 promoter was amplified using the forward primer 5’-GCCAGCTTCACATACTGAGCATCGAGTA-3’ and reverse primer 5’-GCCCTAGGCTGGACTGCTGCT-3’ (introduced Sac I and Nhe I restriction sites in italics). To create the -303/+42, 201/+42, 146/+42 and -91/+42 constructs, various lengths of the ALDH1A1 promoter were amplified using the same reverse primer together with the forward primers 5’-GCCAGCTTCACATACTGAGCATCGAGTA-3’ and reverse primer 5’-GCCCTAGGCTGGACTGCTGCTG-3’ (introduced Sac I and Nhe I restriction sites). The coding regions of human TLX1 and TFIIB were amplified by PCR using the primers 5’-GCCCTAGGCTGGACTGCTGCTG-3’ and 5’-GCCCTAGGCTGGACTGCTGCTG-3’, 5’-GCCAGCTTCACATACTGAGCATCGAGTA-3’, and 5’-GCCAGCTTCACATACTGAGCATCGAGTA-3’, respectively. The -303/+42 and -1/+42 constructs were directionally cloned into the Sac I/Nhe I restriction sites in the luciferase reporter vector pGL3-Basic (Promega, Madison, WI, USA). Insert identities were confirmed by automated DNA sequencing. The FHL1 -821 promoter construct has been previously described.8

 Luciferase and β-galactosidase reporter gene assays

Plasmid DNAs (1 µg/µL), prepared using a purification kit (Plasmid Maxi, Qiagen, Hilden, Germany), were transiently transfected as previously described.10 In brief, PER-117 or HEL cells (1×10⁶) were co-transfected by electroporation (300V, 960 µF) with 15 µg of luciferase reporter plasmid (or as negative control, pGL3-Basic) and 5 µg of pSV-β-Gal control plasmid. Cells were harvested 24 h later followed by measurement of luciferase and β-galactosidase activities using the Tropix Dual-Light luminescent reporter gene assay system (Applied Biosystems, Foster City, CA, USA). Transcriptional activity was defined as the ratio of luciferase activity (in relative light units; RLU) from pGL3-Basic derivatives relative to β-galactosidase activity from pSV-β-Gal, which reflected the efficiency of transfection. All experiments were repeated a total of three times on different days.

For measuring the effect of TLX1 on ALDH1A1 promoter activity, transfections were similarly performed with the additional inclusion of 15 µg of expression plasmid, either pEF-BOS/T LX1, pEF-BOS/T LX1ΔH3 or pEF-BOS as control.10 In this case, transcriptional activity was defined as the log (base 2) transformation of the ratio of luciferase activity (in relative light units; RLU) from pGL3-Basic derivatives relative to β-galactosidase activity from pSV-β-Gal. Statistical analysis was performed in SPSS 2000 using a mixed effects model with day of experiment as a random effect and luciferase reporter plasmid and expression plasmid as fixed effects model with day of experiment as a random effect. Interactions between contrasts comparing pEF-BOS/T LX1 with pEF-BOS, and contrasts comparing ALDH1A1 constructs with pGL3-Basic, were examined. This revealed the extent to which the effect of adding TLX1 was different for the various promoter constructs compared to pGL3-Basic.

Preparation of nuclear extracts

Cells (1×10⁶) were washed twice with 10 mL of cold PBS, resuspended in 400 µL Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) with protease inhibitors (100 µg/mL aprotinin, 5 µg/ml leupeptin, 1 µg/mL pepstatin A, 0.5 mM PMSF) and incubated on ice for 30 min. Pre-cleared extracts were incubated at 4°C for 30 min and then analyzed by electrophoresis on 4% native polyacrylamide gels in 0.5 x TBE buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA) including buffer (20 mM HEPES, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) with 0.5 µg poly(dI-dC) (ICN, Costa Mesa, CA, USA) and 6 µg of nuclear extract in a final volume of 15 µL. The samples were incubated at 4°C for 30 min and then analyzed by electrophoresis on 4% native polyacrylamide gels in 0.5 x TBE at 10 V/cm. For competition experiments, an excess of unlabelled competitor oligonucleotide was added to reaction mixtures. Additional competitor oligonucleotides used were those containing TLX1 (5’-TTCCATTCGATAATTCGAG-3’ and reverse primer 5’-GCCCTAGGCTGGACTGCTGCTG-3’) and GATA5 (5’-GAAACCTGACATATTGTTGCTTCC-3’). Bandshifts were performed in the presence of anti-TLX1 by adding 2 µL of polycytonal rabbit antiserum raised against the C-terminus (sc-880, Santa Cruz Biotechnology, Santa Cruz, CA). Normal rabbit serum was used for the no antibody control. Following electrophoresis, the gels were transferred to 3MM paper (Whatman, Maidstone, UK), dried and autoradiographed at -80°C.

Western blotting and immunoprecipitation of TLX1 complexes

Western blotting was performed with 50 µg of nuclear extract electrophoresed through a 12% SDS-PAGE gel and transferred to a Hybond ECL membrane (Amersham Biosciences) and made double stranded by annealing with an equimolar amount of complementary oligonucleotide in 1 x annealing buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA). Probes were incubated in 1 x binding buffer (20 mM HEPES, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) with 0.5 µg poly(dI-dC) (ICN, Costa Mesa, CA, USA) and 6 µg of nuclear extract in a final volume of 15 µL. The samples were incubated at 4°C for 30 min and then analyzed by electrophoresis on 4% native polyacrylamide gels in 0.5 x TBE at 10 V/cm. For competition experiments, an excess of unlabelled competitor oligonucleotide was added to reaction mixtures. Additional competitor oligonucleotides used were those containing TLX1 (5’-TTCCATTCGATAATTCGAG-3’ and reverse primer 5’-GCCCTAGGCTGGACTGCTGCTG-3’) and GATA5 (5’-GAAACCTGACATATTGTTGCTTCC-3’). Bandshifts were performed in the presence of anti-TLX1 by adding 2 µL of polycytonal rabbit antiserum raised against the C-terminus (sc-880, Santa Cruz Biotechnology, Santa Cruz, CA). Normal rabbit serum was used for the no antibody control. Following electrophoresis, the gels were transferred to 3MM paper (Whatman, Maidstone, UK), dried and autoradiographed at -80°C.
ed with 5 µg of affinity-purified rabbit anti-TLX1 polyclonal antibody (sc-880; Santa Cruz Biotechnology), or no antibody (as control), for 4 h at 4°C with constant gentle rocking. Immune complexes were bound to protein A/G agarose beads, centrifuged and washed with 1.2 mL of cold IP wash buffer (100 mM Tris-HCl pH 7.4, 1% NP40 and 1% deoxycholic acid) containing 500 mM LiCl (once) and 1.2 mL of IP wash buffer (four times).

MALDI-TOF mass spectrometric analyses

Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by silver staining. Bands were excised directly from gels into 96-well microtiter plates (Titertek, Huntsville, Al, USA), destained, and in-gel trypsin digestions performed according to Shevchenko et al. Peptides were extracted with 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid, and aliquots of 0.5 µL applied directly onto a target plate and allowed to air dry. Tryptic peptide masses were then obtained using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Known trypsin autolysis peptide masses (842.51, 1045.56 and 2211.1 Da) were used for internal calibration of each spectrum.

GST-pulldown assay

TLX1 and TFIIB cDNAs in pCIneo were transcribed in vitro with T7 RNA polymerase. The products were labeled with [35S]-methionine (Amersham Biosciences) using the TNT coupled transcription-translation system (Promega). GST-TLX1 fusion proteins were expressed from pGEX-6P-1 as described previously. The GST-pulldown assay was performed by incubating 15 µg of GST, GST-TLX1 or GST-TLX1AH3 immobilized on glutathione sepharose beads (20 µL) with 5 µL of proteins translated in vitro and labeled with [35S]-methionine in 500 µL of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, 0.05% BSA) for 18-20 h at 4°C with continuous rotation. Bound proteins were washed three times with 500 µL of cold binding buffer and eluted in SDS sample buffer. The eluted proteins were resolved on 12% SDS-PAGE gels and visualized by autoradiography.

Results

Functional analysis of the human ALDH1A1 promoter

Ectopic expression of TLX1 was previously shown to modulate endogenous ALDH1A1 expression. To pursue the molecular mechanism(s) underlying this regulatory effect, we cloned 1020 bp of the human ALDH1A1 5'-flanking region into the pGL3-Basic luciferase reporter vector. This sequence comprised nucleotides -978 to +42 relative to the transcriptional start site (Figure 1). Among the conserved promoter elements identified were a TATA-like sequence (GATA box) at -33 and a single CCAAT box at -74, which has previously been shown to be functionally important for ALDH1A1 expression. Transient transfection of a series of deletion constructs (978/42, 303/42, 201/42, 146/42, 91/42, +42, -50/42 and +1/42; Figure 1) into PER-117 and HEL cells (which both lack TLX1 expression) showed that the ALDH1A1 promoter was functional in both cell lines and that the sequences between -50 and -91 and between -50 and -146 were required for maximal promoter activity in PER-117 and HEL cells, respectively (Figure 2A). Consistent with previous results in Hep3B liver cells, deletion of the CCAAT box at -74 caused a dramatic reduction in promoter activity, suggesting that it is a key element for ALDH1A1 expression in multiple cell types.

Regulation of the ALDH1A1 promoter by TLX1

To determine the effect of TLX1 on the transcriptional activity of the ALDH1A1 promoter, transactivation assays were carried out in PER-117 and HEL cells co-transfected with an expression plasmid containing TLX1 or empty vector (pEF-BOS) as a negative control. The results were expressed as a ratio of the normalized transcriptional activity of each of the two promoter constructs with and without TLX1 (Figure 2B). Western blot analysis confirmed that TLX1 protein was expressed in the transfected cells (Figure 2C). Consistent with our previous findings, the effect of TLX1...
on ALDH1A1 was reversed in the transcriptional assay as compared to regulation of the endogenous gene. In PER-117 cells, TLX1 repressed ALDH1A1 promoter activity (Figure 2B), and this required the sequence between -50 and -91, which contains the CCAAT box. Deletion of the CCAAT box (-91CAT) completely abolished TLX1-mediated repression (Figure 2B), suggesting that downregulation may possibly occur via this site. Interestingly, the shortest constructs (-50/+42 and +1/+42) were mildly stimulated by TLX1 in both PER-117 and HEL cells, (Figure 2B) indicating the presence of a separate, positive-acting TLX1-response sequence localized to the region between +1 and +42. No effect by TLX1 was observed on the region between -201 and -303, which contains a conserved TLX1 in vitro binding site at -257 (Figure 1), suggesting that TLX1 binds to a distinct recognition sequence in the ALDH1A1 promoter in vivo, or alternatively, that it acts via a non-DNA binding mechanism. In either case, these data indicate that TLX1 operates via at least two mechanisms in respect to the ALDH1A1 promoter; a general transactivating activity via an element located between +1 and +42 and a strong, cell line-specific repressive activity via an element located between -91 and -50.

The homeodomain is required for TLX1-mediated repression
To assess whether the homeodomain of TLX1 is required for the transcriptional activities of TLX1, a mutant TLX1 expression vector (TLX1ΔH3) was employed, which lacks the DNA recognition helix (helix 3) of the homeodomain. Whereas, TLX1 repressed the activity of the basal ALDH1A1 promoter (-91/+42) 4-fold in PER-117 cells, TLX1ΔH3 not only lacked the ability to negatively regulate ALDH1A1, but switched to become an activator, stimulating transcription by approximately 5-fold (Figure 3). A similar result was obtained when the promoter of another target gene, FHL1, was used (Figure 3). By contrast, the positive regulation of the -91/+42 construct observed in HEL cells was comparable when either TLX1 or TLX1ΔH3 was used (5.5- and 8-fold induction, respectively). Thus, the homeodomain is crucial for TLX1-mediated repression but is not required for TLX1-mediated activation, indicating that these two activities are distinct.

TLX1 does not affect formation of transcriptional complexes at the -74 CCAAT site
We opted to focus on the mechanism by which TLX1 mediates CCAAT-dependent repression. Mobility shift assays were therefore performed to identify whether TLX1 directly binds to the CCAAT box or interferes with the binding of other factors at this site. Nuclear extracts derived from PER-117 or HEL cells, either expressing empty pEF-BOS (as control) or pEF-BOS/TLX1, were incubated with a radiolabeled probe (ALDH CAT) spanning the CCAAT motif (-85 to -60; Figure 1). As shown in Figure 4A, incubation of nuclear extract from PER-117 or HEL cells with ALDH CAT resulted in the appearance of at least two specific DNA-protein complexes, together with a third non-specific complex (C2). The strongest, complex C1, appeared to be common to the two cell types, while additional complexes P and H were unique to PER-117 and HEL cells, respectively. These complexes were inhibited by the addition of a 35-fold molar excess of unlabeled self-competitor (ALDH CAT) but not by a 35-fold molar excess of CCAAT mutant competitor (ALDH CATMut; CCAAT to GACCTG). Contrary to expectation, in both PER-117 and HEL cells the mobility shift pattern was identical regardless of TLX1 expression, suggesting that TLX1 does not affect the formation of specific DNA-protein complexes at the CCAAT site.
expression status (or addition of TLX1 antibody; data not shown), suggesting that TLX1 does not affect the formation of DNA-protein complexes at the CCAAT box.

**TLX1 alters DNA-protein complex formation at the -33 GATA box**

TLX1 may directly or indirectly inhibit CCAAT-dependent transcription via the basal apparatus. The *ALDH1A1* promoter lacks a canonical TATA box but does possess a related GATA box (GATAAA) utilized by a number of genes whose transcriptional initiation involves interplay between TFIID and GATA factors. EMSAs were thus performed to identify whether TLX1 directly binds to the GATA box or interferes with the binding of other factors at this site. Nuclear extracts derived from PER-117 or HEL cells, either expressing empty pEF-BOS (as control) or pEF-BOS/TLX1, were incubated with a radiolabeled probe (ALDH GATAB) spanning the GATA box (-42 to -19; Figure 1). As shown in Figure 4B, incubation of nuclear extract from PER-117 cells lacking TLX1 expression with ALDH GATAB resulted in the appearance of four specific DNA-protein complexes, C1, C2, P1 and P2. HEL nuclear extracts only produced three specific complexes (C1, C2 and H), the strongest of which (C2) appeared to migrate similarly in both cell types. These complexes were inhibited by the addition of a 35-fold molar excess of unlabeled self-competitor (ALDH GATAB) but not by a 35-fold molar excess of GATA box mutant competitor (ALDH GATAABMut; GATAAA to CTCAGC). Strikingly, in PER-117 but not HEL cells, expression of TLX1 resulted in a significant alteration in complex formation. Of the four specific PER-117 complexes, the formation of two (C1 and P1) was completely inhibited by TLX1, while the intensity of the remaining two (C2 and P2) was enhanced. Thus, TLX1-mediated regulation of *ALDH1A1* in T cells, but not erythroid cells, is associated with an alteration of transcription factor binding at the GATA box.

Complex formation at the GATA box in both PER-117 and HEL cells was also strongly inhibited when a 35-fold molar excess of an unlabeled consensus GATA oligonucleotide (GATA; containing WGATAR with different flanking sequences to ALDH GATAB) was used (Figure 5A). This indicated that GATA factors are present in all complexes formed in this assay. However, TLX1 does not appear to bind DNA at this site, since a 35-fold molar excess of an unlabeled consensus satellite 2 oligonucleotide (TLX1), capable of being bound by TLX1,24 could not compete for complex formation (Figure 5A). In agreement with this conclusion, no supershift or inhibition of complex formation was observed following addition of TLX1 antibody (Figure 5B). Notably, however, PER-117 complex C1, which is abolished by TLX1, re-formed in the presence of TLX1 antibody, indicating that TLX1 directly contributes to the disruption of this low mobility complex.

**TLX1 interacts with TFIIB**

To help understand the transcriptional regulatory function of TLX1, we searched for binding partners using an immunoprecipitation strategy in leukemic T cells (ALL-SIL) that...
aberrantly express TLX1 as a consequence of a 10q24 chromosomal translocation. A TLX1 antibody was employed to isolate naturally occurring nuclear protein complexes, which were separated by SDS-PAGE and silver stained (Figure 6A). Excised gel slices representing discrete molecular mass intervals were digested with trypsin and analyzed by MALDI-TOF mass spectrometry in order to determine the identity of the protein bands. Table 1 summarizes the eight proteins detected. Among these were the centromeric proteins CENP-E and CENP-F, an intriguing finding given that TLX1 has previously been localized to centromeric regions. Of particular interest was the detection of the general transcription factor TFIIB, which was identified by the presence of 15 peptides with sequence coverage of 49% (Table 1). To confirm that TLX1 could physically interact with TFIIB in a specific manner, we performed a GST pulldown assay (Figure 6B). Glutathione-Sepharose beads containing GST-TLX1, GST-TLX1ΔH3, or GST alone were incubated with in vitro translated 35S-labeled TFIIB or TLX1 protein. The latter was included as a positive control since TLX1 has previously been shown to homodimerize. Following extensive washing, retention of TFIIB was observed with GST-TLX1 but not with control GST beads (Figure 6B), demonstrating that TLX1 has the capacity to interact with TFIIB. Interestingly, retention of TFIIB, but not TLX1 control, was greatly diminished when GST-TLX1ΔH3 beads were used, indicating that helix 3 of the TLX1 homeodomain contributes strongly to the TFIIB interaction.

Discussion

TLX1 has previously been characterized as a DNA-binding factor that preferentially associates with the core sequence TAAT/GTG in vitro and the similar sequence TA/GATTC present in satellite 2 DNA. In addition, TLX1 can switch, in a cell type- and promoter context-dependent manner, between roles as activator and repressor, however the mechanism(s) responsible for these divergent roles is poorly understood. In this study, we further investigated the function of TLX1 by analyzing its ability to transcriptionally regulate ALDH1A1, a gene previously identified as being TLX1-dependent in developing mouse spleen as well as in several cell lines (PER-117, HEL, NIH-3T3), and which contains a predicted TLX1 binding site, conserved between human and mouse, at -257 upstream of the transcriptional start site. In the first instance, our data confirmed ALDH1A1 as a regulatory target of TLX1, with the polarity of effect observed in terms of activation/repression being heavily dependent upon cell type. In PER-117 T cells, transient luciferase reporter assays with nested deletions of the ALDH1A1 promoter revealed that TLX1-mediated repression occurred in a CCAAT box-dependent manner involving an element located between -91 and -50. By contrast, TLX1 stimulated transcription in a CCAAT-independent manner from a proximal location (-91/+42) in human erythroleukemic

![Figure 5. Perturbation of GATA-containing complexes by TLX1. (A) GATA factor(s) but not TLX1 binds to the ALDH1A1 promoter GATA box. EMSA using a 32P-labeled double-stranded oligonucleotide containing the GATAAA site at -33 of the human ALDH1A1 promoter (ALDH GATAB). The assay was performed with nuclear extracts prepared from PER-117 (left panel) or HEL cells (right panel) with and without overexpression of TLX1. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells). Overbars denote the addition of a 35-fold molar excess of unlabeled double-stranded self (ALDH GATAB), satellite 2 DNA (TLX1) or GATA consensus (GATA) probe as competitor. Asterisks denote complexes altered by TLX1. (B) TLX1 directly disrupts the low mobility ALDH1A1 promoter GATA box complex C1. EMSA performed as above using the -33 GATAAA site oligonucleotide (ALDH GATAB) in the absence (-) or presence (+) of TLX1 antibody. The migration of the free probe is indicated along with the positions of specific DNA-protein complexes (C1, C2, P1 and P2 in PER-117 cells; C1, C2 and H in HEL cells). Complex C1 reappears in PER-117 cells expressing TLX1 in the presence of anti-TLX1 antibody (asterisk).](image)
(HEL) cells. Transactivation, which was also observed in the T-cell background when a minimal promoter sequence was used (-50/+42), was abolished in HEL cells with the addition of extra DNA sequence (-146/-92) suggesting that a specific factor bound to this region is capable of abrogating the stimulatory potential of TLX1. Thus, TLX1 possessed two activities in respect to the \textit{ALDH1A1} promoter, namely \textit{CCAAT}-dependent repression, which was cell type-specific and cryptic \textit{CCAAT}-independent activation, which was unmasked by deleting upstream regulatory sequences. Neither of these activities involved the TLX1 recognition site at -257, which initially suggested that TLX1 either bound a distinct sequence \textit{in vivo}, as is the case with the regulation of its own promoter,\textsuperscript{25} or that it acted via a non-DNA binding mechanism.

Remarkably, the effect of TLX1 on \textit{ALDH1A1} promoter activity in the transient reporter assays was inversely related to its previously observed effect on endogenous \textit{ALDH1A1} levels in PER-117 and HEL cells.\textsuperscript{24} This phenomenon has been observed previously in respect to other putative gene targets of TLX1, namely \textit{NRA43, KIT} and \textit{FHL1}.	extsuperscript{15,29,38} Although puzzling, the finding that a homeoprotein can act as a repressor or activator of transcription depending on promoter context is a common one. In many cases the activity that predominates has been found to be highly dependent on the nature of the cis-regulatory DNA sequence since this can, in turn, affect the interaction of homeoproteins with co-regulatory molecules such as TALE (three amino acid loop extension) homeoproteins, CBP (CREB-binding protein) or Groucho.\textsuperscript{50-54} CCAAT-dependent repression by TLX1 was found to require the DNA recognition helix (helix 3) of the homeodomain. Intriguingly, the TLX1ΔH3 mutant lacking this helix was not only incapable of repressing \textit{ALDH1A1} transcription in PER-117 cells but switched to become an activator of transcription. This indicated that the repression/activation activities of TLX1 are separable with a structurally intact homeodomain being absolutely required for transcriptional repression, but not activation.

Given these findings, a reasonable assumption was that TLX1 would repress \textit{ALDH1A1} transcription by directly binding promoter DNA at or near the \textit{CCAAT} box. This crucial element is likely generally required for \textit{ALDH1A1} expression, since it was also identified as the major positive element in the Hep3B cell line and in Hepa-1 mouse hepatoma cells where it was bound by NF-Y and CCAAT/enhancer binding protein β (\textit{CEBPβ}), respectively.\textsuperscript{44,55} Moreover, the CCAAT box is capable of being bound by the CCAAT binding transcription factor (CTF1/NFIC) with which TLX1 is known to interact in a functional manner.\textsuperscript{56} However, EMSA assays using PER-117 (or HEL) nuclear extracts revealed that TLX1 did not directly bind the CCAAT box, nor did it affect protein

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**Table 1. Proteins identified by immunoprecipitation and peptide mass fingerprinting.**

| Identified protein                                      | Matched peptide cover (%) | Mass (kDa) | UniProtKB/Swiss-Prot accession number |
|--------------------------------------------------------|---------------------------|-----------|---------------------------------------|
| General transcription factor IIB (TFIIB)                | 49                        | 34.83     | Q00403                                |
| Centromere protein F (CENPF)                            | 39                        | 367.76    | P49454                                |
| Centromere protein E (CENPE)                            | 35                        | 316.42    | Q02224                                |
| RAD50 homolog (RAD50)                                   | 31                        | 153.89    | Q92878                                |
| Retinoblastoma binding protein 9 (RBBP9)                | 28                        | 21.00     | O75884                                |
| NK homeobox 6-1 (NKX6-1)                                | 28                        | 37.55     | P78426                                |
| Lymphoblastic leukemia 1 (LYL1)                          | 23                        | 28.63     | P12980                                |
| Zinc finger protein 29 (ZNF20)                          | 20                        | 61.57     | P17024                                |

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![Figure 6. TLX1 interacts with TFIIB.](image-url)

(A) Isolation of TLX1-associated proteins. Nuclear extracts from ALL-SIL leukemic T cells were immunoprecipitated using TLX1-specific antibody (Ab), or no antibody (No Ab) as negative control. Coprecipitated proteins were resolved by SDS-PAGE and stained. (B) GST pulldown assay. GST, GST-TLX1 and GST-TLX1ΔH3 immobilized on glutathione-Sepharose beads were incubated with in vitro-translated, \textsuperscript{35}S-labeled TLX1 as positive control, and TFIIB. Bound proteins were washed, eluted, resolved by 12% SDS-PAGE and detected by autoradiography: 50% of the labeled input proteins TLX1 and TFIIB are shown on the left.
that TLX1, like many other homeoproteins, to activation observed with the TLX1 results provide one potential mechanism to TLX1-TFIIB interaction was reduced in the DNA binding manner, both in this study and GATA box, by inhibiting the rate/extent of PIC (PIC) assembly, providing a bridge between promoter-bound TFIIID and RNA polymerase II. This points to a mechanism to account for how TLX1 can inhibit basal transcription in a non-DNA binding manner, both in this study and GATA1 is heavily dependent on its acetylation status. Taken together, we suggest a model in which interactions between TLX1 and both TFIIIB and CBP lead to the transcriptional repression observed in our transient luciferase assays. Whereas contacts with TFIIIB may inhibit the formation of a functional PIC, binding to CBP may prevent it functioning as a co-activator and acting to acetylate GATA factors. This would explain the substantially altered formation of GATA-containing protein complexes observed at the ALDH1A1 promoter GATA box, despite the lack of DNA binding by TLX1. It is conceivable that a general ability of TLX1 to indirectly regulate target genes by altering GATA factor activity, whether via CBP or an alternative mechanism, may be consequential for its role as an oncoprotein. This is particularly in view of evidence linking both a blockage in T-cell development and leukemogenesis to incapacitated GATA function. Interestingly, a non-DNA binding mode of action for TLX1 that involves activity regulation of other transcription factors is reminiscent of another important T-ALL oncoprotein, SCL/TAL1, which induces leukemia by recruiting the co-repressor/histone deacetylase mSin3A to inhibit the transcriptional activity of E47/HEB.44 There is little evidence to date showing that TLX1 can bind directly to natural DNA sequences to regulate target gene expression. Instead, TLX1 has been shown to operate indirectly by interacting with other proteins, most notably the phosphatases PP1 and PP2A to regulate gene cascades in various pathways such as RB/E2F and p107/MYC.45 Consistent with this paradigm, our data confirm that TLX1 is capable of regulating ALDH1A1 expression in a non-DNA binding manner by affecting transcriptional complexes at the proximal ALDH1A1 promoter, although clearly, additional cis-regulatory elements (that may influence TLX1 protein interactions) are required to recapitulate the effect of TLX1 on endogenous ALDH1A1 gene expression. We showed that TLX1, like other homeodomain transcription factors including tinman/Nkx2-5, abdominal-A, Nkx6.1 and Vnd/Nkx2.2 is capable of acting as a bi-functional transcriptional regulator, whose activation and repression activities operate in a cell-type specific manner and via two distinct mechanisms. The first involves the ability of TLX1 to repress transcription, possibly by perturbing interactions between CCAAT box-binding transcriptional activators and proteins (GATA factors/basal transcriptional machinery) assembled at a non-canonical TATA (GATA) box. This activity does not appear to involve direct DNA binding, although a structurally intact homeodomain is required, presumably in order for TLX1 to interact with TFIIIB and/or CBP. Chromatin immunoprecipitation assays to identify the specific transcription factor(s) bound at this composite CCAAT-box/TLI responsive element and at the GATA box before and after TLX1 expression are required to substantiate this hypothesis. The second activity involves the ability of TLX1 to stimulate transcription through as yet unidentified regulatory elements in the proximal ALDH1A1 promoter and does not require an intact homeodomain. Our understanding of the role of TLX1 in normal development and in T-cell leukemogenesis is crucially dependent on deciphering the mechanisms by which TLX1 is capable of regulating gene expression. Future work to characterize TLX1 target genes and to fully define the protein participants involved in TLX1-mediated gene regulation will represent important steps towards this goal.

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