MEIS proteins as partners of the TLX1/HOX11 oncoprotein

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Abstract

Aberrant expression of the *TLX1*/*HOX11* proto-oncogene is associated with a significant subset of T-cell acute lymphoblastic leukemias (T-ALL). Yet the manner in which *TLX1*contributes to oncogenesis is not fully understood. Since, typically, interactions of HOX and TALE homeodomain proteins are determinant of HOX function, and HOX/MEIS co-expression has been shown to accelerate some leukemias, we systematically examined whether TLX1 interacts with MEIS and PBX proteins. Here, we report that TLX1 and MEIS proteins both interact and are co-expressed in T-ALL, and suggest that co-operation between TLX1 and MEIS proteins may have a significant role in T-cell leukemogenesis.

Keywords: TLX1; MEIS; PBX; Homeodomain; Interaction; Leukemia

Introduction

Aberrant expression of the *TLX1*/*HOX11* NK-like homeobox gene is strongly associated with T-cell acute lymphoblastic leukemia (T-ALL), where it occurs at both high and low levels owing to translocations involving *TCR* loci and as yet undefined events, respectively [1], [2] and [3]. In sharp contrast, *TLX1* expression is not detected in B-lineage ALL or in primary lymphocytes [1], [4] and [5]. This specificity of *TLX1* expression in a high proportion of T-ALL cases argues that *TLX1* is important to oncogenesis in these patients.

Studies using murine models of *TLX1*-driven tumorigenesis have provided ancillary evidence of its oncogenic potential. TLX1 can immortalise hematopoietic precursors [6], block cell differentiation [6], [7] and [8] and prompt the development of B-cell lymphomas after long latency [9]. Altogether, this body of evidence suggests that *TLX1* over-expression in lymphocytes contributes to cellular immortalisation and potentiates oncogenesis. However, extended latency periods indicate that *TLX1* expression alone is insufficient for tumorigenesis, and that additional factors or events are required for malignant transformation.

Much attention has been focused on the function of the TLX1 homeoprotein in order to understand its oncogenic role. TLX1 is a DNA-binding homeodomain transcription factor [10] that is essential for splenogenesis in the developing animal [11], and which regulates the expression of target genes such as *Aldh1a1* and *Wt1* [12] and [13]. In addition, TLX1 has been implicated in non-transcriptional oncogenic mechanisms, namely G1/S cell cycle progression through inhibition of protein phosphatases 1 and 2A [14] and [15] and promotion of chromosome missegregation through co-operation with factors such as the Ubr1 ubiquitin ligase [16].

A number of studies on oncogenic homeoprotein function have concluded that co-operating proteins are significant for HOX function, as they increase the affinity and specificity of DNA binding of the heterodimer compared to the HOX protein alone [17] and [18]. TLX1 has been detected in transcriptional complexes with a number of factors including CTF1, CBP and Gro/TLE1 [19], [20] and [21]. One of the most abundant and important classes of HOX co-factors is

the TALE homeodomain superclass, comprised of PBX, MEIS and PREP proteins [22]. Heterodimeric and trimeric complexes between HOX, PBX and MEIS proteins and co-operation between *HOX* and *TALE* genes is central to the development of many leukemias [23], [24] and [25]. Initial *in vitro* studies have shown that TLX1 can interact with the PBX TALE homeodomain family [26]. But while PBX–HOX interactions are important for the function of HOX proteins in normal cellular regulatory processes and in oncogenesis, so too are MEIS–HOX interactions [25].

We hypothesized that if TLX1 interacts with MEIS homeodomain proteins, such interactions could be significant for the onset or maintenance of TLX1-dependent oncogenesis. Therefore, this study investigated whether TLX1, like members of HOX paralog groups 9 and 10, can interact with MEIS, as well as PBX proteins. Our results demonstrated that TLX1 and MEIS1/2 not only interact, but also are co-expressed in T-ALL and therefore have the potential to co-operate in leukemogenesis.

Materials and methods

Yeast two-hybrid interactions

Full-length coding sequences for human *TLX1*, *MEIS1*, *MEIS2A*, *MEIS2B* and *PBX* were amplified from cell line cDNA or plasmid constructs and directionally cloned in-frame into the pGilda bait (Origene, USA) and/or pJG4-5 prey vectors of the LexA-based two-hybrid system. Partial fragments of *TLX1* were also expressed as two-hybrid bait or prey fusions from the same vectors: TLX1-AD (51–331aa); TLX1N (1–196aa); TLX1C (190–331aa); TLX1H (151–273aa). All clones were verified by sequence analysis. In a LexA-based two-hybrid system, haploid *Saccharomyces cerevisiae* (*S. cerevisiae*) strains of opposite mating types containing baits (strain EGY48, pre-transformed with the *LacZ* reporter pSH18–34) or preys (strain RFY206) were mated in grid format [27], and then replica-plated to selective minimal media: yeast synthetic minimal media containing X-gal, and lacking uracil, histidine, tryptophan (UHW^{-}), and containing either 2% dextrose or 2% raffinose/2% galactose for induction of two-hybrid fusion proteins. For interaction experiments using TLX1 baits,

two-hybrid fusion proteins were induced with 0.2% galactose to minimise autoactivation of the *LacZ* reporter by TLX1 baits. Activation of the *LacZ* reporter was observed after incubation on selective minimal media from 2 days onwards, and was scored by relative color intensity. Scorings were corrected for background activation (bait construct + prey vector) before tabulation.

Co-immunoprecipitation (Co-IP) from yeast cells

S. cerevisiae diploids containing different bait and prey combinations were inoculated into UHW$^-$ 2% raffinose broth for overnight growth. These cultures were used to seed YPR (standard YPD medium with 2% dextrose replaced by 2% raffinose) at 0.5×10^7 cells/ml. YPR cultures were incubated for 6 h to achieve log phase growth (2×10^7 cells/ml), whereupon expression of tagged bait and prey proteins (LexA-bait and HA-prey protein fusions) were fully induced with 2% galactose for 8 h. For each co-IP, protein lysates from 1×10^8 cells were immunoprecipitated with 10 μl of α-rat-Dynalbeads (Dynal Biotech, Norway) pre-bound with α-hemagglutinin epitope (HA) (3F10, Roche, USA) rat monoclonal antibody. Controls were performed in parallel using α-rat-Dynalbeads without primary antibody.

Co-immunoprecipitation from transfected mammalian cells

Human coding sequences for *TLX1* and *MEIS* were cloned, with a 3′ HA tag, into the pEF-BOS vector; *TLX1* was also cloned into the same vector without the HA tag. HSB-2 T-lymphoblastoid cells were transiently co-transfected by electroporation (0.3 kV, 200 Ω, 975 μF in 0.4 cm-gap cuvette) with 20 μg each of either: (a) MEIS-HA + TLX1; (b) TLX1; (c) MEIS-HA; or (d) TLX1-HA. Co-IPs were performed in accordance with manufacturers' instructions: nuclear proteins were extracted from 3×10^7 cells at 48 h post-transfection, incubated with 2 μg α-HA (3808-1, Clontech, USA), mixed for 1 h at 4 °C, and then further incubated overnight with 50 μl washed α-rabbit Dynalbeads (Dynal Biotech).

Immunoblots

Denatured proteins were separated on 10% polyacrylamide gels and transferred under Towbin buffer to Hybond C$^+$ membrane (Amersham, USA). Membranes were probed with primary and HRP-

conjugated secondary antibodies and visualised with the ECL detection system (Amersham) and autoradiograph exposure. Blocking, dilutions and incubations of all commercial antibodies were used in accordance with manufacturers' suggestions. In yeast co-IP analysis, membranes were probed with α-LexA (sc-7544, Santa Cruz Biotechnology, USA) or α-HA (12CA5, Roche). In mammalian co-IP analysis, membranes were probed with α-TLX1 antibody used at 1:4000 dilution [28].

RT-PCR

Pediatric T-ALL cell lines [29], including PER-117 stably transfected with *TLX1* [30], were screened by RT-PCR for *TLX1*, *MEIS1*, *MEIS2* and *GAPDH* expression. To facilitate detection of multiple transcripts, primers pairs were targeted to the 3′ UTR of *MEIS2*(Fwd: 5′ TGGAATGACTATGTCAGCACAGAG; Rev: 5′ GAATTGGCTTATGAAGCACGAACT) and *MEIS1* (Fwd: 5′ ATGCATTGTCTGCAATGGTGACTG; Rev: 5′ TAAGACACTGCCTGCAACAGCTGA). Primer pairs were targeted to span exon splices for *TLX1* (Fwd: 5′ CATGCCGGGCGTCAACAACCT; Rev: 5′ TCACTCGCAGGCCGACGCCAC) and *GAPDH* (Fwd: 5′ ATGGTGAAGGTCGGTGTGAACGGA; Rev: 5′ GAGGTCCACCACCCTGTTGCTGTA). The cDNA was prepared with either Thermoscript (Life Technologies, USA) or Omniscript (Qiagen, USA) kits, and amplified in standard PCR reactions for 30 cycles.

Real-time quantitative RT-PCR

The real-time quantitative RT-PCR methodology, RNA extraction and cDNA synthesis have been previously described in detail [2], along with the primers and probes used for *TLX1* analysis. Primers and 6FAM-labelled probe sets for *MEIS* genes were as follows. MEIS1: fwd primer 5′ GCTCCTCTGTCAATGACGCTTT; rev primer 5′ CATTTCTCAAAAATCAGTGCTAAGAGA; probe ATGCCATTTATGGACACCCCCTCTTCC. MEIS2: fwd primer 5′ CAGTATGGGATCCGCTGTCA; rev primer 5′ AAACAACGGGTGCCCATAGA; probe CGCGTCCTTGTCCCGCTTCAA. The cohort of pediatric T-ALL patients has been previously

described [31]; IRB approval and parental consents were obtained prior to collection of patient specimens.

Results

TLX1 interacts with multiple TALE proteins

TALE homeoproteins have previously been implicated as partners of TLX1 [26], [32] and [33]. To systematically assess the comparative ability of TALE family members to interact with TLX1, a yeast two-hybrid system was employed, since the relative ranking of protein interaction strength in two-hybrid experiments, judged by reporter activation levels, has been reported generally to correlate with *in vitro* measurements of the affinity of protein interaction [34]. SCL and GATA3, important non-homeodomain transcription factors in hematopoiesis and leukemogenesis, were included as negative controls. TLX1/TALE interactions were tested in both orientations (TLX1 constructs as both bait and prey), since protein interactions can be affected by the orientation of the assessment [34]. However, full-length TLX1 was only used as a prey protein because it was found to strongly auto-activate when used as bait. Overall, multiple TLX1–TALE interactions were found, which were of differing strengths. The results of independent mating experiments were averaged and are summarised in Table 1 while Fig. 1 shows a representative interaction test involving TLX1 with MEIS1 and 2A.

These yeast-mating experiments confirmed TLX1 interaction with PBX1/2/3 proteins and also identified the two shorter isoforms of PBX3 (PBX3C and PBX3D) as novel TLX1 partners. This analysis also specifically identified MEIS1 and MEIS2 as protein partners for TLX1. Indeed, MEIS2, together with PBX3B, appeared to be the highest affinity TLX1 interactors in the panel of TALE proteins tested. The observed reporter activation by MEIS2/TLX1 and PBX3B/TLX1 was comparable to that of SCL/LMO2 complexes in similar experiments, signifying that these TLX1 interactions may be of similar affinity to SCL/LMO2 interaction. Deletion constructs, included to map the TLX1 interaction domains, showed that the amino-terminus of TLX1 is required for interaction with MEIS

proteins and that the homeodomain plus YPWMR motif is required for interaction with PBX proteins (Table 1 and Fig. 1).

Confirmation of MEIS proteins as TLX1 interactors

Strong interaction between TLX1 and MEIS proteins was a novel finding. To confirm the yeast two-hybrid assay results involving MEIS1 and MEIS2A, both yeast and mammalian co-immunoprecipitation (co-IP) experiments were performed. Firstly, physical interaction of TLX1 and MEIS proteins was tested in co-IP from yeast cells, using TLX1 interaction with PBX1B as a positive control. HA-tagged TLX1 was immunoprecipitated from protein extracts of fully induced yeast cultures, and co-IP of several constructs, provided as LexA fusions, were assessed by immunoblotting (Fig. 2). MEIS1 and MEIS2A (LexA fusion proteins) specifically co-immunoprecipitated with HA-TLX1. In control immunoprecipitations using magnetic beads without primary antibody, neither MEIS1 nor MEIS2A fusion proteins were precipitated. These results have been independently supported by GST-pulldown [33], suggesting that the TLX1/MEIS interaction does not require a nuclear adapter protein. TLX1/MEIS physical interactions were also established in co-IPs from transfected human T-ALL cells (Fig. 3). HSB-2 cells were co-transfected with TLX1 and HA-tagged MEIS1 or MEIS2A. HA-MEIS proteins were immunoprecipitated, and co-IP of TLX1 was detected by immunoblotting. The mammalian co-IP of TLX1 with both MEIS1 and MEIS2A established that interactions of TLX1 and MEIS proteins occur in the mammalian cell environment as well as in yeast cells.

Co-expression of TLX1 and MEIS in T-ALL cell lines

For TLX1 and MEIS proteins to have functional relevance in leukemia, it is not only necessary that they interact, but also that the proteins are co-expressed in leukemic cells. Consequently, using RT-PCR, we assessed the co-expression of *TLX1* and *MEIS* mRNA in a panel of T-ALL cell lines, normal lymphoid cell populations, and a positive control cell line for *MEIS* expression, K562 (Fig. 4A). PER-255 and ALL-SIL are T-ALL cell lines that endogenously express *TLX1* as a result of *TCR–TLX1* translocations. Here, they were also shown to express both *MEIS1* and *MEIS2* transcripts.

Indeed, overall, *MEIS* expression in the T-ALL cell line panel was high, with *MEIS1* and *MEIS2*expressed, respectively, in 7/9 (78%) and 6/9 (67%) of the cell lines tested. Both *MEIS* genes were also expressed in mature T-cells, as well as tonsil, peripheral blood lymphocytes (PBLs), and the cell line K562. One T-ALL cell line, PER-117, had also been stably transfected with *TLX1*. RT-PCR analysis showed that both *MEIS1* and *MEIS2*transcripts were expressed before and after *TLX1* transfection (Fig. 4A), establishing that *TLX1* expression did not silence expression of *MEIS1* or *MEIS2*.

Co-expression of TLX1 and MEIS in primary leukemic specimens from T-ALL patients

The co-expression studies were extended to include patient specimens. Forty independent pediatric T-ALL bone marrow specimens, obtained at diagnosis, were assessed for expression of *TLX1*, *MEIS1* and *MEIS2* by qRT-PCR. Reflecting the expression pattern seen in the T-ALL cell lines, *MEIS1/2* expression was detected in the majority of patient specimens (37/40 or 92%). Specifically, *MEIS1* expression was detectable in 88% (35/40), *MEIS2* expression was detectable in 62% (25/40), with co-expression of both *MEIS* genes detectable in 58% (23/40) of the paediatric T-ALL specimens. Of all patient specimens, 25% (10/40) had detectable *TLX1* expression. *MEIS* was co-expressed in all but one *TLX1*-positive patient specimen (Fig. 4B). Of 10 *TLX1*-positive patient specimens, 90% (9/10) had detectable *MEIS* expression: 20% (2/10) co-expressed *TLX1* and *MEIS1*, 10% (1/10) co-expressed *HOX11* and *MEIS2*, while 60% (6/10) expressed *TLX1* and both *MEIS* genes. Taken together, the results of this study show that TLX1 interacts with MEIS proteins, and that both transcripts are co-expressed in T-ALL and cell lines and tumors.

Discussion

Identifying the interacting protein components of TLX1-containing complexes can provide insight into its functional networks and thereby help to elucidate its role in tumorigenesis [14], [19], [20], [21], [26] and [33]. Abundant research has shown that HOX/MEIS and

HOX/MEIS/PBX protein interactions play critical roles in both development and oncogenesis [24], [25] and [35]. Accordingly, we investigated whether PBX and MEIS proteins could function as co-factors of the TLX1 homeoprotein. Using yeast two-hybrid interaction studies the interactions of TLX1 with MEIS were found to be of comparable affinity to interactions of TLX1 with various PBX proteins tested in parallel. Moreover, co-IP studies confirmed MEIS proteins as novel partners for the TLX1 oncoprotein. Consistent with the principles established for HOX/TALE interactions, TLX1 interaction with MEIS proteins required the amino-terminal portion of the TLX1 protein (excluding the first 50 amino acids), while interactions with PBX proteins required both the homeodomain and YPWMR motif of TLX1.

Previously, PBX proteins, including the long isoforms of PBX1/2/3, were reported to interact with TLX1 in gel-shift assays [26]. The yeast interaction results reported here agree with these findings, and expand these TLX1 interactors to include the shorter PBX3 isoforms, PBX3C and PBX3D. However, Allen et al. [26] were unable to detect TLX1 interaction with MEIS proteins on DNA. Technical considerations are the likely reason for the difference in results. For example, gel-shift assays require optimal DNA sequences to bind protein complexes successfully.

Having established that MEIS proteins are novel partners for TLX1, we investigated the co-expression patterns of *TLX1*, *MEIS1* and *MEIS2* in T-ALL cell lines and patient specimens. Both *TLX1*-expressing cell lines co-expressed both *MEIS* genes, and nearly all (90%) of the *TLX1*-positive T-ALL patient specimens also expressed either *MEIS1*, *MEIS2*, or both genes. The co-expression of *MEIS* with *TLX1* in T-lineage neoplasms potentially enables the interaction of these proteins to play a pivotal role in *TLX1*-driven oncogenesis. In the oncogenic collaboration between HOX and MEIS proteins, there is mounting evidence that the initiating oncogenic factor is the aberrant expression of the *HOX* gene. Also called the “co-operative differentiation arrest” model [36], this theory proposes that deregulation of the *HOX* gene arrests cell differentiation, thus committing the cell to oncogenic transformation along a particular lineage. TLX1 interaction with MEIS proteins could also be of significance in the normal context since, like *Meis*, *Tlx1* is involved in the development of the central nervous system [37].

In murine models, deregulation of *TLX1* alone has failed to recapitulate leukemic disease analogous to the T-ALL phenotype seen in humans [6], [7] and [9], indicating a requirement for co-operating factors or events. Similarly, the biological pathways controlled by TLX1 are only partially characterized, yet are critical to understanding TLX1 function. Notably, *MEIS* expression in T-lineage neoplasms has not been widely examined to date. Instead, the majority of studies have focused on myeloid leukemia, where MEIS proteins can function co-operatively with HOX proteins in leukemogenesis[25], and neuroblastoma [38], where *TLX1* transcripts have also been identified [39]. Here, assessment of expression of *TLX1* and *MEIS* genes in T-ALL cell lines and primary patient specimens established a strong pattern of co-expression and suggested that TLX1 and MEIS have the potential to co-operate in T-cell leukemogenesis. Further studies are now warranted to functionally confirm whether MEIS factors are critical co-factors of normal and/or oncogenic TLX1 function.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

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Author Contributions. NM, NG, JF and DD performed the experiments and contributed to data analysis and interpretation. NM and WG wrote the manuscript. PM, WG and UK designed the study, interpreted the data, and critically revised the manuscript.

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Table 1. TLX1 interacts with multiple TALE homeoproteins.

Bait (pGilda)	Prey (pJG4-5)						
	Vector	PREP1	TLX1-AD[a]	TLX1N	TLX1H	TLX1C	TLX1[a]
Vector							
MEIS1		±	+	+			+
MEIS2A		+	++	+++			++
PREP1							
PBX1A		+	±	+			±
PBX1B		+	+	++			++
PBX2		++++	++	+	+		++
PBX3A							
PBX3B		++++	+++	+++	+	±	+++
PBX3C			±				No growth
PBX3D			±				No growth
SCL							
GATA3							

Prey (pJG4-5)	Bait (pGilda)					
	Vector	PREP1	TLX1-AD	TLX1N	TLX1H	TLX1C	SCL
Vector							
MEIS1			+	+			
MEIS2A			+	+			
PREP1							
PBX1A		+++++					
PBX1B		++++		+			
PBX2		+++++	+				
PBX3A							
PBX3B		+++++	++++	+	++		
PBX3C			++++	+	++		
PBX3D			+	+			
SCL							
GATA3							

Replica-plated yeast diploids were assessed for protein interactions as indicated by *LacZ* reporter activation (scored by color intensity after 2 days). The results were averaged across three (TLX1 as preys, upper half) or two (TLX1 as baits, lower half) independent experiments, and scorings were corrected for background activation (bait plus prey vector) before tabulation. Assessment of activation was performed as follows: ++++ = very strong; +++ = strong; ++ = intermediate; + = weak; ± = minimal; blank = none. TLX1, 1–331aa (full-length); TLX1-AD, 51–331aa; TLXN, 1–196aa; TLX1H, 151–273aa; TLX1C, 190–331aa.

[a]TLX1 and TLX1-AD matings demonstrated poor growth compared to other diploid patches.

Fig. 1. TLX1/MEIS interactions in yeast. MEIS bait transformants were mated with TLX1 and PREP1 prey transformants and the resulting diploids examined for activation of the *LacZ* reporter on selective medium containing X-gal. All TLX1 preys interacted with the MEIS1 and MEIS2A baits, with varying intensities of reporter activation, measuring the relative strength of the interaction. TLX1, 1–331aa (full-length); TLX1-AD, 51–331aa; TLXN, 1–196aa.

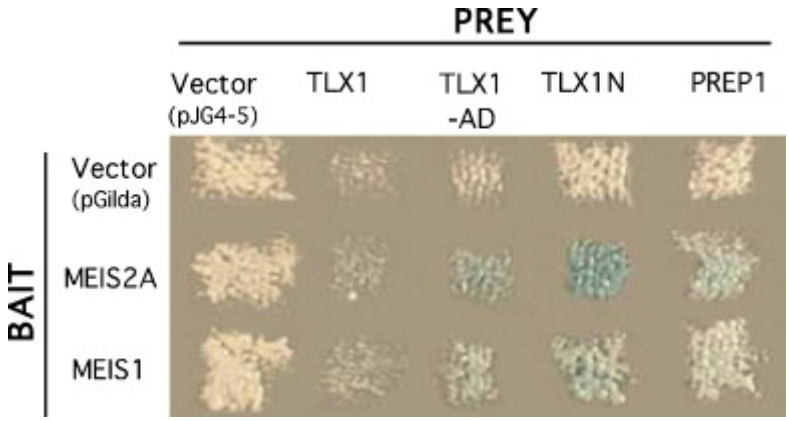

Fig. 2. Association of TLX1 and MEIS proteins in yeast cells. (A) HA-tagged TLX1 protein extracted from induced yeast cultures was immunoprecipitated with α-HA (pre-bound to magnetic beads). Co-immunoprecipitated MEIS1 (M1), MEIS2A (M2) or PBX1B (P1b) LexA-fusion proteins were detected in immunoblots using α-LexA antibody (left panel). Immunoprecipitations with control beads (no antibody) were performed and blotted in parallel (right panel). (B) Protein lysates equivalent to 15% of co-IP input were probed with α-LexA (top panel) and α-HA (bottom panel) to determine the relative expression levels from MEIS/PBX and TLX1 constructs in the different cell extracts.

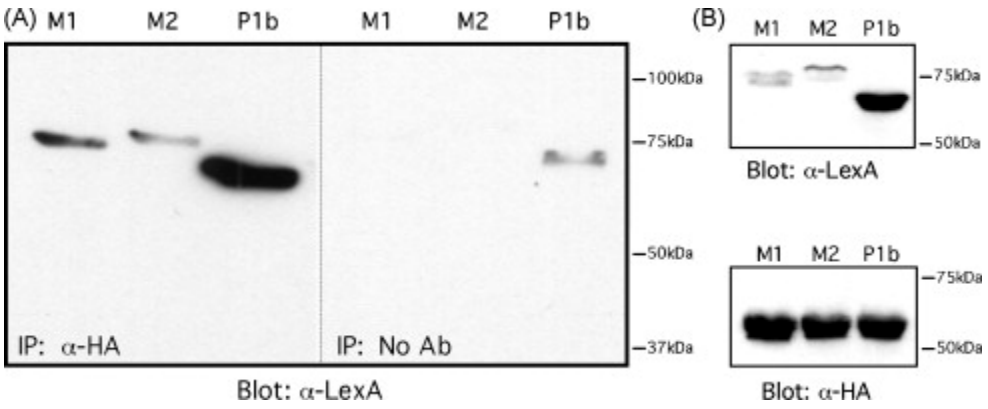

Fig. 3. Association of TLX1 and MEIS proteins in mammalian cells. (A) HA-tagged MEIS proteins were immunoprecipitated from transfected HSB-2 cells and co-immunoprecipitated TLX1 was detected in immunoblots using α-TLX1 antibody. Control immunoprecipitations without antibody were performed and blotted in parallel. Upper panel, MEIS1; lower panel, MEIS2A. (B) Protein lysates equivalent to 20% of co-IP input were probed with α-HA or α-TLX1 to demonstrate antibody specificity. Upper panel, MEIS1 transfectants; lower panel, MEIS2A transfectants.

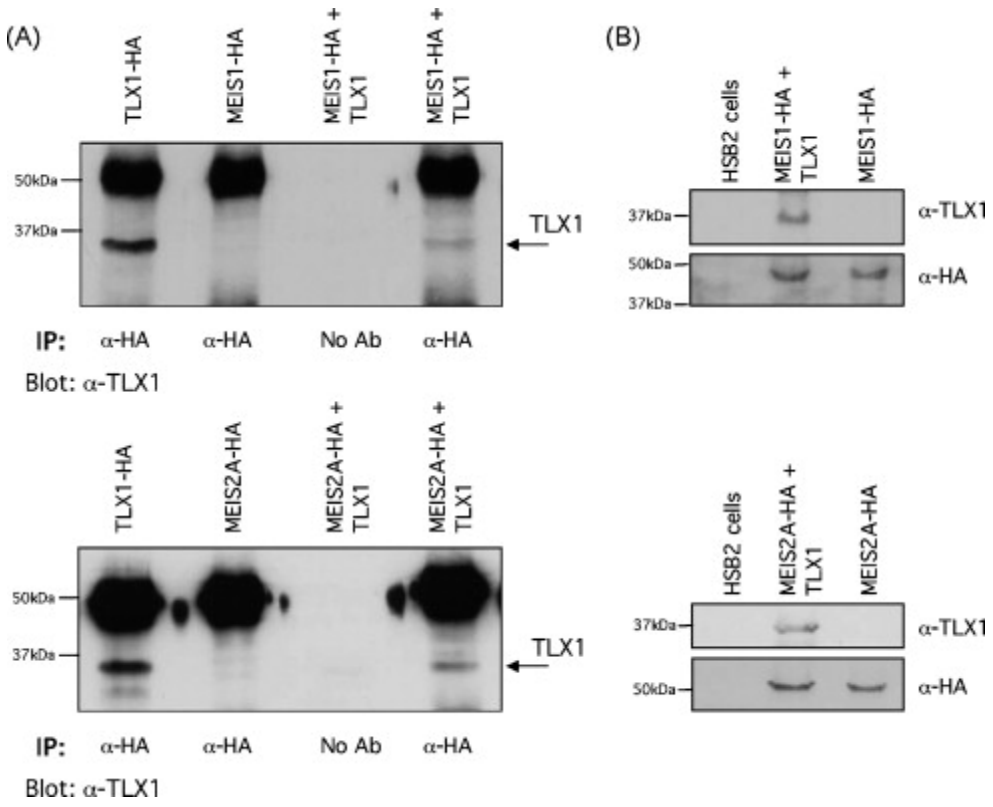

Fig. 4. Co-expression of *TLX1* and *MEIS* genes in T-ALL cell lines and primary patient specimens. (A) T-ALL cell lines and normal lymphoid cells were screened by RT-PCR for *TLX1*, *MEIS1* and *MEIS2* expression. *GAPDH* was amplified as a control for the amount of cDNA used in each reaction. Total RNA from K562 cells and PER-117 cells stably transfected with *TLX1*, were included as positive controls for *MEIS1*/*2*expression and *TLX1* expression, respectively. (B) Frequency histogram showing *MEIS1* and *MEIS2*expression status as determined by qRT-PCR analysis in 10 primary T-ALL tumors positive for *TLX1*expression.