Cancer Cell
Article

TLX Homeodomain Oncogenes Mediate T Cell Maturation Arrest in T-ALL via Interaction with ETS1 and Suppression of TCRα Gene Expression

Saïda Dadi,[1,3,9] Sandrine Le Noir,[1,9] Dominique Payet-Bornet,[3,9] Ludovic Lhermitte,[1] Joaquin Zacarias-Cabeza,[3] Julie Bergeron,[1] Patrick Villarèse,[1] Elodie Vachez,[3] Willem A. Dik,[4] Corinne Millien,[1] Isabelle Radford,[2] Els Verhoeyen,[5] François-Loïc Cosset,[5] Arnaud Petit,[6] Norbert Ifrah,[7] Hervé Dombret,[8] Olivier Hermine,[1] Salvatore Spicuglia,[3] Anton W. Langerak,[4] Elizabeth A. Macintyre,[1,10] Bertrand Nadel,[3,10] Pierre Ferrier,[3,10,*] and Vahid Asnafi[1,10,*]

[1]Department of Hematologye, Université de Médecine Paris Descartes Sorbonne Cité, Centre National de la Recherche Scientifique (CNRS) UMR8147

[2]Department of Cytogenetics, Université Paris 5-Descartes
Assistance Publique-Hôpitaux de Paris, Hôpital Necker-Enfants-Malades, Paris, 75015, France

[3]Centre d'Immunologie de Marseille-Luminy (CIML), Institut National de la Santé et de la Recherche Médicale (Inserm U1104), CNRS UMR7280, Université de la Méditerranée, 13009 Marseille, France

[4]Department of Immunology, Erasmus MC, University Medical Center, 3016 Rotterdam, Netherlands

[5]Université de Lyon, F69000; Inserm, EVIR, U758, Human Virology Department, F-69007; Ecole Normale Supérieure de Lyon, F-69007; Université Lyon 1, F-69007, Lyon, France

[6]Department of Hematology, AP-HP Hôpital Armand Trousseau, Paris 75012, France

[7]Department of Hematology, Centre Hospitalier, Angers 49933, France

[8]Department of Hematology, AP-HP Hôpital St-Louis, Paris 75010, France

[9]These authors contributed equally to this work

[10]These authors contributed equally to this work

*Correspondence: vahid.asnafi@nck.aphp.fr (V.A.), ferrier@ciml.univ-mrs.fr (P.F.)

DOI 10.1016/j.ccr.2012.02.013

SUMMARY

Acute lymphoblastic leukemias (ALLs) are characterized by multistep oncogenic processes leading to cell-differentiation arrest and proliferation. Specific abrogation of maturation blockage constitutes a promising therapeutic option in cancer, which requires precise understanding of the underlying molecular mechanisms. We show that the cortical thymic maturation arrest in T-lineage ALLs that overexpress TLX1 or TLX3 is due to binding of TLX1/TLX3 to ETS1, leading to repression of T cell receptor (TCR) α enhanceosome activity and blocked TCR-Jα rearrangement. TLX1/TLX3 abrogation or enforced TCRαβ expression leads to TCRα rearrangement and apoptosis. Importantly, the autoextinction of clones carrying TCRα-driven TLX1 expression supports TLX "addiction" in TLX-positive leukemias and provides further rationale for targeted therapy based on disruption of TLX1/TLX3.

INTRODUCTION

Acute leukemias are characterized by a multistep oncogenic process leading to the maturation arrest and malignant transformation of a hematopoietic precursor (Pui et al., 2004). Understanding of the molecular mechanisms leading to this block in maturation is a prerequisite for the development of therapeutic approaches aiming to unblock differentiation of leukemic blasts (Look, 1997). Transcription factors (TFs) that are involved in the control of cell differentiation or proliferation of normal

Significance

Targeted therapy, including abrogation of a block to cell maturation, is a promising therapeutic approach in leukemias but has so far proved difficult to implement, partly due to their multioncogenic nature, especially in T-ALL where few oncogenes/tumor suppressors have emerged as tangible candidates. Among T-ALL patients, aberrant expression of TLX1/TLX3 defines a large subgroup of leukemias with a cortical thymic developmental arrest. Our data show that this differentiation block is due to failure to rearrange TCRα and that sustained TLX expression is required for leukemic maintenance despite the acquisition of a variety of additional genetic abnormalities. Taken together, our results provide further rationale for targeted therapy based on disruption of TLX1/TLX3 in this T-ALL subset.

hematopoietic progenitors and are deregulated in acute leukemias could represent promising therapeutic targets, as described for the treatment of PML-RARA$^+$ acute promyelocytic leukemia (Degos, 1992).

Human T-lymphocyte ontogeny is a hierarchical process occurring in the thymus in which the ordered somatic recombination of V, D, and J gene segments at the TCRδ, TCRγ, TCRβ, or TCRα loci determine the development into either γδ or αβ T cell lineages (Dik et al., 2005; Spits, 2002) (Figure S1 available online). Progressive lineage restriction and acquisition of T cell potential following migration from the bone marrow to the thymus involve successive differentiation steps defined by the acquisition of a number of surface molecules, including CD5, CD1a, CD34, CD3, CD4, and CD8. TCRδ rearrangement is the first to occur, at the CD5$^+$, CD1a$^-$ CD4/8 double-negative (DN) stage, followed by concurrent TCRγ and TCRβ rearrangements coinciding with CD1a expression (Dik et al., 2005). While the occurrence of productive TCRγ and TCRδ rearrangements will determine the assembly of a γδ receptor, a complete productive TCRβ gene rearrangement will first allow surface expression of a pre-TCR complex formed by the assembly of the TCRβ chain with a pre-Tα (pTα) invariant chain. Pre-TCR surface expression, referred to as the β-selection process, is marked by arrest of TCRβ gene rearrangements and extensive cellular expansion. It is mandatory to the progression of αβ T cell precursors to the CD4/CD8 double-positive (DP) cortical thymic cell stage, and to the initiation of Vα-Jα rearrangements (von Boehmer et al., 1998). The TCRδ locus being interspersed between the Vα and Jα segments, it is deleted out during Vα-Jα recombination, marking definitive engagement to the TCRαβ lineage. The "frontiers" of the TCRδ locus may be regarded as being defined by the 5′δRec and 3′ψJα elements, as all 5′V and 3′Jα gene segments located outside these segments contribute to the functional repertoire of αβ T cells, including a few Vα/Vδ gene segments that occasionally recombine with Jδ gene segments (Krangel et al., 1998). The TCRα rearrangement is a highly regulated process, in which the TCRα enhancer (Eα) plays a primary role (Bassing et al., 2003; Sleckman et al., 1997). The molecular regulation of Eα has been intensively studied (Hawwari and Krangel, 2005; Ho et al., 1989, 1990; McMurry and Krangel, 2000). The minimal Eα core contains binding sites for three TFs, LEF-1, RUNX1/AML1, and ETS1, which have been demonstrated to be crucial for the transcriptional and *cis*-chromatin opening activities of the so-called Eα enhanceosome (Giese et al., 1995; Ho et al., 1989, 1990; Roberts et al., 1997).

T-ALL is a heterogeneous group of acute leukemias that are arrested at various stages of normal thymic-cell differentiation (Asnafi et al., 2003; Ferrando et al., 2002). Recognized T-ALL oncogenic events include transcriptional activation of proto-oncogenes, submicroscopic deletion of tumor suppressor genes, and activation of the Notch1 pathway by *NOTCH1* or *FBXW7* mutations (Aifantis et al., 2008). Among the various T-ALL oncogenic alterations reported to date, TCR chromosomal translocations represent a recurrent oncogenic hallmark of T-ALL (Cauwelier et al., 2006). Such translocations are generally believed to result from illegitimate V(D)J recombination events that lead to the ectopic activation of oncogenes owing to their relocation to the vicinity of potent *cis*-activating elements within the involved TCR locus.

Overexpression of the orphan homeobox (HOX) proteins TLX1 and TLX3 represents the most frequent oncogenic event due to chromosomal translocation in human T-ALL. TLX1 and TLX3 belong to the NKL subtype of HOX proteins. They contain a highly conserved homeodomain (HD) that is known to be involved in DNA and protein-protein interactions (Holland et al., 2007). In T-ALL, both proteins are associated with specific gene expression profiles, a variety of additional genetic mutations, and differentiation arrest at an early cortical stage of thymocyte maturation (Asnafi et al., 2004; Ferrando et al., 2002; Soulier et al., 2005). Physiological expression of *TLX1* and *TLX3* is restricted to embryonic development (Roberts et al., 1994; Shirasawa et al., 2000), and no specific function of these genes in the T cell lineage has been reported. Transgenic expression of human *TLX1* in mice induces an initial DN2 thymic block followed by development of aneuploid T-ALL, mitotic checkpoint defects, clonal TCRβ rearrangements, a mostly cortical phenotype, and a transcriptional profile similar to that observed in human TLX1$^+$ T-ALLs (De Keersmaecker et al., 2010). Corresponding data are not yet available for TLX3, and the molecular mechanisms underlying the observed developmental arrest in TLX1$^+$ and TLX3$^+$ T-ALLs remain elusive. We therefore undertook to determine if and how TLX oncoproteins were linked to the cortical thymic maturation arrest and what the impact of this specific oncogenic function was on the initiation, development, and maintenance of TLX$^+$ T-ALLs.

RESULTS

TLX-Expressing T-ALLs Undergo VβDJβ Rearrangements but Display a Strong Bias against Vα-Jα Joints

A series of 230 T-ALLs were analyzed by real-time quantitative PCR (RT-qPCR) for *TLX1* and *TLX3* expression. Applying a TCR-based classification (Asnafi et al., 2003), 52 cases were immature/uncommitted (surface (s) and cytoplasmic (c) TCRβ negative) and comprised IM0, IMδ, and IMγ subtypes (i.e., harboring, respectively, a germline configuration of all three TCRβ, δ, and γ loci, or a TCRδ- or TCRγ-rearranged locus, and in some cases with an incompletely rearranged DJβ locus); 103 cases were early cortical IMβ/pre-αβ and included IMβ and pre-αβ subtypes (displaying VβDJβ rearrangement and, respectively, either a cTCRβ$^-$ or sTCR$^-$/cTCRβ$^+$ phenotype); 39 cases were sTCRαβ$^+$; and 36 were sTCRγδ$^+$ (of which 20 also harbored a VβDJβ-rearranged locus) (Table S1). Sixty-five cases (28%) demonstrated *TLX1* or *TLX3* overexpression in a mutually exclusive manner, and were henceforth referred to as TLX$^+$. Among TLX$^+$ T-ALLs, all but one demonstrated at least one VβDJβ rearrangement; this included all TCRγδ-expressing cases ($p < 0.001$). Strikingly, none of the TLX$^+$ samples displayed a sTCRαβ$^+$ phenotype, implying a uniform arrest in maturation at an early cortical cell stage, prior to TCRα chain expression.

To determine whether the lack of TCRαβ expression in TLX$^+$ T-ALLs was due to a defect in TCRα gene rearrangement or TCRα chain expression, we analyzed, by Southern blotting and DNA PCR, the status of the TCRδ locus (which is deleted during Vα-to-Jα rearrangement) in 52 phenotypically matched IMβ/pre-αβ T-ALLs (Asnafi et al., 2003), including 26 TLX$^+$ (13 each of TLX1 and TLX3) and 26 TLX$^-$ T-ALLs (Table 1). Remarkably,

Table 1. TCRδ Allele Rearrangement Status in IMβ/Pre-αβ TLX$^+$ (n = 26) and TLX$^-$ (n = 26) T-ALL

IMβ/Pre-αβ T-ALL TCRδ Allelic Status	TLX$^+$ (n = 52)	TLX$^-$ (n = 52)	p Value
GL, D-D, D-J or V-D	6 (11%)	5 (10%)	ns
VDJ	37[a] (71%)	10[b] (19%)	< 0.001
Deletion	3 (6%)	31 (60%)	< 0.001
Translocated alleles	6 (11%)	6 (11%)	ns

See also Table S1 and Figures S1 and S2.
[a]Includes 15 atypical rearrangements (seven Vα-Jδ, three Vδ8-Jδ1, one Vδ6-Jδ2, and one Vδ7-Jδ1) and 20 typical rearrangements using conventional Vδ1–3 segments.
[b]Only one atypical rearrangement (Vδ5-Jδ1).

49 of 52 (94%) TCRδ alleles were readily detected (i.e., were not deleted) in TLX$^+$ T-ALLs compared with 21 of 52 (40%) TCRδ alleles in TLX$^-$ controls ($p < 0.001$). The TCRδ locus may be regarded as defined by the 5′δRec and 3′ψJα elements (Figure S1), as all functional 5′V and 3′Jα gene segments located outside these limits were found to potentially undergo rearrangement in αβT cells, including a few Vα/Vδ gene segments that occasionally recombine with Jδ gene segments (Krangel et al., 1998). Thorough analysis of TCRδ rearrangements in TLX$^+$ versus TLX$^-$ samples by PCR cloning and sequencing demonstrated that the majority of the former samples harbored complete VδDJδ joints with, intriguingly, a high proportion involving TCRδ-specific, distal 5′ Vδ gene segments (Vδ4–6), dual TCRδ/α (Vδ7, Vδ8), or even TCRα-specific (Vα) gene segments, accounting for a total of 41% of the VDJ rearranged alleles compared to only 10% in TLX$^-$ cases (Table 1). Overall, TLX1$^+$ and TLX3$^+$ T-ALL samples displayed no significant difference in their profiles of TCRδ locus rearrangement (data not shown). These results, coupled with the lower level of TCRδ locus deletion and absence of TCRαβ expression, strongly argue for a block in V-to-Jα rearrangement in TLX$^+$ versus TLX$^-$ T-ALL samples. As Eα is required for optimal Vα-to-Jα recombination (Bassing et al., 2003; Sleckman et al., 1997), we hypothesized that Eα activity could be compromised during the course of TLX$^+$ T-ALL leukemic transformation in humans.

TLX$^+$ T-ALL Show Reduced Accessibility of the TCRα Locus

In the mouse, Eα is occupied by dedicated TFs from the CD44^{lo}CD25$^+$ (DN3) stage of thymocyte development onward, well before transcriptional and recombinational activation of—and, indeed, establishment of chromosomal accessibility at—the TCR-Jα/Cα locus, arguing that Eα binding by TFs is dissociated from its functional activity (Hernández-Munain et al., 1999; Mauvieux et al., 2003; Spicuglia et al., 2000). In order to assess the chromosomal status of the 3′ part of the TCRα locus, including Eα, in human TLX$^+$ T-ALL, we used the formaldehyde-assisted isolation of regulatory elements (FAIRE) assay, which allows the isolation of nucleosome-depleted (hence, mainly accessible) genomic DNA regions (Giresi et al., 2007). Using two primary TLX$^+$ T-ALLs, we recovered large amounts of Eα-overlapping chromosomal DNA, but the recovery of upstream DNA sequences containing the TEA promoter or the Jα58 or Jα28 segments was within the range observed for the unrelated T-ALL repressed genes PCDHGA12 and RPIB9 (Figure 1A and data not shown). In addition, quantification of the expression by RT-qPCR demonstrated a drastic decrease (~100-fold on average) of Cα transcripts in TLX$^+$ T-ALL compared with TLX$^-$ T-ALL (Figure 1B), corroborating microarray data on gene-expression analysis of similar leukemia samples (Ferrando et al., 2002). Finally, using chromatin-immunoprecipitation (ChIP)-on-chip assays we found that the H3K27me3 mark, a hallmark of silent chromatin, is significantly enriched in the TCR-Jα/Cα genomic region in TLX$^+$ T-ALL compared to TLX$^-$ T-ALL (Figures 1C and S2). In contrast, similar H3K27me3 profiles were observed in all T-ALL samples in the repressed (*GATA1*; H3K27me3-enriched) or activated (*GAPDH*; H3K27me3-depleted) control loci. Consistent with the FAIRE data of nucleosomal depletion along Eα-containing sequences, H3K27me3 enrichment in TLX$^+$ T-ALL closely surrounded but spared this discrete region. Overall, these findings imply that the unrearranged TCR-Jα/Cα alleles typically found in TLX$^+$ T-ALL blasts are poorly transcribed and most likely embedded within repressive chromatin, despite localized accessibility of Eα DNA. They further suggest that in the presence of TLX, Eα is unable to confer chromosomal accessibility to the adjacent genomic regions, notably the TCR TEA/Jα-containing region.

TLX1 and TLX3 Repress Eα Activity via Their Homeodomain

To further test the possibility that the TLX1 and TLX3 proteins interfere with Eα transcriptional activity, we used an Eα-dependent reporter that expressed chloramphenicol acetyl transferase (Eα-CAT). We found that ectopic expression of either GFP-TLX1 or GFP-TLX3 repressed Eα-CAT expression by approximately 6-fold, and that this expression was partly rescued by the co-expression of TLX1 or TLX3 siRNA, respectively (Figure 2A). We therefore evaluated the level of Eα-CAT repression exerted by TLX1 and TLX3 mutants with or without their HD (HD$^+$ and HD^{del}, respectively), in reference to that of the corresponding full-length (FL) proteins. As shown in Figure 2B, both TLX1 HD^{del} and TLX3 HD^{del} exerted a reduced repressive activity compared with their respective full-length (FL) proteins. Conversely, TLX1 HD$^+$ exerted an activity roughly equivalent to FL TLX1, whereas TLX3 HD$^+$ retained about half the repressive effect of FL TLX3. We exclude the possibility that the reduced repression activity by TLX1 HD^{del} and TLX3 HD^{del} was due to their lack of nuclear localization by demonstrating that both predominantly localized to the nucleus (Figures 2C and 2D). We conclude that TLX1 and TLX3 repress Eα transcriptional activity primarily in an HD-dependent manner.

TLX1 and TLX3 Exert Their Eα Repressive Activity by Interacting with ETS1

The Eα-mediated transcriptional activity depends on the cooperative binding of the ETS1 and RUNX1 TFs to Eα, together with the lymphoid-specific HMG domain protein LEF1. To test whether TLX1/TLX3-mediated Eα repression is ETS1, RUNX1, or LEF1 dependent, we repeated the reporter assays and removed each TF individually. The fact that Eα-CAT expression was not totally abolished in the absence of any one of these TFs (Giese et al., 1995) makes it possible to quantify repression of the residual activity after individual TF removal. In these conditions,

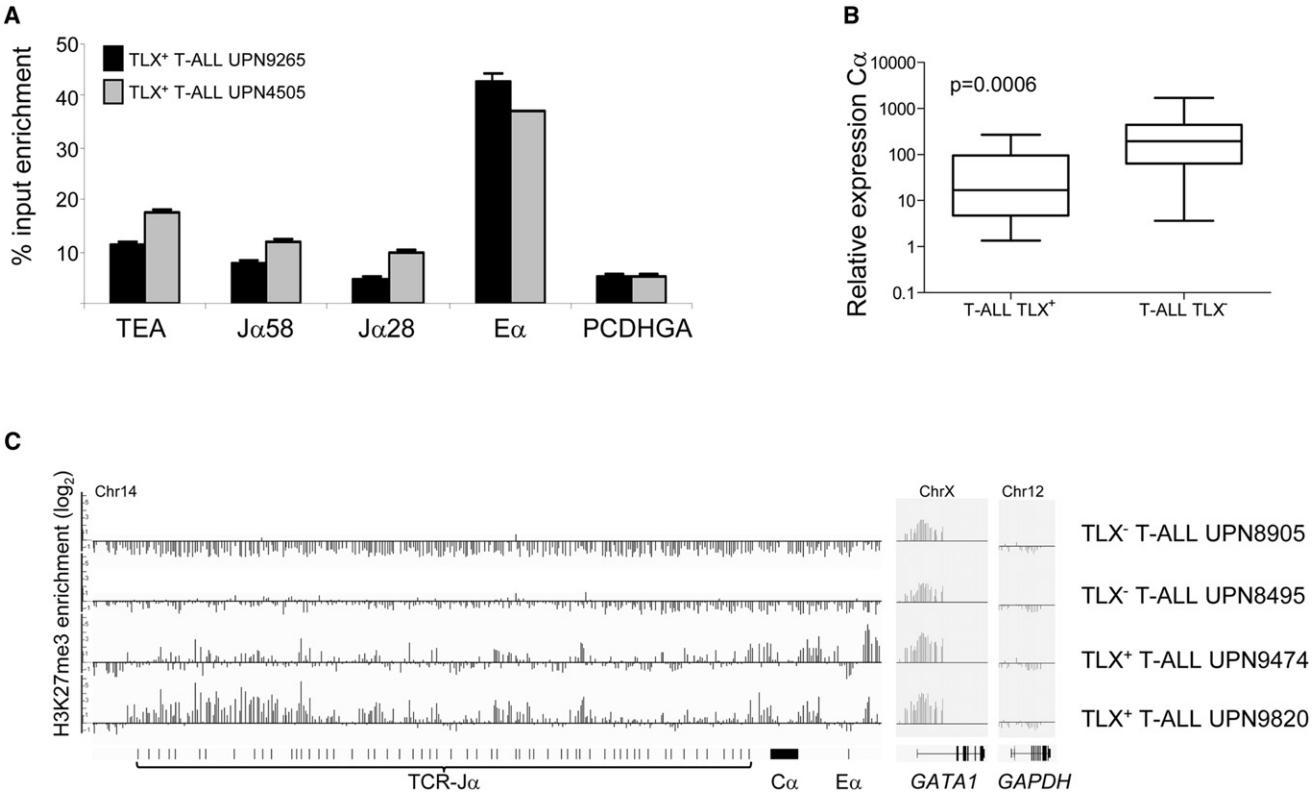

Figure 1. TLX+ T-ALLs Show Reduced Accessibility of the TCRα Locus

(A) Graph showing formaldehyde-assisted isolation of regulatory elements (FAIRE) signals on two TLX+ T-ALL primary cell samples (UPN, unique patient number), as assessed in using RT-qPCR and TEA, Jα58-, Jα28-, Eα-, or PCDHGA (a gene not expressed in T cells)-specific primers. Data represent means of duplicate measurements whereby amplification signals were normalized to those of the corresponding input DNA with error bars to represent ±SD.

(B) Box plots for relative TCR-Cα gene expression normalized to housekeeping gene Abelson1 (ABL) in phenotypically matched TLX+ (n = 23) and TLX− (n = 18) T-ALLs, as assessed by RT-qPCR.

(C) Plots of ChIP-on-chip signals for H3K27me3 relative enrichment in two TLX+ and two TLX− T-ALL cell samples. Black horizontal lines represent the no-change lines. Black vertical bars indicate H3K27me3 peaks. The positions of the TCR-Jα, Cα, and Eα genomic regions, as well as those of exons and upstream promoter sequences in the silent (*GATA1*) and expressed (*GAPDH*) control genes are indicated (exons depicted as vertical traits or black boxes). All four T-ALL samples harbor at least one unrearranged TCRα allele (not shown).

See also Figure S2.

the omission of ETS1 and, to a lesser extent, RUNX1, but not LEF1, diminished TLX1/TLX3-mediated Eα-CAT repression (Figure 2E). We also found that serial diminution of transfected ETS1-expression vector led to a progressive decrease of TLX/TLX3-mediated repression (Figure 2F). Altogether, our data imply that TLX1/TLX3-mediated repression occurs mostly via ETS1.

We therefore carried out in vitro GST-pull-down assays and found that immobilized TLX1 and TLX3 retained the ETS1 FL protein but not LEF1 (Figure 3A). The ETS1 DNA binding domain (DBD) and the TLX1 and TLX3 HD-containing regions appeared to be most important for this interaction (Figure 3A). We further performed streptavidin precipitation experiments using HeLa cells transfected with expressing vectors encoding ETS1-HA-His, LEF1-HA, and TLX1- or TLX3-Flag-SBP tagged proteins. Precipitation of TLX1/TLX3 only retained ETS1 but not LEF1 (Figure 3B). To confirm an interaction between endogenous proteins, we carried out co-immunoprecipitation (Co-IP) using TLX1+ ALL-SIL and TLX3+ DND41 cell lines and anti-TLX1 or TLX3 monoclonal antibody (mAbs), which recovered both ETS1 and the corresponding TLX in the precipitated material (Figure 3C). Finally, we investigated the subcellular localization of TLX1, TLX3, and ETS1 in TLX+ T-ALL clinical samples by cell fractionation and immunostaining. Both analyses demonstrated that TLX1, TLX3, and ETS1 localize in the nucleus (Figures 4A and 4B). Moreover, both TLX+ and TLX− T-ALL expressed similar amounts of ETS1 protein and RNA (Figure 4A and data not shown). Strikingly, the nuclear distribution of ETS1 was diffuse in TLX− patient leukemic cells and cell lines (Figure 4B) but was irregular in TLX+ nuclei and overlapped significantly with that of the TLX proteins (Figure 4C; see figure legend for statistical information). Using ALL-SIL, we showed that, as anticipated, the nuclear distribution of ETS1 became more diffuse following TLX1 downmodulation (Figure 4D).

ETS1 Mediates TLX1 and TLX3 Recruitment to Eα-Associated DNA Sequences

Given the data above in favor of an interaction between TLX1/TLX3 and ETS1 proteins impinging upon Eα activity, we next tested whether TLX1/TLX3 directly interacts with the ETS1

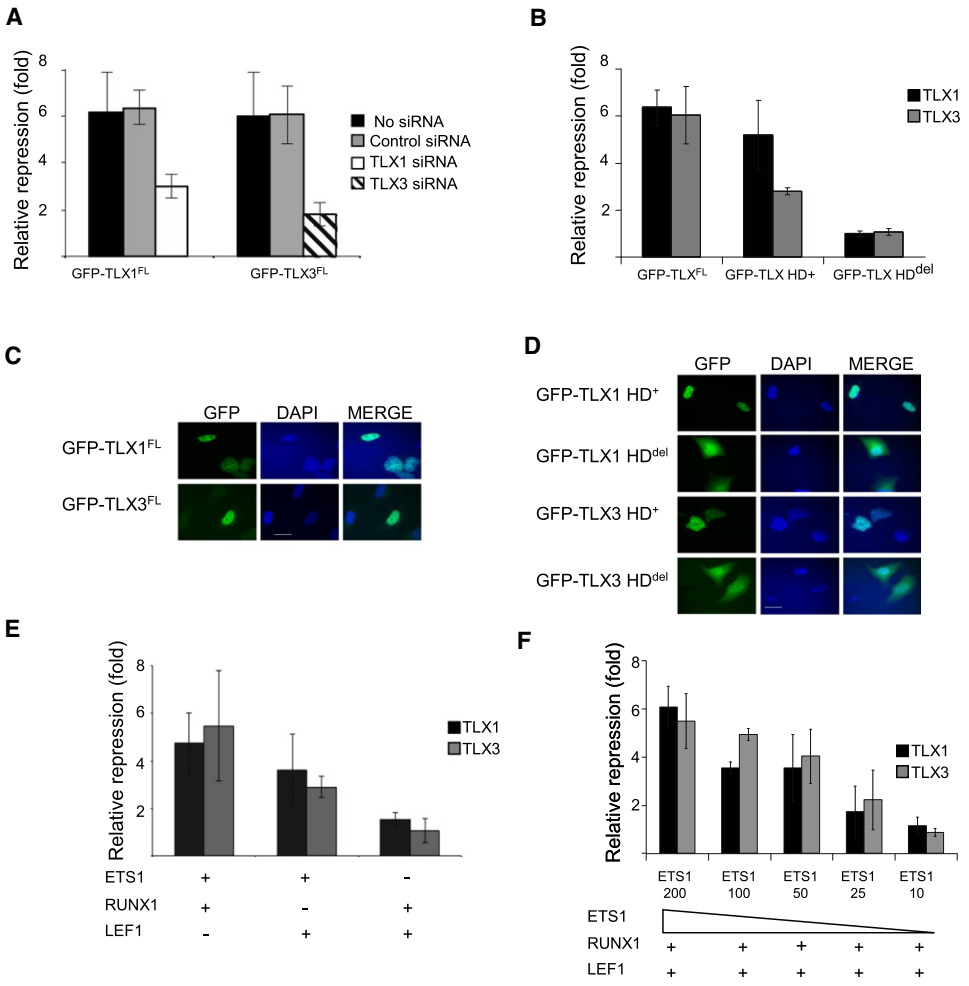

Figure 2. TLX1 and TLX3 Repress Eα-CAT Activity via Their Homeodomain

(A) Graph showing Eα-CAT fold repression in HeLa cells following transfection with GFP-TLX1^{FL} or GFP-TLX3^{FL} encoding vectors, with or without cotransfection of the indicated small interfering (si) RNAs. Data represent means of triplicate measurements whereby Eα-CAT signals were normalized to those of control cells transfected with a GFP vector, with error bars to represent ±SD.

(B) As in (A), but with HeLa cells transfected with GFP-TLX^{FL} vectors alone, or with similar vectors carrying or lacking the corresponding TLX homeodomain (GFP-TLX HD^{+} and GFP-TLX HD^{del}, respectively).

(C and D) Examples of fluorescence microscopic analysis of GFP-TLX expression in transfected HeLa cells depicted in (A) and (B). White scale bar is 10 μm.

(E) As in (A), but with HeLa cells transfected with GFP-TLX^{FL} vectors, plus (+) or minus (−) additional vectors encoding the indicated ETS1, RUNX1, or LEF1 TFs.

(F) As in (E), but using decreasing amounts (200–10 ng) of ETS1-expression vector.

binding sequences (EBS) of human Eα. Using electrophoretic mobility shift assays (EMSA) we observed no significant shift of the EBS probe when incubated with TLX1/TLX3 alone (Figure 5A, lanes 2–7). As expected, incubation with purified recombinant ETS1 led to a major shift of the labeled probe (Figure 5B, lane 1). Strikingly, the addition of TLX1 (lanes 2–4) or TLX3 (lanes 5–7) to ETS1 and EBS produced a dose-dependent super-shifted complex.

To ascertain Eα occupancy by endogenous TLX1 and TLX3 in vivo, we performed ChIP assays using ALL-SIL (TLX1^{+}), DND41 (TLX3^{+}), and, as a control, RPMI-8412 (TLX^{-}) cell lines. The ChIPed DNA was RT-qPCR amplified using Eα-specific oligonucleotide primers and primers specific for the unrelated Actin gene promoter (not bound by either ETS1 or TLX TF; data not shown), used here as a mock control for data normalization. As expected, anti-ETS1 enriched Eα-associated sequences from all three cell lines (Figure 5C). In contrast, anti-TLX1 and anti-TLX3 enriched Eα-associated sequences from only ALL-SIL and DND41, respectively, demonstrating in vivo recruitment of TLX1 and TLX3 onto Eα. Using the DND41 and ALL-SIL cell lines, we verified that knockdown of ETS1 reduced Eα-binding by both ETS1 and TLX1/TLX3 (Figure 5D). Taken together, these data indicate that TLX1 and TLX3 were recruited to Eα via their interaction with ETS1 in TLX^{+} leukemic cells.

TLX Downmodulation and Enforced TCRαβ Expression Both Lead to Redifferentiation Linked with Massive Cell Apoptosis

Our results suggest that one of the major oncogenic functions of TLX1/TLX3 in T-ALL would be to block αβ T cell development by

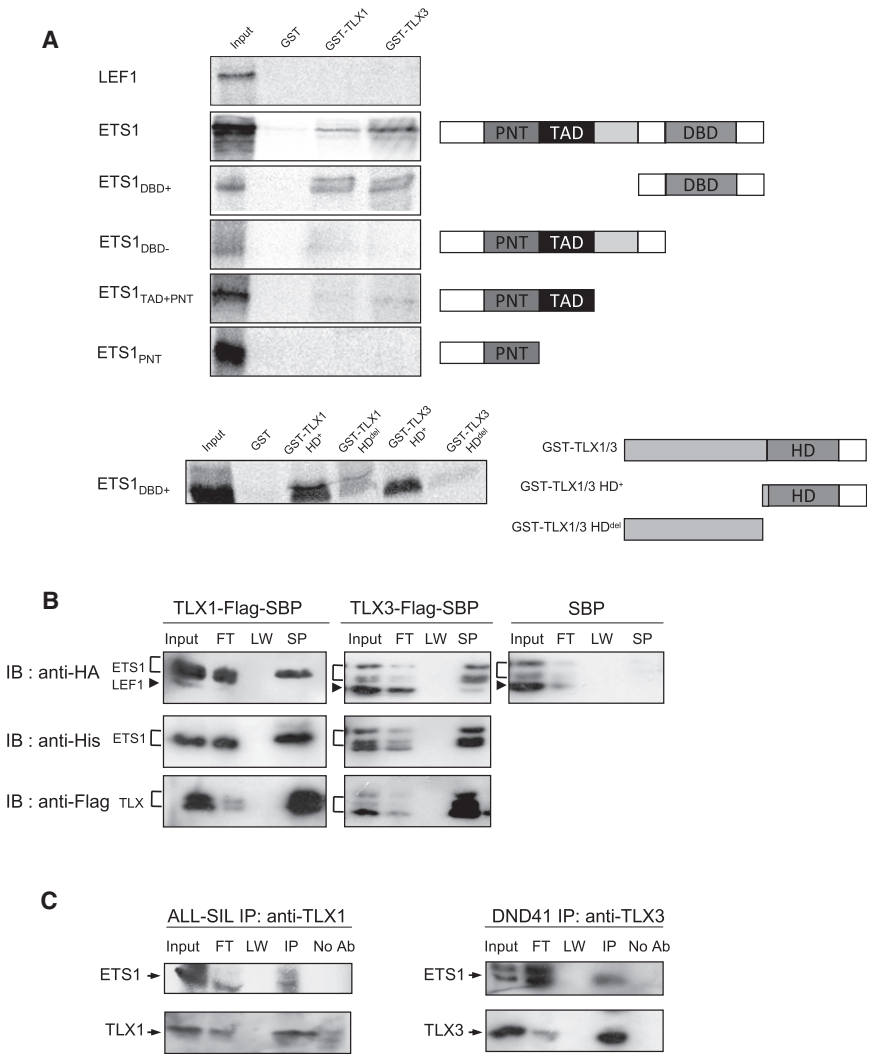

Figure 3. TLX1 and TLX3 Interact with ETS1

(A) GST, GST-TLX1, and GST-TLX3 pull-down of ^{35}S-labeled in vitro translated LEF1, ETS1, or truncated versions of ETS1: ETS1$_{DBD+}$, ETS1$_{DBD-}$, ETS1$_{TAD+PNT}$, and ETS1$_{PNT}$. The various versions of ETS1 are depicted on the right: PNT, pointed domain; TAD, transactivation domain; DBD, DNA binding domain. (Bottom) GST, GST-TLX HD^{+}, and GST-TLX HD^{del} pull-down of ^{35}S-labeled ETS1$_{DBD+}$. TLX and its truncated forms are depicted on the right: HD, homeodomain. The "Input" lanes correspond to 5% of the ^{35}S-labeled protein used for a pull-down experiment.

(B) Cell lysates from HeLa cells cotransfected with vectors expressing ETS1-HA-His, LEF1-HA, and TLX1-(or TLX3-) Flag-SBP were precipitated with streptavidin (SP) beads and then immunoblotted (IB) with either an anti-HA antibody (to reveal the fusion proteins ETS1-HA-His and LEF1-HA), an anti-His antibody (to reveal the ETS1-HA-His), or an anti-Flag antibody (to reveal TLX and to evaluate SP efficiency). Input represents 10% of cell lysate used for SP. FT, flow-through; LW, last wash.

(C) Cell lysates from ALL-SIL and DND41 cells were immunoprecipitated (IP) using anti-TLX1 (left) or anti-TLX3 (right) antibody followed by immunoblotting with indicated antibodies. The "Input" lanes correspond to 10% of cell extracts used in the Co-IP. No Ab, control IP experiment performed without antibody; FT, flow-through; LW, last wash.

inhibiting Eα activity. To further explore this possibility, we knocked down TLX1 and TLX3 expression in ALL-SIL and DND41 cell lines, respectively. While the control cells grew normally, the knockdown TLX1/ALL-SIL and TLX3/DND41 cells demonstrated massive apoptosis (Figure 6A and 6B). Strikingly, both Cα and TEA-Cα transcripts, as markers of TCR-Jα locus activation (Hernández-Munain et al., 1999; Monroe et al., 1999), were upregulated in the TLX knockdown cells (Figure 6C). Moreover, unlike transduced mock controls, the two TLX knockdown cells harbored Vα-Jα rearrangements, although with a restricted polyclonal pattern, as expected (Figure 6D). The knockdown cells further demonstrated a maturation shift, as shown by increased cell size and CD5 expression (Figure 6E). Most important, a small proportion of these cells became sTCRαβ^{+} (Figure 6F).

We then assessed whether such a redifferentiation process, including the triggering of cell death, could be induced by forced expression of sTCRαβ, thus bypassing TLX/ETS1-mediated suppression of Vα-Jα rearrangement, in a TLX^{+}/TCR^{-} T-ALL cell line. TLX1^{+} ALL-SIL cells were transduced using lentiviral multicistronic vectors enabling expression of GFP with or without TCRβ and TCRα (Figure 6G, top). GFP^{+} sTCRαβ^{+} ALL-SIL cells exhibited reduced viability and cell growth compared to GFP^{+} sTCRαβ^{-} controls when cultured in the OP9-DL1 stromal-cell system (Figure 6G, bottom). This correlated with increased apoptosis of the sTCRαβ^{+} cells, as evidenced by propidium iodide (PI)/annexin V dual staining (Figure 6H; note that Jurkat cells carrying an identical sTCRαβ were not affected). It is important to note that massive apoptosis was not observed when transduced cells were cultured in a stromal-cell-free standard culture system (Figures 6G and 6H). These data demonstrate the key role of TCR expression in mediating cell death in TLX^{+} T-ALLs and strongly suggest that the apoptosis observed upon TLX inhibition is a consequence of redifferentiation.

Overall, ectopic expression of TLX1/TLX3 in cortical thymocytes appears to be required to maintain leukemic proliferation, survival, and failure to differentiate; and defect in TCRαβ expression—via ETS1-mediated TLX recruitment onto Eα—is a major mediator in oncogenic addiction, even after acquisition of a variety of additional genetic abnormalities.

TCRα/δ Translocations Occur 5′ to TLX1 Leading to Eα-Independent TLX1 Expression

While chromosomal translocations that lead to *TLX3* deregulation in T-ALLs virtually always involve genomic partners other than *TCRα/δ* (Bernard et al., 2001; Soulier et al., 2005), those leading to *TLX1* deregulation predominantly involve *TCRα/δ*.

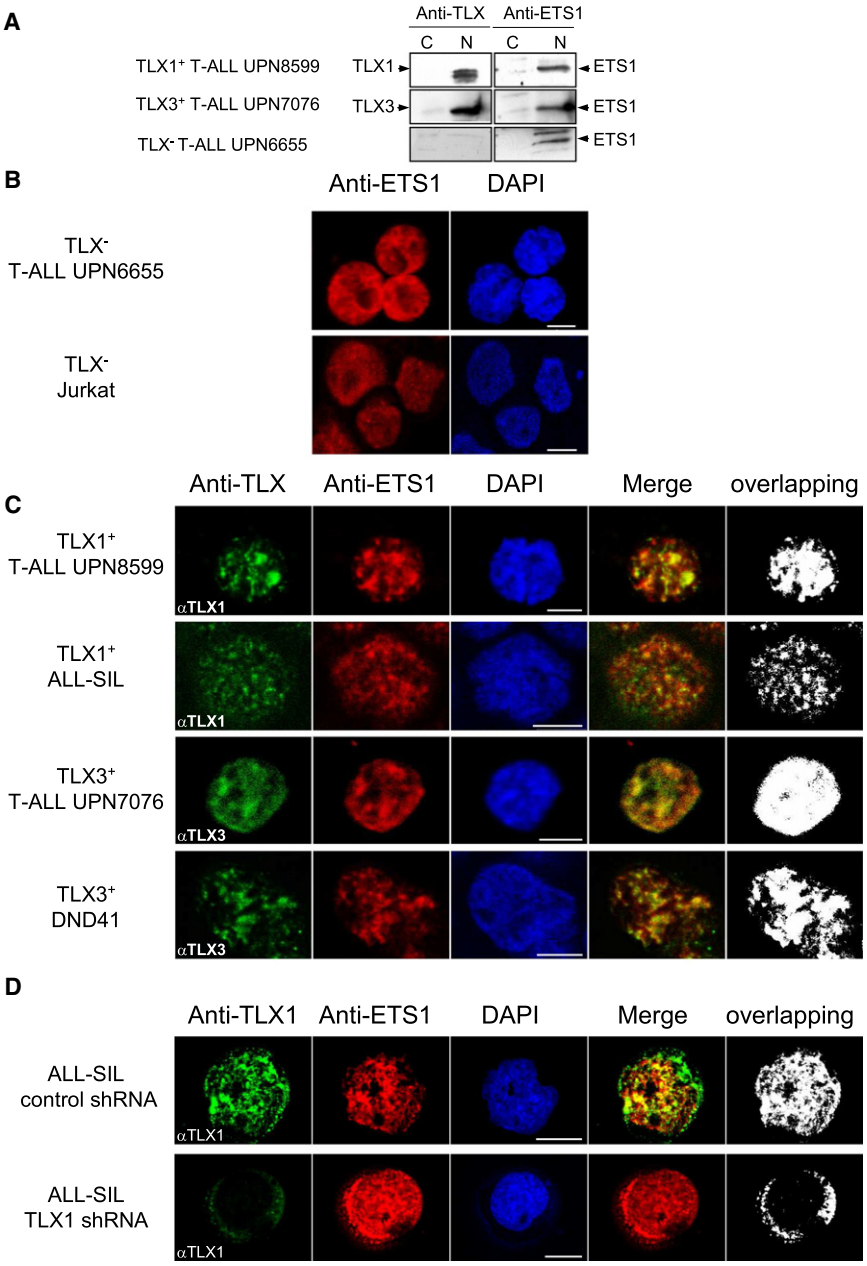

Figure 4. TLX1, TLX3 and ETS1 Colocalize in TLX$^+$ T-ALL Blast Nuclei

(A) Western blot analysis of cytoplasmic (C) and nuclear (N) extracts from TLX1$^+$, TLX1$^+$, and TLX$^-$ T-ALL cells. Blots were probed with anti-TLX and anti-ETS1 antibodies as indicated.

(B) Confocal microscopy analysis of TLX$^-$ T-ALL and cell line (Jurkat) labeled with the indicated fluorescent anti-ETS1 mAb (Alexa 647, red) or stained by DAPI.

(C) Confocal microscopy analysis of the TLX$^+$ T-ALL and cell lines ALL-SIL (TLX1$^+$) and DND41 (TLX3$^+$) labeled with the indicated fluorescent anti-TLX mAb (Alexa 488, green) and anti-ETS1 mAb (Alexa 647, red), or stained by DAPI. The merged images and overlapping areas (determined using ImageJ software) are also shown. Pearson's coefficients for the overlapping areas were as follows: $r = 0{,}851$ (UPN480); $r = 0{,}608$ (ALL-SIL); $r = 0{,}929$ (UPN364); and $r = 0{,}816$ (DND41).White scale bars are 5 μm.

(D) As in (C), but the ALL-SIL cells were transduced with TLX1-specific (TLX1 small-hairpin [sh]RNA) or nonspecific (control shRNA) RNAs as indicated. White scale bars are 5 μm.

However, if a critical oncogenic function of TLX1 is to repress Eα activity, as supported by the data above, how could Eα possibly provide sustained TLX1 expression if it is juxtaposed to and drives TLX1 expression? To get further insight into the mechanism of *TLX1* transcriptional activation, we first analyzed its expression by RT-qPCR in 526 adult and pediatric T-ALLs and identified 61 cases as TLX1$^+$. We then identified *TLX1* translocation into either the *TCRδ* locus (35 cases) or the *TCRβ* locus (12 cases) in the 47 TLX1$^+$ cases with enough material for carrying out FISH- and/or ligation-mediated (LM) PCR analysis. All informative cases tested for allelic transcripts (12 *TCRδ-TLX1* and 2 *TCRβ-TLX1*) demonstrated monoallelic *TLX1* expression, in line with a "standard" regulatory element substitution mechanism of oncogene deregulation in *cis*. We mapped the breakpoints from 8 out of 12 TCRβ-TLX1 alleles and 30 out of 35 *TCRδ-TLX1* alleles (Figure S3). While all 8 breaks from *TCRβ-TLX1* translocations mapped 3′ to *TLX1*, all 30 breaks from TCRδ-TLX1 translocations mapped 5′ to *TLX1* (Figure 7A, top lane). Consequently, whereas *TLX1* activation in *TCRβ-TLX1* fusions was consistent with a classical scenario of TCR-gene-enhancer-mediated activation (in this case, the TCRβ gene enhancer Eβ; Figure 7A, middle lanes), the *TCRδ-TLX1* translocations separated the *TLX1* gene and Eα element on the two derivative chromosomes (Figure 7A, bottom lanes), implying Eα-independent activation and/or maintenance of *TLX1* oncogenic expression.

To investigate whether *TLX1* overexpression resulted from its juxtaposition to a *cis*-regulatory element(s) within and/or upstream to the TCRδ promoter, we performed clonospecific RT-PCR across the breakpoints of both *TCRδ-TLX1* and *TCRβ-TLX1* translocations. Given the structure of these translocations, the detection of fusion transcripts in all the *TCRδ-TLX1* samples, but not in the *TCRβ-TLX1* samples (Figure 7B), was consistent with the presence of a positive regulatory element(s) within and/or upstream to the TCRδ locus that drives *TLX1* overexpression.

Eα Repression Results in a TLX1 Feed-Forward Repression Loop

Despite the obvious difference in the origin of *TLX1* transcriptional activation in *TCRβ* versus *TCRδ* translocations, we observed no significant disparity in their levels of *TLX1* expression

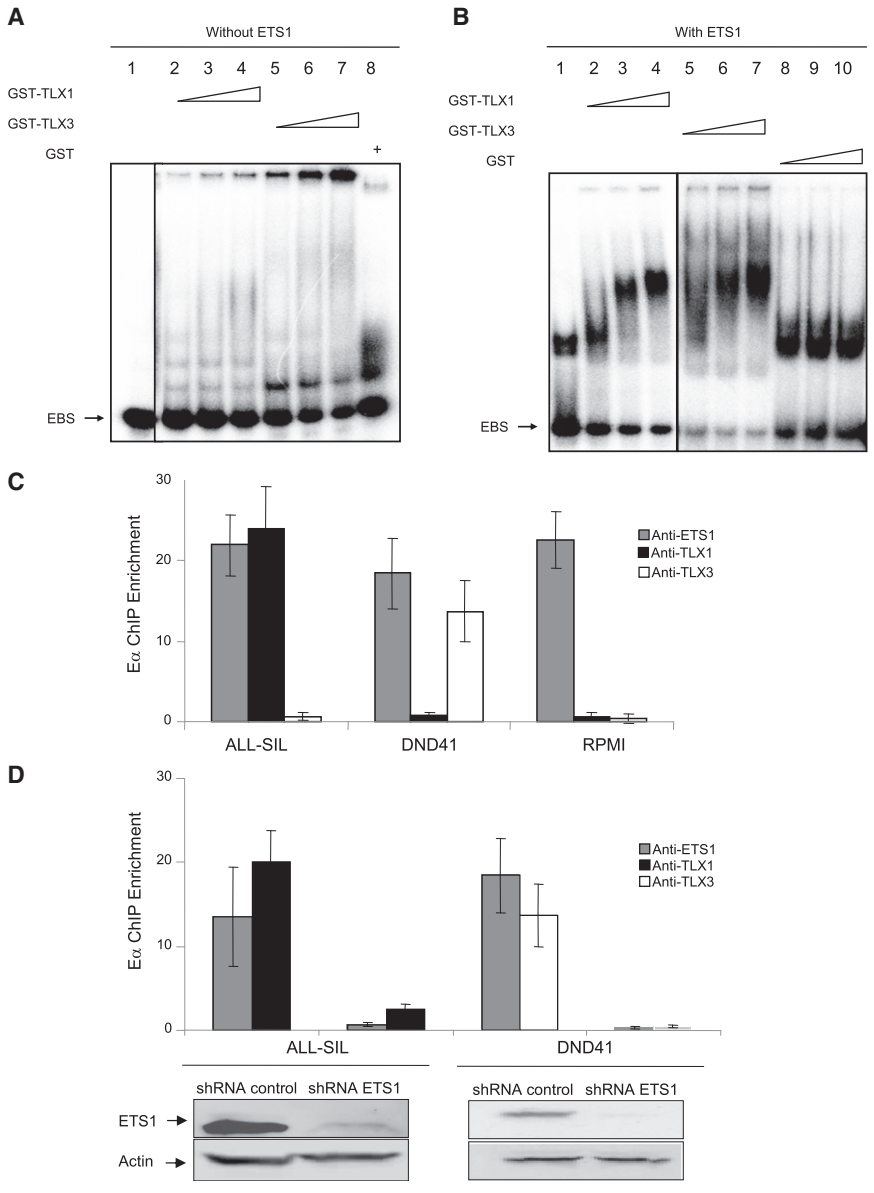

Figure 5. ETS1 Mediates TLX1 and TLX3 Recruitment to Eα-Associated DNA Sequences

(A) EMSA of the ^{32}P-labeled EBS oligonucleotide probe comprising core Eα nucleotide sequences (Giese et al., 1995) with purified recombinant GST-TLX1 and GST-TLX3 proteins. Lane 1, free EBS probe; lanes 2–4 and lanes 5–7 EBS incubated with increasing amounts (10, 50, and 100 ng) of GST-TLX1 or GST-TLX3, respectively; lane 8, EBS incubated with 100 ng of GST.

(B) As in (A), but with the presence of 20 ng of purified recombinant ETS1 protein (lanes 1–7) and ETS1 with increasing amounts (10, 50, and 100 ng) of GST (lanes 8–10).

(C) Graphs of ChIP signals for Eα from the ALL-SIL (TLX1$^+$), DND41 (TLX3$^+$), and RPMI (TLX$^-$) cell lines using anti-ETS1, anti-TLX1, and anti-TLX3 antibodies, as indicated. ChIPed DNA was qPCR amplified using Eα and actin (negative-control)-specific oligonucleotide primers. Enrichment level was determined by comparison to a standard curve from input DNA. ChIPed signal enrichments correspond to the ratios between the Eα signal and actin signal. IgG isotype control was performed to assess absence of nonspecific Eα ChIP enrichment (not shown). Data represent means of triplicate measurements with error bars to represent ±SD.

(D) As in (C), but with the ALL-SIL and DND41cells transduced with ETS1-specific or nonspecific shRNAs. Shown beneath the graph are western blots for ETS1 or actin expression from mock transduced (shRNA control) and knockdown (shRNA ETS1) cells.

(data not shown). This may reflect oncogenic selection of only those rearrangements with sufficient/optimal *TLX1* expression. We also searched for possible differences in the stage of maturation arrest and/or kinetics of *TLX1* activation. TLX1$^+$ T-ALLs are commonly arrested at a cortical TCRαβ negative stage of maturation (Ferrando et al., 2002). In keeping with this, all TLX1$^+$ T-ALL cases (whether *TCRδ* or *TCRβ* translocated) display a uniform cortical CD1a$^+$/CD34^{neg} phenotype (Asnafi et al., 2003; Ferrando et al., 2002). Furthermore, sequence analysis demonstrated that both TCRδ and TCRβ translocations occurred at a similar stage of early thymic-cell differentiation with respect to rearrangement events, since all *TCRδ*- and *TCRβ-TLX1* junctions resulted from repair mistakes introduced during, respectively, a Dδ2-Dδ3 (or Dδ3-Jδ1) rearrangement, or a Dβ-Jβ rearrangement (Figure S3). These data further implied that *TLX1* oncogenic activation uniformly took place at an immature DN/CD1a$^-$/CD34$^+$ stage of thymic cell development, while cell maturation arrest occurred at a later (cortical) stage.

We therefore hypothesized that 3′ *TCRδ-TLX1* translocations do not undergo oncogenic selection because of a feed-forward inhibitory effect exerted by TLX1 itself, inducing autorepression of Eα transcriptional activity. This would infer that TCRα/δ driven translocations with 3′ *TLX1* breakpoints could occur but would fail to be selected within an oncogenic context. As several examples of potentially oncogenic TCR translocations have previously been reported in healthy thymi (Dik et al., 2007; Marculescu et al., 2003, 2006), we searched normal thymus for putative TCRδ-TLX1 translocations with breakpoints involving either side of *TLX1* (Figure 7C, top lanes). We set up a highly sensitive double-nested qPCR allowing the recovery of rare translocation events (10^{-8}–10^{-9}) and applied this assay to screen the DNA equivalent of 10^9 thymocytes from 10 healthy postnatal thymi. Even though no *5′ type* junction was observed, two *3′ type* junctions were reliably detected (Figure 7C, bottom lanes). This demonstrates that *3′ type* TCRδ-TLX1 translocations do occur in the normal thymus and are at least no less common than *5′* translocations. The main difference between the 3′ *TCRβ-TLX1* translocations observed in T cell

leukemias and the 3′ *TCRδ-TLX1* exclusively found in nonleukemic thymi consists in their association with the Eβ or Eα element, respectively. Given the aforementioned inhibition of Eα activity by TLX1, these data support the intriguing possibility that deregulation of *TLX1*, when driven by Eα, will lead not to oncogenic selection but rather to autonomous counterselection of the chromosomal translocation due to feed-forward repression (Figure 7D).

DISCUSSION

HOX proteins in general, and TLX in particular, exert a repressive activity on transcriptional events during embryonic development (Mann et al., 2009; Merabet et al., 2005; Owens et al., 2003; Shen et al., 2001). Our study identifies TCR Eα as a target for such a repressive activity upon ectopic expression in T cell development, offering molecular insight into the stage of maturation arrest and oncogenesis of TLX$^+$ T-ALLs.

The onset of V(D)J recombination is primarily regulated at the level of chromatin and access of the RAG1/RAG2 recombinase apparatus to its DNA targets, a process that depends on the activity of transcriptional enhancers in TCR and Ig loci, including Eα and Eδ in the TCRα/δ locus (Krangel et al., 1998). Our data pinpoint a suppressive chromatin configuration around Eα and a molecular explanation to the strong bias against Vα-to-Jα rearrangement observed in TLX$^+$ T-ALLs. The TCRα repressed structure appears to specifically affect the TEA/Jα-containing region, since in cases where unrearranged TCRα/δ alleles could be analyzed for H3K27me3, the proximal, 3′ part of the TCR-Vα locus behaved similarly in TLX$^+$ and TLX$^-$ blasts (Figure S2). Although proximal Vα segments lie within the range of long-distance chromatin regulation by Eα, an active, derepressed chromatin configuration would be expected at these sequences in early cortical thymocytes due to the activity of the nearby Eδ (Hawwari and Krangel, 2005). In short, the aberrant TCRα/δ recombination patterns seen in TLX$^+$ T-ALL faithfully mirror the chromatin opening function that exclusively relies on Eα.

We have identified the ETS1 TF as a mediator, via protein-protein interaction, of this Eα-suppressing function of TLX1/TLX3. There is precedent for inhibition of a cell-developmental pathway by ETS1, MafB-ETS1 interaction having been reported to result in a block to erythroid differentiation (Sieweke et al., 1996). The precise mechanisms for the repression by TLX remain to be elucidated, but our current data provide interesting clues. Recruitment of both TLX and ETS1 onto Eα render unlikely a basic model whereby TLX sequesters ETS1 away from the enhanceosome; however, more complex, locally induced, opposing molecular switches dependent on the nature of ETS1-interacting TF partners could not be excluded (Sieweke et al., 1996). Likewise, our unpublished findings that TLX1/TLX3 still suppresses transcription on cell treatment with a histone deacetylase (HDAC) inhibitor suggest that HDAC is not involved. The creation of a distinct heterochromatin-promoting complex is plausible, since HOX proteins have been shown to fix polycomb (PcG) repressive components (Papp and Müller, 2006). Our finding of H3K27me3 enrichment along Eα-flanking genomic regions in TLX$^+$ T-ALL supports this, as H3K27me3, in addition to being a marker of repressive chromatin, is associated with activity of the PcG complex PRC2 (Sauvageau and Sauvageau, 2008). This suppressive function for TLX on Eα activity via ETS1 does not exclude protein-protein interaction with and mediation of a suppressive effect by, other TFs, such as RUNX1 (Hollenhorst et al., 2009).

ETS1 plays an important role in cell developmental controls and neoplastic processes (Dittmer, 2003). TLX1/TLX3 overexpression would therefore be expected to deregulate multiple biological networks via its interaction with ETS1. The TLX1/TLX3-ETS1-mediated Eα inhibition described here unlikely would explain the whole T-ALL oncogenic program induced by TLX1/TLX3, since Eα deletion in mice has not been associated with the development of T cell leukemia (Sleckman et al., 1997) and transgenic TLX1-driven murine leukemias demonstrate a variety of somatic genetic abnormalities (De Keersmaecker et al., 2010). TLX1/TLX3-ETS1-mediated Eα inhibition, however, likely accounts for the early cortical block in cell maturation around β-selection characteristic of these leukemias.

Inactivation of ETS1 impairs, but does not abolish, the development of DN3 thymic cell differentiation into DP cells and the defect appears to be specific to the αβ T cell lineage, as γδ T cells mature normally (Eyquem et al., 2004). In line with this, TLX$^+$ T-ALLs often express a TCRγδ, albeit in conjunction with the cytoplasmic TCRβ chain. In this context, it is noteworthy that, despite the presence of ETS1 and RUNX binding sites within Eβ, ETS1 deficiency did not affect Eβ activity (Eyquem et al., 2004), pointing to different requirements for the activity of Eβ and of Eα. As mentioned, an alternative and non-mutually-exclusive candidate for mediation of the maturation arrest might be BCL11b, recently reported to be a direct target downregulated by TLX1 (De Keersmaecker et al., 2010). Loss of BCL11b, a transcriptional repressor required for T lymphoid specification, leads, however, to a much earlier DN1/2 block (Ikawa et al., 2010; Li et al., 2000, 2004). Our data would suggest that abrogation of ETS1 activity on Eα is more likely than loss of BCL11b to explain the cortical arrest seen in human T-ALLs. As in vivo confirmation of the capacity of TLX1 to inhibit Eα-enhanceosome-driven transcription, we also show here that *TCRδ-TLX1* genomic products resulting from a t(10;14) translocation will lead to either a cell-maturation arrest and leukemogenesis or autoextinction of translocated clones, depending on the configuration of the translocation breakpoints. One interesting aspect of the autoextinction model is that in order to efficiently repress its own sustained expression *in-cis*, the TLX1 protein has to be produced prior to disrupting activity of the Eα enhanceosome on the *TLX1-TCRδ*/Eα translocated allele (Figure 7C). If so, it seems reasonable to assume that the same repression also occurs *in-trans* on the normal (nontranslocated) *TCRα* allele, leading to inhibition of *TCRα* rearrangement and a block in thymocyte differentiation. The fact that these cells do not undergo malignant transformation implies that sustained (and/or higher-level) TLX1 expression is required for leukemic transformation. Such a TLX1 "oncogene addiction" argues in favor of TLX1 being the initiating and causative oncogenic event in TLX1$^+$ T-ALL cases. This model obviously does not exclude oncogenic synergy with other abnormalities.

In line with a major role of ETS1/TLX-mediated repression of Eα in this TLX-induced oncogenic addiction, knockdown of TLX1/TLX3 led to apoptosis, concomitant TCRα transcription and rearrangement, cell-maturation, and sTCRαβ$^+$ expression

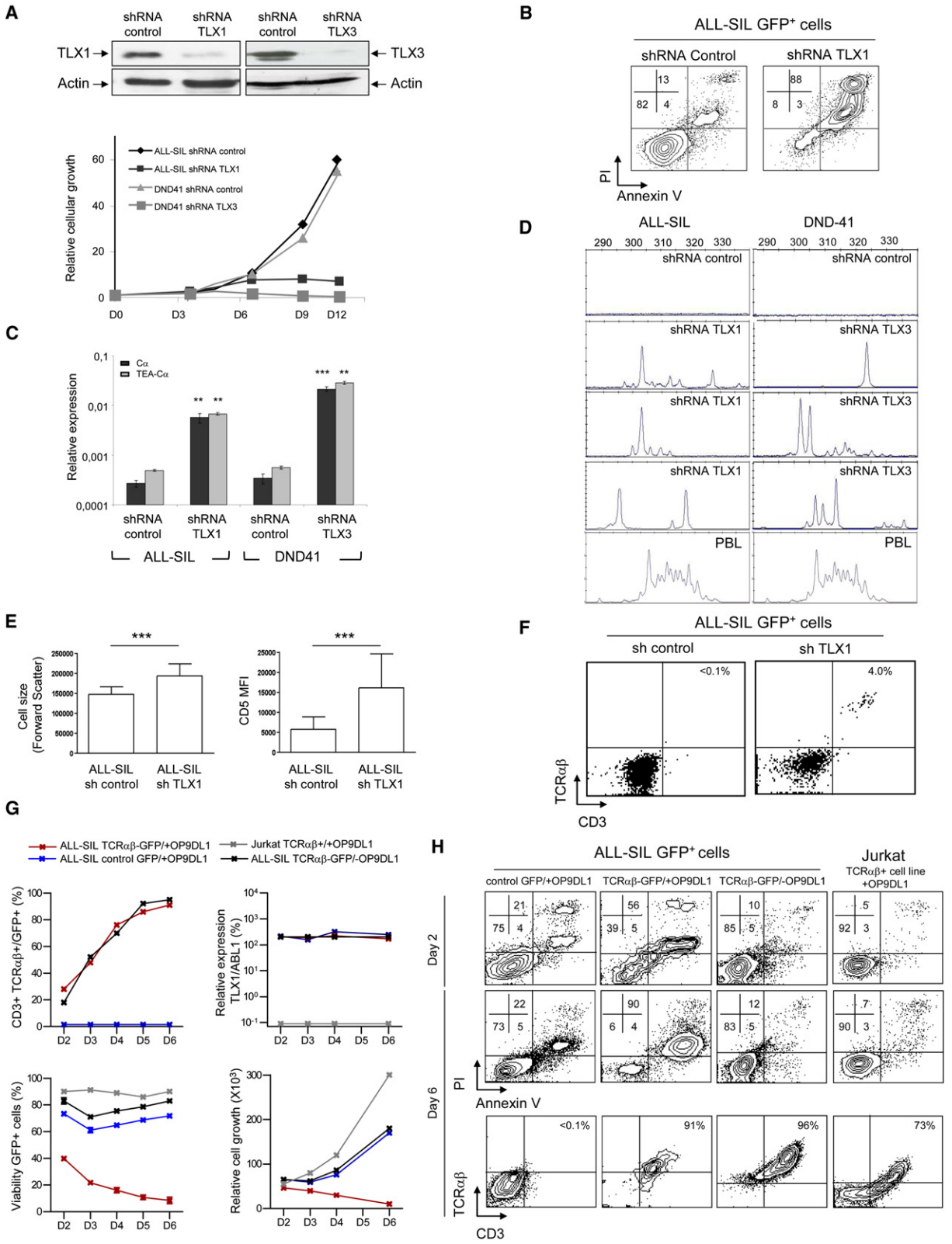

Figure 6. Abrogation of the T Cell Maturation Block Induces Cell Death

(A) Western blots for expression of TLX1 (top left), TLX3 (top right), and the actin control (bottom) in ALL-SIL and DND41 cells transduced with specific or nonspecific (control) shRNA. The graph shows cellular growth at days (D) 3, 6, 9, and 12 of cell culture.

in a significant proportion of cells. This implies that continuous TLX expression is required for maintenance of leukemic cell survival and blocked differentiation, and that both may be intricately linked. The fact that the apoptotic effect of TLX1 abrogation was mimicked by transducing a TCRαβ transgene in TLX1/ALL-SIL cells in the presence, but not in the absence, of stroma supports these conclusions. Thus, as predicted from a bypass of the ETS1/TLX-mediated Eα inhibitory control, both enforced sTCRαβ expression leading to exit from β-selection and TLX abrogation lead to a similar outcome (i.e., apoptosis). Different outcomes (cell death versus proliferation) depending on the presence or absence of OP9-DL1 stroma are likely to reflect a role for stromal (or other ligand) interaction in cell death following TCRαβ expression and differentiation. In general, T cell responses require costimulation-engagement of the clonotypic TCR together with that of distinct coreceptors and cognate ligands. Our current findings offer a unique opportunity to explore such a partnership in TLX$^+$ T-ALL tumorigenesis in prospective studies.

Taken together, our results demonstrate that the maturation block observed in TLX$^+$ T-ALLs is in large part due to ETS1-mediated TLX recruitment to the Eα core, leading to repression of Eα and blocked Vα-Jα rearrangement. Failure to express a TCRα gene arrests development of αβ-committed thymocytes around β-selection, when a variety of cell-proliferation signals are likely to be maintained, hence contributing to oncogenesis. This blockage can be overcome by TLX1/3 abrogation or by downstream TCRαβ expression within an appropriate cellular context. These observations have fundamental consequences both for targeted therapy in TLX$^+$ T-ALLs and for the role of aberrant TCR expression in T lymphoid oncogenesis.

EXPERIMENTAL PROCEDURES

Full Experimental Procedures and any associated references are available in the Supplemental Experimental Procedures.

Patient Analysis and Clinical Diagnosis

Diagnostic samples of peripheral blood or bone marrow from T-ALL patients included in the GRAALL or FRALLE protocols were investigated. Approval was obtained from institutional review boards of institutions participating in this study; the full list of participating centers is given in Supplemental Experimental Procedures. The age cut-off between pediatric and adult cases was 18 years. All samples contained ≥80% blasts. Informed consent was obtained according to the declaration of Helsinki. DNA and RNA extraction and identification of TCRδ, TCRγ, and TCRβ clonal rearrangement were identified as described (Asnafi et al., 2003).

CAT-Reporter Assays

CAT-transactivation assays were performed as described (Giese et al., 1995).

Streptavidin Precipitations and Immunoprecipitations

HeLa cells were cotransfected using expression vectors for ETS1-*HA*-*His*, LEF1-*HA*, and either TLX1-SBP-Flagx3 or TLX3-SBP-Flagx3, or the empty expression vector SBP-Flagx3 as a control. ALL-SIL (TLX1$^+$) and DND41 SIL (TLX3$^+$) cells were used for protein IPs. Nuclear extracts were prepared and incubated with streptavidin agarose beads or with anti-TLX1 (16F6) or anti-TLX3 (10A5) mAbs, respectively, covalently linked to protein G agarose beads. After washes, precipitated proteins were detected by western blot analysis.

Fluorescence and Immunofluorescence Analyses

For EGFP fluorescence analysis, HeLa cells plated on coverslips were transiently transfected with EGFP-fusion constructs and further cultured for 48 hr. Imaging was performed using a Zeiss (LSM-510) confocal microscope. For immunofluorescence analysis, cell lines or primary blast cells (1×10^5) were cytospun onto glass slides. Images were obtained on a Leica TCS SP5 confocal laser scanning microscope and merged using Leica LAS AF software.

Chromatin Immunoprecipitation

ChIPs from the ALL-SIL, DND41, and RPMI cell lines or from T-ALL samples were performed according to the Agilent protocol version 10.0 (http://www.chem.agilent.com), using anti-TLX1 (16F6), anti-TLX3 (10A5), anti-H3K27me3 (05851, Abcam), and anti-ETS1 (sc-350, C-20X, Santa Cruz) mAbs.

TLX1 and TLX3 Knockdown

MISSION TRC shRNA Target Set vectors for TLX1 (TRCN0000014995), TLX3 (TRCN0000018030), and ETS1 (TRCN0000231917) were purchased from Sigma. Knockdown of the corresponding endogenous RNA transcripts was performed by transduction of the ALL-SIL and DND41 cell lines.

Screening for TCR-TLX1 junctions

Screening for 3′ *type* t(10;14)(q24;q11) translocations was performed using pooled thymocytes from 10 healthy children undergoing cardiac surgery.

ACCESSION NUMBER

The ChIP-on-chip data have been deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with the accession number E-MEXP-3527.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table, three figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2012.02.013.

(B) Flow cytometric analysis of TLX1$^+$ ALL-SIL cells following transduction with retroviruses encoding the GFP protein and either control (nonspecific) or TLX1-specific shRNAs. GFP$^+$-transduced cells were gated for analysis of AnnexinV/PI staining.
(C) RT-qPCR quantification of Cα and TEA-Cα transcripts in ALL-SIL/DND41 following shRNA (TLX-specific versus mock control) transduction. Cα and TEA-Cα transcript levels are shown relative to those of ABL control transcripts (**p value ≤ 0.01; ***p value ≤ 0.001), with error bars to represent ±SD.
(D) Multiplex RT-PCR analysis of Vα-Jα-Cα rearrangements from ALL-SIL (left) and DND41 (right) cDNA, following mock control (top) or TLX-specific (second to fourth panel pairs) shRNA transduction and 9 days of cell culture. Normal TCRαβ rearranged repertoires give a Gaussian distribution of variable length Vα-Jα-rearranged PCR fluorescent products, as found in normal PBLs (bottom). Abrogation of TLX1/TLX3 allows a variety of Vα-Jα rearrangements.
(E) Histograms of forward scatter and CD5 mean of fluorescence of GFP$^+$ ALL-SIL cells assessed by flow cytometry, following shRNA transduction. Error bars represent ±SD. ***p ≤ 0.001, Student test.
(F) Flow cytometric analysis of GFP$^+$ ALL-SIL cells for CD3 and TCRαβ surface expression.
(G) Kinetics of CD3 and TCRαβ surface expression (top left), and cell viability and growth (bottom), analyzed for GFP$^+$ cells in the indicated cell lines, with or without transduction of TCRαβ cDNAs and with or without OP9-DL1 stromal culture conditions. RT-qPCR analysis for TLX1 transcription in Jurkat and ALL-SIL cells is also shown (top right).
(H) Top and middle cytographs are as in (B), but with the ALL-SIL cells treated as in (G). Bottom cytographs are as in (F), but with the ALL-SIL cells treated as in (G). Jurkat cells are shown as TCRαβ$^+$ TLX1$^-$ controls cultured in identical conditions.

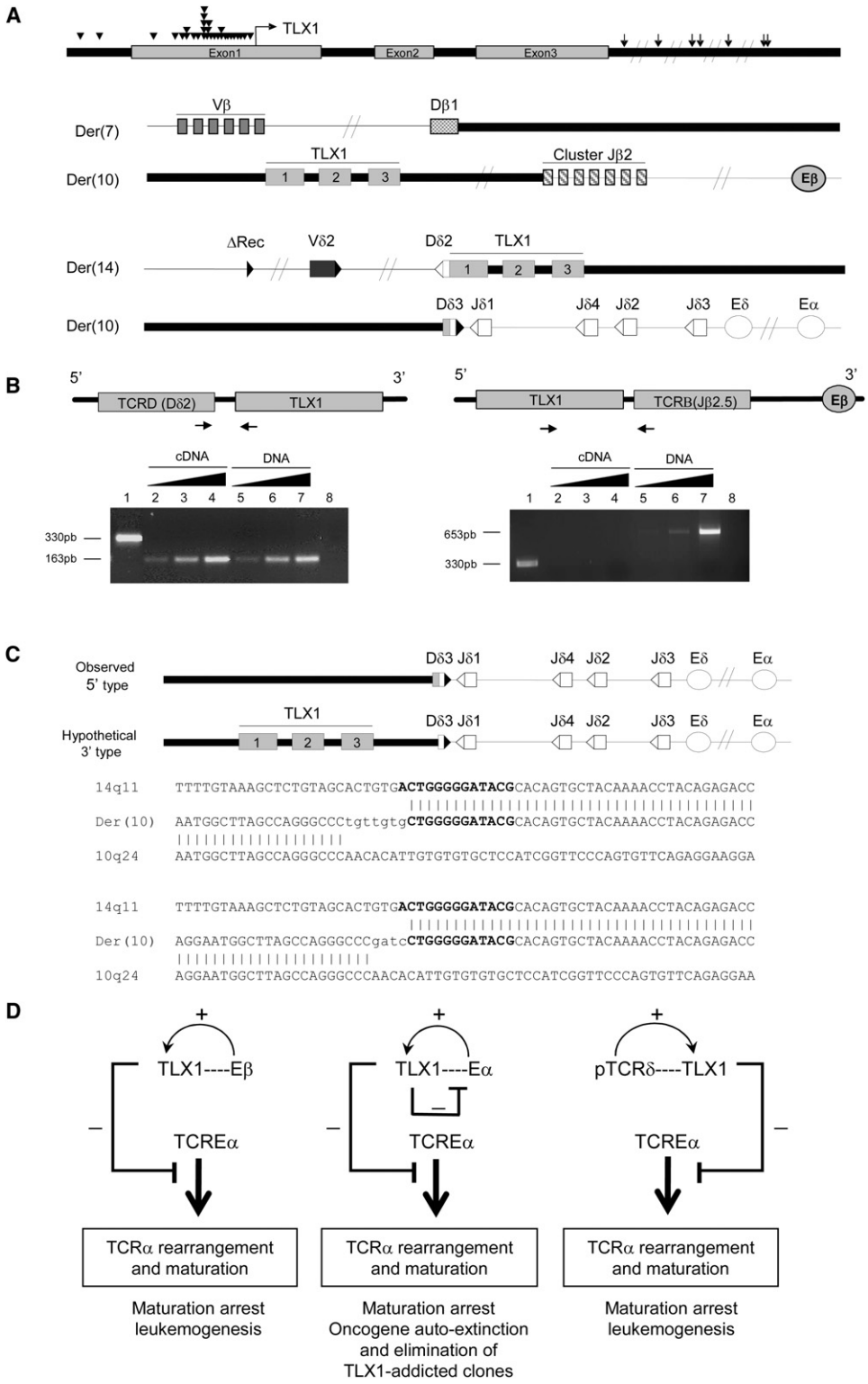

Figure 7. Molecular Analysis of *TCRβ-TLX1* and *TCRδ-TLX1* Translocations

(A) Top lane, schematic representation of the *TLX1* locus (10q24). Arrowheads and arrows indicate the relative positions of breakpoints in *TCRβ*- and *TCRδ-TLX1* translocations, respectively; middle and bottom lanes, representations of *TCRβ*- and *TCRδ-TLX1* typical translocations. Thick and thin lines depict the 10q24 and 7q34 chromosomal regions, respectively (sequences of the *TCR-TLX* breakpoint junctions are reported in Figure S3).

(B) *TCRδ-TLX1* translocations, but not *TCRβ-TLX1* translocations, generate TCR-TLX fusion transcripts. Fusion sequences resulting from *TCRδ-TLX1* translocations (left) or *TCRβ-TLX1* translocations (right) are depicted and PCR primers indicated for 150 ng of cDNA amplified using TLX1-specific primers (lane 1), 15,

ACKNOWLEDGMENTS

We thank participants in the LALA-94, GRAALL-2003/05, FRAALLE-93/2000, Dutch Childhood Oncology Group (DCOG) (project OC2001/003), and COG ALL (project #2002-09) study groups for collecting and providing biological samples and data sets. Samples were collected and processed by the AP-HP "Direction de Recherche Clinique" Tumor Bank at Necker-Enfants Malades. We are grateful to: V. Lheritier for her help in collecting clinical samples; Dr. M. Pierres (CIML) for the generation of anti-TLX1/3 mAbs; the Paris-Descartes Institute for Research at Necker-Enfants Malades (IRNEM) Cell Imaging and Cell sorting plateforms; Dr. R. Grosschedl (University of Munich, Germany) for the gift of materials for TCRαenhCAT reporter assays; and Dr. Emmanuelle Six for cell culture assistance.

S.D. was supported by grants from the Ministère de l'Enseignement Supérieur et de la Recherche, the Fondation pour la Recherche Médicale (FRM), and the Société Française d'Hématologie (SFH). J.Z.-C. was supported by a fellowship from the Agence Nationale de la Recherche (ANR). J.B. was supported by the National Cancer Institute of Canada (NCIC), Terry Fox Foundation (TFF) award. A.W.L. and W.A.D. were supported by the Dutch Cancer Society, Grant EMCR 2002-2707. S.L.N. was supported by the Institut National du Cancer (INCa-DHOS) and a grant from the Association pour la Recherche sur le Cancer (ARC). This work was supported by grants from ARC, the Fondation de France/Comité Leucémie and Enfants et Santé, in association with the Société Française de Lutte contre les Cancers et Leucémies de l'Enfant et de l'Adolescent (SFCE). Work in the P.F. laboratory is supported by institutional grants from Inserm and CNRS, and by grants from the Commission of the European Communities, the ANR, INCa, and the Fondation Princesse Grace de Monaco. Work in the BN laboratory is supported by institutional grants from Inserm and CNRS, and grants from INCa and the Fondation de France.

S.D., S.L.N., D.P.B., L.L., E.Va., J.C.Z., P.V., and S.S. performed cellular and molecular biology experiments; W.A.D. and A.W.L. provided material and data; E.Ve. and F.L.C. provided lentiviral shRNAs; J.B., C.M., and I.R. contributed to LM-PCR and FISH analysis; A.P., N.I., H.D., and O.H. provided clinical materials and data; S.D., S.L.N., D.P.B., B.N., E.A.M., P.F., and V.A. analyzed data and wrote the manuscript; D.P.B., B.N., E.A.M., P.F., and V.A. oversaw conceptual development of the project, analyzed data, and wrote the manuscript.

Received: April 2, 2010
Revised: January 3, 2012
Accepted: February 13, 2012
Published: April 16, 2012

REFERENCES

Aifantis, I., Raetz, E., and Buonamici, S. (2008). Molecular pathogenesis of T-cell leukaemia and lymphoma. Nat. Rev. Immunol. *8*, 380–390.

Asnafi, V., Beldjord, K., Boulanger, E., Comba, B., Le Tutour, P., Estienne, M.H., Davi, F., Landman-Parker, J., Quartier, P., Buzyn, A., et al. (2003). Analysis of TCR, pT α, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment. Blood *101*, 2693–2703.

Asnafi, V., Beldjord, K., Libura, M., Villarese, P., Millien, C., Ballerini, P., Kuhlein, E., Lafage-Pochitaloff, M., Delabesse, E., Bernard, O., and Macintyre, E. (2004). Age-related phenotypic and oncogenic differences in T-cell acute lymphoblastic leukemias may reflect thymic atrophy. Blood *104*, 4173–4180.

Bassing, C.H., Tillman, R.E., Woodman, B.B., Canty, D., Monroe, R.J., Sleckman, B.P., and Alt, F.W. (2003). T cell receptor (TCR) α/δ locus enhancer identity and position are critical for the assembly of TCR δ and α variable region genes. Proc. Natl. Acad. Sci. USA *100*, 2598–2603.

Bernard, O.A., Busson-LeConiat, M., Ballerini, P., Mauchauffé, M., Della Valle, V., Monni, R., Nguyen Khac, F., Mercher, T., Penard-Lacronique, V., Pasturaud, P., et al. (2001). A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. Leukemia *15*, 1495–1504.

Cauwelier, B., Dastugue, N., Cools, J., Poppe, B., Herens, C., De Paepe, A., Hagemeijer, A., and Speleman, F. (2006). Molecular cytogenetic study of 126 unselected T-ALL cases reveals high incidence of TCRbeta locus rearrangements and putative new T-cell oncogenes. Leukemia *20*, 1238–1244.

De Keersmaecker, K., Real, P.J., Gatta, G.D., Palomero, T., Sulis, M.L., Tosello, V., Van Vlierberghe, P., Barnes, K., Castillo, M., Sole, X., et al. (2010). The TLX1 oncogene drives aneuploidy in T cell transformation. Nat. Med. *16*, 1321–1327.

Degos, L. (1992). All-trans-retinoic acid treatment and retinoic acid receptor α gene rearrangement in acute promyelocytic leukemia: a model for differentiation therapy. Int. J. Cell Cloning *10*, 63–69.

Dik, W.A., Pike-Overzet, K., Weerkamp, F., de Ridder, D., de Haas, E.F., Baert, M.R., van der Spek, P., Koster, E.E., Reinders, M.J., van Dongen, J.J., et al. (2005). New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J. Exp. Med. *201*, 1715–1723.

Dik, W.A., Nadel, B., Przybylski, G.K., Asnafi, V., Grabarczyk, P., Navarro, J.M., Verhaaf, B., Schmidt, C.A., Macintyre, E.A., van Dongen, J.J., and Langerak, A.W. (2007). Different chromosomal breakpoints impact the level of LMO2 expression in T-ALL. Blood *110*, 388–392.

Dittmer, J. (2003). The biology of the Ets1 proto-oncogene. Mol. Cancer *2*, 29.

Eyquem, S., Chemin, K., Fasseu, M., and Bories, J.C. (2004). The Ets-1 transcription factor is required for complete pre-T cell receptor function and allelic exclusion at the T cell receptor β locus. Proc. Natl. Acad. Sci. USA *101*, 15712–15717.

Ferrando, A.A., Neuberg, D.S., Staunton, J., Loh, M.L., Huard, C., Raimondi, S.C., Behm, F.G., Pui, C.H., Downing, J.R., Gilliland, D.G., et al. (2002). Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell *1*, 75–87.

Giese, K., Kingsley, C., Kirshner, J.R., and Grosschedl, R. (1995). Assembly and function of a TCR α enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes Dev. *9*, 995–1008.

Giresi, P.G., Kim, J., McDaniell, R.M., Iyer, V.R., and Lieb, J.D. (2007). FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. Genome Res. *17*, 877–885.

75, or 150 ng of cDNA (lanes 2–4, respectively) and 1, 10, or 100 ng of genomic DNA (lanes 5–7, respectively) amplified using *TCR-TLX1* hybrid primers. The absence of genomic DNA contamination in the cDNA fractions was tested by qPCR using albumin-specific primers (lane 8).

(C) Schematic representations of the observed 5′ (Der[14]) and hypothetical 3′ (Der[10]) types of *TCRδ-TLX1* translocations. The nucleotide sequences of two 3′ *type TCRδ-TLX1* junctions amplified from healthy thymi are shown; N regions and Dδ3-specific nucleotides are indicated in bold and lowercase letters, respectively.

(D) Model of how TLX1 addiction may drive autoselection of chromosomal translocations via *trans* repression of the Eα enhanceosome. (Left) In t(7;10) translocation, TLX1 ectopic expression is driven by the TCRβ gene enhancer (Eβ), leading to Eα repression and inhibition of *TCRα* gene rearrangement. (Right) An identical scenario occurs when TLX1 ectopic expression is driven by TCRδ regulatory elements, i.e., in a subset of t(10;14) in which TLX1 and Eα are segregated on different derivative chromosomes. (Middle) In a distinct subset of t(10;14), TLX1 segregates on der(10) and is linked to Eα. Eα-driven TLX1 expression leads to Eα repression and the extinction of its own expression. This form of t(10;14) can be found in the thymus from hematologically healthy individuals, but not in T-ALL samples, implying that the resulting unsustained/cyclic TLX1 expression may not be sufficient for full leukemic transformation.

See also Figure S3.

Hawwari, A., and Krangel, M.S. (2005). Regulation of TCR δ and α repertoires by local and long-distance control of variable gene segment chromatin structure. J. Exp. Med. *202*, 467–472.

Hernández-Munain, C., Sleckman, B.P., and Krangel, M.S. (1999). A developmental switch from TCR delta enhancer to TCR α enhancer function during thymocyte maturation. Immunity *10*, 723–733.

Ho, I.C., Yang, L.H., Morle, G., and Leiden, J.M. (1989). A T-cell-specific transcriptional enhancer element 3′ of C α in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA *86*, 6714–6718.

Ho, I.C., Bhat, N.K., Gottschalk, L.R., Lindsten, T., Thompson, C.B., Papas, T.S., and Leiden, J.M. (1990). Sequence-specific binding of human Ets-1 to the T cell receptor α gene enhancer. Science *250*, 814–818.

Holland, P.W., Booth, H.A., and Bruford, E.A. (2007). Classification and nomenclature of all human homeobox genes. BMC Biol. *5*, 47.

Hollenhorst, P.C., Chandler, K.J., Poulsen, R.L., Johnson, W.E., Speck, N.A., and Graves, B.J. (2009). DNA specificity determinants associate with distinct transcription factor functions. PLoS Genet. *5*, e1000778.

Ikawa, T., Hirose, S., Masuda, K., Kakugawa, K., Satoh, R., Shibano-Satoh, A., Kominami, R., Katsura, Y., and Kawamoto, H. (2010). An essential developmental checkpoint for production of the T cell lineage. Science *329*, 93–96.

Krangel, M.S., Hernandez-Munain, C., Lauzurica, P., McMurry, M., Roberts, J.L., and Zhong, X.P. (1998). Developmental regulation of V(D)J recombination at the TCR α/δ locus. Immunol. Rev. *165*, 131–147.

Li, A., Rue, M., Zhou, J., Wang, H., Goldwasser, M.A., Neuberg, D., Dalton, V., Zuckerman, D., Lyons, C., Silverman, L.B., et al; Dana-Farber Cancer Institute ALL Consortium. (2004). Utilization of Ig heavy chain variable, diversity, and joining gene segments in children with B-lineage acute lymphoblastic leukemia: implications for the mechanisms of VDJ recombination and for pathogenesis. Blood *103*, 4602–4609.

Li, R., Pei, H., and Watson, D.K. (2000). Regulation of Ets function by protein-protein interactions. Oncogene *19*, 6514–6523.

Look, A.T. (1997). Oncogenic transcription factors in the human acute leukemias. Science *278*, 1059–1064.

Mann, R.S., Lelli, K.M., and Joshi, R. (2009). Hox specificity unique roles for cofactors and collaborators. Curr. Top. Dev. Biol. *88*, 63–101.

Marculescu, R., Vanura, K., Le, T., Simon, P., Jäger, U., and Nadel, B. (2003). Distinct t(7;9)(q34;q32) breakpoints in healthy individuals and individuals with T-ALL. Nat. Genet. *33*, 342–344.

Marculescu, R., Vanura, K., Montpellier, B., Roulland, S., Le, T., Navarro, J.M., Jäger, U., McBlane, F., and Nadel, B. (2006). Recombinase, chromosomal translocations and lymphoid neoplasia: targeting mistakes and repair failures. DNA Repair (Amst.) *5*, 1246–1258.

Mauvieux, L., Villey, I., and de Villartay, J.P. (2003). TEA regulates local TCR-Jalpha accessibility through histone acetylation. Eur. J. Immunol. *33*, 2216–2222.

McMurry, M.T., and Krangel, M.S. (2000). A role for histone acetylation in the developmental regulation of VDJ recombination. Science *287*, 495–498.

Merabet, S., Pradel, J., and Graba, Y. (2005). Getting a molecular grasp on Hox contextual activity. Trends Genet. *21*, 477–480.

Monroe, R.J., Sleckman, B.P., Monroe, B.C., Khor, B., Claypool, S., Ferrini, R., Davidson, L., and Alt, F.W. (1999). Developmental regulation of TCR δ locus accessibility and expression by the TCR δ enhancer. Immunity *10*, 503–513.

Owens, B.M., Zhu, Y.X., Suen, T.C., Wang, P.X., Greenblatt, J.F., Goss, P.E., and Hawley, R.G. (2003). Specific homeodomain-DNA interactions are required for HOX11-mediated transformation. Blood *101*, 4966–4974.

Papp, B., and Müller, J. (2006). Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev. *20*, 2041–2054.

Pui, C.H., Relling, M.V., and Downing, J.R. (2004). Acute lymphoblastic leukemia. N. Engl. J. Med. *350*, 1535–1548.

Roberts, C.W., Shutter, J.R., and Korsmeyer, S.J. (1994). Hox11 controls the genesis of the spleen. Nature *368*, 747–749.

Roberts, J.L., Lauzurica, P., and Krangel, M.S. (1997). Developmental regulation of VDJ recombination by the core fragment of the T cell receptor α enhancer. J. Exp. Med. *185*, 131–140.

Sauvageau, M., and Sauvageau, G. (2008). Polycomb group genes: keeping stem cell activity in balance. PLoS Biol. *6*, e113.

Shen, W.F., Krishnan, K., Lawrence, H.J., and Largman, C. (2001). The HOX homeodomain proteins block CBP histone acetyltransferase activity. Mol. Cell. Biol. *21*, 7509–7522.

Shirasawa, S., Arata, A., Onimaru, H., Roth, K.A., Brown, G.A., Horning, S., Arata, S., Okumura, K., Sasazuki, T., and Korsmeyer, S.J. (2000). Rnx deficiency results in congenital central hypoventilation. Nat. Genet. *24*, 287–290.

Sieweke, M.H., Tekotte, H., Frampton, J., and Graf, T. (1996). MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. Cell *85*, 49–60.

Sleckman, B.P., Bardon, C.G., Ferrini, R., Davidson, L., and Alt, F.W. (1997). Function of the TCR α enhancer in αβ and γδT cells. Immunity *7*, 505–515.

Soulier, J., Clappier, E., Cayuela, J.M., Regnault, A., Garcia-Peydro, M., Dombret, H., Baruchel, A., Toribio, M.L., and Sigaux, F. (2005). HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood *106*, 274–286.

Spicuglia, S., Payet, D., Tripathi, R.K., Rameil, P., Verthuy, C., Imbert, J., Ferrier, P., and Hempel, W.M. (2000). TCRalpha enhancer activation occurs via a conformational change of a pre-assembled nucleo-protein complex. EMBO J. *19*, 2034–2045.

Spits, H. (2002). Development of αβ T cells in the human thymus. Nat. Rev. Immunol. *2*, 760–772.

von Boehmer, H., Aifantis, I., Azogui, O., Feinberg, J., Saint-Ruf, C., Zober, C., Garcia, C., and Buer, J. (1998). Crucial function of the pre-T-cell receptor (TCR) in TCR β selection, TCR β allelic exclusion and αβ versus γδ lineage commitment. Immunol. Rev. *165*, 111–119.