The TLX1 oncogene drives aneuploidy in T-cell transformation

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Author Contributions
KDK performed cellular, genetic and molecular characterization of TLX1-induced tumors and preleukemic thymocytes, identified BCL11B mutations in mouse and human tumors and wrote the manuscript. PJR generated the TLX1 transgenic mice and characterized the tumor phenotype. GDG analyzed ChIP-chip data and gene expression signatures in human and mouse tumors. TP performed ChIP-chip. VT characterized mouse thymocytes. PVV and KDK analyzed array CGH data. MLS performed mouse tumor microarray analysis. KB and MH analyzed TLX1 transgenic lines. MC performed histological and immunohistochemical studies. JL, PA, MK, BK, PP, DK and FG provided mouse tumor samples. HP provided gene expression data on normal mouse thymocytes. XS analyzed ChIP-chip data. JVM and FS analyzed BCL11B mutations in human T-ALL samples. SR, HC, ND, JS and DA provided cytogenetic data on human T-ALLs. EP, JR, PW and JR provided human T-ALL specimens from ECOG clinical trials. JM generated human expression profiling data and characterized human T-ALL samples. CCC supervised histological and immunohistochemical studies. AC supervised the bioinformatic data analysis. AAF designed the study, supervised research and wrote the manuscript.

Accession codes
Microarray data are available at Gene Expression Omnibus (accession numbers GSE19499 and GSE10609).
Abstract

The TLX1 transcription factor oncogene plays an important role in the pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL). However, the specific mechanisms of T-cell transformation downstream of TLX1 remain to be elucidated. Here we show that forced expression of TLX1 in transgenic mice induces T-ALL tumors with frequent deletions and mutations in Bcl11b, and identify the presence of recurrent mutations and deletions in BCL11B in 16% of human T-ALLs. Most notably, mouse TLX1 tumors were typically aneuploid and showed a marked defect in the activation of the mitotic checkpoint. Mechanistically, TLX1 directly downregulates the expression of CHEK1 and additional mitotic control genes and induces loss of the mitotic checkpoint in non-transformed preleukemic thymocytes. These results identify a novel mechanism contributing to chromosomal missegregation and aneuploidy active at the earliest stages of tumor development in the pathogenesis of cancer.

T-ALL is an aggressive hematologic tumor resulting from the malignant transformation of T-cell progenitors. The TLX1 transcription factor oncogene is translocated and aberrantly expressed in 5% to 10% of pediatric and up to 30% of adult T-ALL cases. In addition, TLX3, a highly related TLX family member, is overexpressed as result of the t(5;14) (q35;q32) translocation in about 25% of pediatric TALLs and in 5% of adult T-ALL cases. TLX1 expression defines a distinct molecular group of T-ALL characterized by a differentiation block at the early cortical stage of thymocyte development and favorable prognosis. Moreover, TLX1 and TLX3 leukemias seem to constitute a distinct oncogenic group with specific genetic alterations rarely found in non-TLX induced T-ALLs including the rearrangement of the NUP214-ABL1 oncogene and mutations in the WT1 and PHF6 tumor suppressor genes. However, little is known about the specific mechanisms that mediate T-cell transformation downstream of TLX1. To address this question we have used an integrative genomic approach to characterize the transcriptional programs and oncogenic pathways active in human and mouse TLX1-induced leukemia.
RESULTS

T-ALL development in TLX1 transgenic mice

In order to investigate the mechanisms of T-cell transformation driven by TLX1, we generated p56Lck-TLX1 transgenic mice in which the Lck proximal promoter drives expression of TLX1 in T-cell progenitors. TLX1 transgenic mice from three founder lines showed accelerated mortality due to the development of lymphoid tumors with a median latency of 27, 32 or 46 weeks, respectively ($P<0.0001$) (Fig. 1a). Tumor-bearing mice showed enlarged thymi populated by immature lymphoblasts and leukemic infiltration of bone marrow and peripheral organs including lymph nodes, spleen, liver and kidneys (Fig. 1b). Overall, 92% of TLX1 transgenic animals developed tumors at 52 weeks. TLX1-induced leukemias showed high levels of TLX1 protein (Fig. 1c–d) and expression of CD3 indicative of a T-cell phenotype (Fig. 1e). Moreover, flow cytometry analysis demonstrated the expression of CD4 and CD8 in most tumors (Fig. 1f–g). In addition, this analysis revealed significant heterogeneity in these leukemias with 53% of the tumors showing two or more immunophenotypically different cell populations (Fig. 1f). Finally, TLX1-induced tumors showed clonal expression of a single TCRB chain in 4/5 tumors examined and only 2 TCRB chains in the remaining sample (Fig. 1h), indicating that despite their heterogeneous immunophenotypes these are monoclonal T-cell tumors.

Mouse and human TLX-induced T-ALLs have a common expression signature

To address the transcriptional programs activated in mouse TLX1 leukemias, we analyzed the expression profiles of mouse tumors from TLX1 transgensics (n=6) and of T-ALLs generated by retroviral insertional mutagenesis or arising in the context of different T-ALL transgenic and knockout models including Tal1, Lmo2 and Olig2/Bhlhb1 transgensics and Tcf3/E2A, Trp53 and Pten knockouts (n=49) (Supplementary Table 1). Significant differentially expressed genes were calculated by Comparative Marker Selection Genepattern tool using t-test statistical test and non-parametric $P$ value calculation (1,000 random permutations). This analysis revealed that TLX1 tumors are characterized by a distinct gene expression signature with upregulation of 114 genes and downregulation of 377 transcripts (Fold change >2, $P<0.05$) (Fig. 2a; Supplementary Table 2). Moreover, Gene Set Enrichment Analysis (GSEA) of human T-ALL (n=82) using these mouse TLX1-signature genes showed significant enrichment in human TLX1 and TLX3 tumors ($P<0.001$) (Fig. 2b; Supplementary Table 3), highlighting the relevance of our mouse model for the analysis of TLX-induced transformation.

TLX1 expression blocks T-cell development and induces apoptosis

Next, we decided to explore the effects and mechanistic role of TLX1-expression in T-cell progenitors in young TLX1 transgenic mice prior to the development of T-ALL. Analysis of TLX1-transgenic mice at 3 and 6 weeks of age showed a drastic reduction in thymic size and cellularity compared with littermate controls (Fig. 3a), but no clear defects in cell proliferation (Fig. 3b). Flow cytometry analysis of thymocyte differentiation markers in TLX1 transgenic animals and littermate controls showed a defect in T-cell development with arrest at the double negative 2 (DN2) stage of thymocyte differentiation (Fig. 3c), which was accompanied by a marked increase in apoptosis (Fig. 3d,e). Consistent with these results,
transgenic expression of BCL2 in Lck-TLX1 VavP-BCL2 double transgenic mice abrogated apoptosis (Fig. 3f) and rescued the defect in thymus size (Fig. 3g) and cellularity (Fig. 3h) observed in preleukemic TLX1-transgenic mice.

**Secondary mutations in the pathogenesis of TLX1-induced T-ALL**

The prolonged latency in the development of T-ALL in TLX1 transgenic mice and the clonal nature of these tumors prompted us to investigate the presence of cooperating mutations involved in the pathogenesis of TLX1-induced leukemias. Activating mutations in NOTCH1 are present in over 50% of human T-ALLs. Similarly, prototypical mutations in Notch1 were present in 3/25 (12%) TLX1-induced mouse tumors (Supplementary Table 4). Moreover, array Comparative Genomic Hybridization (aCGH) analysis revealed the presence of recurrent numerical and structural chromosomal alterations in these tumors (Fig. 4a, Supplementary Fig. 1). Thus, focal homozygous deletions affecting the Pten tumor suppressor gene were present in 4/15 (27%) samples (Fig. 4a, Supplementary Fig. 1, Supplementary Table 5). In addition, immunohistochemical analysis of Pten showed loss of Pten protein expression in 11/24 (42%) tumors (Supplementary Fig. 1). Notably, PTEN deletions, mutations and loss of PTEN protein expression have been reported in 20% of human T-ALLs. Chromosomal deletions involving Trp53 and Ikzf1, which are sporadically mutated and deleted in human T-ALLs, were detected in one tumor each (Fig. 4a, Supplementary Fig. 1, Supplementary Table 5). In addition three mouse TLX1 T-ALL samples harbored heterozygous deletions in chromosome 12 with a common deleted region containing only the Bcl11b gene (Fig. 4a–c, Supplementary Table 5). Moreover, DNA sequence analysis of Bcl11b showed the presence of Bcl11b mutations in 4/15 (27%) mouse TLX1-induced T-ALLs (Fig. 4d–e, Supplementary Table 6), which together with the 3 Bcl11b alterations identified in our aCGH analysis brings the prevalence of Bcl11b alterations in mouse TLX1-induced tumors to 7/15 (47%). Notably, mutation analysis of Bcl11b in T-ALL tumors occurring in Pten knockout animals (n=6), Trp53 knockout mice (n=2), Ctnnb1 transgenic animals (n=4) or induced by retroviral insertional mutagenesis (n=9) failed to detect any Bcl11b mutations in these non-TLX1 transgenic tumors (Fisher’s exact test P <0.001).

**Recurrent deletions and mutations in BCL11B in human T-ALL**

Bcl11b encodes a zinc finger transcription factor with a critical role in the differentiation and survival of T-cell progenitors in the thymus. Given the similarities between our mouse model of TLX1-induced leukemia and human T-ALL, we hypothesized that BCL11B alterations could also be implicated in the pathogenesis of human T-ALL. Most notably, aCGH analysis of human T-ALL samples showed the presence of focal heterozygous deletions encompassing the BCL11B locus in 2/69 (3%) T-ALL cases (Fig. 5a). In addition, mutation analysis of BCL11B in human T-ALL demonstrated the presence of truncating frameshift mutations and missense point mutations in 9/71 (13%) cases analyzed (Fig. 5b). Interestingly, all 6 BCL11B missense mutations involved zinc finger domains (Fig. 5b). One sample showed compound heterozygous BCL11B mutations while the remaining 5 cases harbored heterozygous BCL11B lesions (Supplementary Table 7). Notably, sequence analysis of samples obtained at the time of clinical remission demonstrated the somatic origin of BCL11B mutations in all 6 cases with available material (Fig. 5c). Finally,
expression analysis of TLX1 or TLX3 in 8 T-ALLs harboring mutations or deletions in BCL11B with RNA available showed aberrant expression of these transcription factor oncogenes in 5/8 (63%) [TLX1 (4/8); TLX3 (1/8)] of these samples (Supplementary Table 7). Altogether, these results identify BCL11B as a tumor suppressor gene recurrently deleted and mutated in human T-ALL.

**Interaction between BCL11B and TLX1 in T-cell transformation**

The identification of a high prevalence of Bcl11b mutations and deletions in TLX1-induced tumors suggest a specific genetic interaction between these two genes in T-cell transformation. To test if BCL11B could be a direct transcriptional target of TLX1 in T-ALL, we analyzed the binding of TLX1 to the BCL11B promoter via chromatin immunoprecipitation analysis in ALL-SIL, a T-ALL cell line expressing high levels of TLX1 as result of the t(10;14)(q24;q11) translocation21. This analysis revealed that indeed TLX1 binds to the BCL11B promoter (Fig. 5d). Moreover, siRNA knockdown of TLX1 in this cell line resulted in transcriptional upregulation of BCL11B (Fig. 5e), indicating that BCL11B is a direct transcriptional target downregulated by TLX1 in T-ALL.

**TLX1 T-ALLs are aneuploid and have a defective mitotic checkpoint**

In addition to identifying focal areas of amplification and deletion, our aCGH analysis of mouse TLX1 tumors showed gains and losses of whole chromosomes in most samples analyzed, suggesting a high prevalence of numerical chromosomal abnormalities in these leukemias (Fig. 4a, Supplementary Table 8). Spectral karyotyping (SKY) analysis confirmed these results and showed the presence of trisomy 15 in 3/4 TLX1 tumor samples analyzed (Fig. 6a, Supplementary Table 8). Overall, 78% (14/18) of TLX1-induced leukemias showed chromosomal gains and losses, with 67% (12/18) harboring trisomy 15 (alone or in combination with trisomy 17, gain of chromosome Y, monosomy X or hyperdiploidy) (Fig. 6b, Supplementary Table 8). Previous in vitro studies had associated TLX1 with alterations in the G2/M cell cycle checkpoint22 and forced expression of TLX1 can induce aneuploidy in B-cells after disruption of the mitotic spindle23. To test the function of the mitotic checkpoint in our TLX1-induced leukemias we treated TLX1-T-ALL lymphoblasts with taxol, which impairs microtubule remodeling. This analysis revealed that TLX1 mouse T-ALL lymphoblasts fail to arrest in mitosis following disruption of the mitotic spindle (Fig. 6c). In contrast, tumors induced by the TAL1, TAL1 plus LMO1, TAL1 plus LMO2 and NUP214-ABL1 T-ALL oncogenes showed a marked accumulation of cells in mitosis after taxol treatment (Fig. 6c, Supplementary Fig. 2). Similar results were obtained using nocodazole, which disrupts tubulin polymerization (Fig. 6c), and BrdU incorporation analysis verified that TLX1 mouse tumors proliferate and fail to arrest in M phase following disruption of the mitotic spindle (Supplementary Fig.3).

**TLX1 directly downregulates mitotic genes in T-cell transformation**

The acquisition of chromosomal instability may represent an early event in the pathogenesis of cancer24. To investigate the specific mechanisms involved in the earliest stages of TLX1-induced transformation, we performed microarray gene expression analysis of sorted preleukemic DN2 cells from 3 week old mice. Significant differentially expressed genes

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were calculated by Comparative Marker Selection Genepattern tool12 as described above. This analysis revealed marked differences in gene expression in TLX1 transgenic thymocytes and DN2 cells from normal littermate controls with 175 genes upregulated and 138 genes downregulated in TLX1-expressing cells (Fold change > 1.5, \( P < 0.005 \)) (Supplementary Table 9). Next, and to specifically address the role of direct TLX1 target genes in T-cell transformation, we performed ChIP-chip analysis of TLX1 in ALL-SIL cells. In these experiments, TLX1 chromatin immunoprecipitates were hybridized to human proximal promoter arrays and analyzed using ChIP-chip Significance Analysis (CSA) to identify high confidence TLX1 direct target genes25. This analysis identified 5,549 promoters bound by TLX1 with a significance cutoff of \( P < 0.0001 \) (Supplementary Table 10). Integration of these ChIP-chip results with the gene expression signature associated with aberrant expression of TLX1 in T-cell progenitors revealed a marked enrichment of TLX1 direct target genes involved in mitosis (\( P < 0.001 \)) (Supplementary Table 11) downregulated in TLX1-preleukemic cells (Fig.6d and Supplementary Fig. 4). Notably, these included CHEK1, a key regulator of the mitotic spindle checkpoint and one of the genes most consistently downregulated in both mouse and human TLX-induced leukemias (Supplementary Tables 2 and 3). Consistent with these results, TLX1-knockdown in ALL-SIL cells resulted in transcriptional upregulation of CHEK1 (Fig.6g) and pharmacologic inhibition of CHEK1 in human TALL cell lines abrogated the activation of the mitotic checkpoint upon treatment with taxol (Supplementary Fig. 5). These results suggest a mechanistic role of TLX1 in the control of cell cycle checkpoint genes at the earliest stages of T-cell transformation.

To test if TLX1 expression can induce a defective mitotic checkpoint phenotype in preleukemic cells we isolated viable thymocytes from TLX1-BCL2 double transgenic mice and compared them with cells obtained from BCL2 transgenic controls. This experiment showed a marked defect in the activation of the mitotic checkpoint after taxol treatment in TLX1-BCL2 preleukemic cells compared with BCL2 controls (Fig. 6h). The polyclonal/non transformed nature of TLX1-BCL2 preleukemic cells was verified by RT-PCR analysis of TCRB expression (Supplementary Fig. 6). Overall, these results demonstrate that the impaired control of the mitotic checkpoint in TLX1-transgenic mice is an early event in tumor formation that precedes clonal selection in T-cell transformation.

**Chromosomal alterations in human TLX-induced leukemias**

Overall the results described above support a direct mechanistic role for TLX1 in the induction of chromosomal missegregation suggesting that similar mechanisms may operate in the pathogenesis of human leukemias. Most notably, and consistent with this thesis, the TLX1-positive ALL-SIL cell line showed a selective defect in the mitotic checkpoint (Supplementary Fig. 2). In addition, karyotype analysis of a series of 57 well characterized pediatric T-ALLs2 demonstrated a higher frequency of aneuploid karyotypes (8/13, 62%) in TLX1 and TLX3 positive human T-cell tumors compared with other genetic subgroups of T-ALL (5/44, 11%) (Fisher’s exact test \( P < 0.001 \)) (Supplementary Table 12). Moreover, analysis of an independent series of 229 T-ALLs showed a marked predominance of numerical chromosomal abnormalities in TLX1 and TLX3 positive T-ALLs (aneuploidy/pseudodiploid karyotype ratios: 6/1 for TLX1 tumors; 10/10 for TLX3 leukemias and 24/63...
Discussion

TLX1 plays a central role in the pathogenesis of T-ALL. However the elucidation of the specific mechanisms that mediate T-cell transformation downstream of TLX1 has been hampered by the lack of an animal model of TLX1-induced T-ALL and by the lack of understanding of the direct transcriptional targets controlled by TLX1 in human leukemia. Here we demonstrate that TLX1 transgenic mice develop clonal immature T-cell tumors highly related to human TLX1 and TLX3 T-ALLs in their clinical presentation and gene expression signatures. The extended latency in the development of leukemia in this model and the clonal nature of TLX1-induced tumors suggest that TLX1 overexpression in the thymus is not sufficient for malignant transformation of T-cell progenitor cells. This thesis is further supported by the identification of recurrent cooperating mutations and chromosomal abnormalities in mouse TLX1-induced leukemias. Most notably, TLX1-induced tumors harbored recurrent alterations in oncogenes (Notch1) and tumor suppressor genes (Pten, Cdkn2a) frequently mutated in human T-ALL13–15,26. In addition this analysis revealed the presence of recurrent mutations and deletions centered on the Bcl11b locus in 47% of mouse TLX1-induced tumors analyzed. Based on these results, we hypothesized that BCL11B could play a tumor suppressor role in human leukemia and identified recurrent deletions and mutations in this gene in 16% of human TALLs. Notably, most BCL11B alterations found in mouse TLX1-induced tumors and in human T-ALLs were heterozygous and loss of one copy of Bcl11b in mice accelerates the development of thymic lymphomas after γ-irradiation or in Trp53 heterozygous mice without loss of the wild type Bcl11b allele27. Finally, ChIP and expression analysis demonstrated that BCL11B is a direct transcriptional target downregulated by TLX1 in T-ALL. The model that emerges from these results is that aberrant expression of TLX1 partially downregulates the BCL11B tumor suppressor gene during T-cell transformation and that this negative transcriptional regulatory axis is fixed and reinforced by secondary genetic alterations in the BCL11B locus acquired during tumor progression. Strikingly, PHF6 and WT1, two additional tumor suppressor genes frequently mutated in TLX1-induced T-ALL are also among the TLX1 targets identified in our ChIP-on-chip analysis (Supplementary Table 10), suggesting that this model could also apply to these TLX1-target tumor suppressor genes.

Perhaps the most striking finding in TLX1-induced leukemias was the presence of a very high incidence of numerical chromosomal alterations in these tumors, indicating that alterations in the mitotic machinery could play an important role in TLX1-induced transformation. Consistent with this model, our results demonstrate that TLX1-induced T-ALLs have a defective mitotic checkpoint and fail to arrest in mitosis upon treatment with taxol or nocodazole. Moreover, this mitotic checkpoint defect is readily present in preleukemic thymocytes from TLX1 transgenic mice, suggesting that aneuploidy represents a direct effect of TLX1 overexpression and an early event in the genesis of mouse TLX1-induced tumors.
Mechanistically, analysis of TLX1 direct target genes in the context of TLX1 preleukemic thymocytes revealed that multiple TLX1 direct target genes involved in mitosis and chromosomal segregation are downregulated in T-cell progenitor cells isolated from TLX1 transgenic mice. Among these, CHEK1, a critical checkpoint regulator, is downregulated in mouse and human TLX-induced leukemias and could have a particularly prominent role in the loss of the mitotic checkpoint downstream of TLX1. However, it would be deceiving to attribute the induction of aneuploidy by TLX1 to the dysregulation of a single gene, as multiple transcripts with prominent roles in mitosis seem to be controlled by TLX1 in T-cells. In addition, a transcription independent effect of TLX1 in the control of cell cycle via interaction with the PP2A phosphatase has been proposed.

Overall, our results mechanistically link the aberrant expression of TLX1 with the development of chromosomal instability at the earliest stages of T-cell transformation and illustrate the power of integrative genomic analyses of mouse and human tumors to elucidate basic mechanisms and oncogenic pathways involved in tumor initiation and disease progression.

**Methods**

**Transgenic mice**

The human TLX1 cDNA was amplified by PCR using BamHI restriction site containing primers and cDNA of the human T-cell leukemia cell line ALL-SIL as template and was cloned in the pUC1017 vector, downstream of the mouse T-cell specific p56Lck proximal promoter. C57BL/6J VavP-Bcl2 transgenic mice have been described before.

**Gene expression profiling of mouse tumors**

We profiled a cohort of 6 TLX1 transgenic-derived TALLs and additional 49 T-cell lymphoblastic tumors from different genetic models of T-ALL (see Supplementary Table 1 for details on the genetic background of tumors analyzed). Gene-expression profiling was performed using Affymetrix Mouse Genome 430A 2.0 Array following standard procedures. Raw data is available in GEO (accession number GSE19499). Array normalization was performed by DNA-Chip-Analyzer (dChip). Following interarray normalization we preprocessed and analyzed the microarray data for differential expression using the Genepattern platform for microarray analysis.

**Gene expression profiling human T-ALL**

Affymetrix gene expression data from pediatric T-ALL samples has been reported before (GEO accession # GSE10609). This series encompasses 7 TLX1 positive cases, 23 TLX3 positive samples and 53 other T-ALLs including 33 tumors with aberrant expression of TAL1 and LMO2. Tumors with high levels of expression of HOXA9 constitute a heterogeneous molecular group resulting from translocations involving the HOXA loci, aberrant expression of CALM-AF10, SET-NUP214 and MLL fusion oncogenes and were a priory excluded from this series. Enrichment of the TLX1 direct target genes identified by
ChIP-chip ($P < 10^{-4}$) in the TLX1 and TLX3 class signature was analyzed by GSEA using the signal-to-noise metric and a 1,000 permutations of the class labels.

**Gene expression profiling of normal and TLX1 transgenic thymocytes**

We isolated single cell suspensions of thymi from 3 week old wild type and TLX1 transgenic littermates and isolated CD4$^-$CD8$^-$CD44$^+$CD25$^+$ cells on a FACS ARIA Sorter (Beckton Dickinson). We collected cells in RLT buffer (Qiagen) and extracted RNA with the RNeasy mini kit (Qiagen). We verified RNA quantity and quality on a Nanodrop spectrophotometer and an Agilent Bioanalyzer 2100, respectively. We used the Ovation RNA Amplification System V2 (NuGEN) to generate amplified cDNA, which was subsequently fragmented and labeled with the FL-Ovation cDNA Biotin Module V2 (NuGEN) and hybridized on Mouse Genome 430 2.0 Arrays (Affymetrix).

Array normalization and data analysis was performed as described above. Significant differentially expressed genes were calculated by the Comparative Marker Selection Genepattern tool using t-test statistical test with an asymptotic P value and zero permutations.

For enrichment analysis of mitotic genes controlled by TLX1 in preleukemic cells, we first identified 155 mitotic regulators (GO:0000278–mitotic cell cycle) within the top 5,549 TLX1 targets identified by ChIP-chip ($P < 0.0001$) using the DAVID (http://david.abcc.ncifcrf.gov/) Functional Annotation tool, and used this geneset to run GSEA in TLX1 transgenic versus wild type DN2 cells test using the signal-to-noise metric and 1,000 permutations of the gene list.

**Array comparative genomic hybridization (aCGH)**

We performed aCGH analysis of mouse tumors using the Mouse Genome CGH 244A platform (Agilent Technologies) according to the instructions of the manufacturer. To analyze these data we used DNA Analytics 4.0 software (Agilent Technologies). For Array-CGH analysis of human T-ALLs we used SurePrint G3 Human CGH 1x1M Oligo Microarrays (Agilent Technologies). We analyzed human array CGH data with the Genomic Workbench 5.0.14 software (Agilent Technologies).

**Mitotic checkpoint analysis**

Integrity of the mitotic checkpoint was tested by flow cytometry analysis of cell cycle in cells treated with 1 μM taxol (Sigma) (human cell lines), 0.1 μM taxol (mouse cell lines), 100 ng ml$^{-1}$ nocodazole (Sigma) or vehicle for 24 hours. For analysis of the mitotic checkpoint in pre-leukemic mouse cells, thymocytes from 3 week old VavP-Bcl2 and VavP-Bcl2/TLX1 transgenic littermates were cultured on OP9-DL1 cells for 24 hours followed by taxol treatment and cell cycle analysis as described above.

**TLX1 ChIP-chip analysis**

We performed ChIP-chip analysis of TLX1 direct target genes in the ALL-SIL T-ALL cell line using a rabbit polyclonal TLX1-specific antibody (C-18, sc-880) (Santa Cruz Biotechnology) using Agilent Human Proximal Promoter Microarrays (244K features/array)
as previously described. We identified TLX1 direct targets via ChIP-chip Significance Analysis (CSA) as described before.

**Quantitative ChIP Analysis**

Relative real-time PCR quantitation of promoter sequences was normalized to β-actin levels in chromatin immunoprecipitates performed with a TLX1-specific antibody (C-18 rabbit polyclonal antibody (sc-880), Santa Cruz Biotechnology), or an IgG antibody. Primer sequences are listed in Supplementary Table 15.

**siRNA knockdown**

In TLX1 siRNA knockdown experiments we electroporated ALL-SIL cells (275V, 1,000 μF) using a Gene pulser MXcell electroporator (Biorad) with 200 nM of Silencer Select Negative Control #1 siRNA (Ambion, cat #4390844) or TLX1 siRNA1 (5’-GAUGGAGAGUAACCGCAGAtt-3’). Knockdown efficiency was measured by quantitative RT-PCR and Western blot.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. 
TLX1-induced T-cell leukemias in mice. (a) Kaplan-Meier survival curves of p56Lck:TLX1 transgenic mice and littermate controls from three independent founder lines. Accelerated mortality in TLX1 transgenic mice was associated with the development of immature T-cell tumors. (b) Infiltration of thymus, bone marrow and liver by immature lymphoblasts. (c) Western blot analysis of TLX1 expression in mouse T-cell tumors. (d-e) Immunohistochemical analysis of TLX1 (d) and CD3 expression in mouse TLX1-induced T-ALL cells (e). Scale bar 100 μm. (f,g) Immunophenotype distribution (f) and representative
flow cytometry plots (g) showing heterogeneous expression of CD4 and CD8 in Tlx1-induced leukemias. DN, double negative; DP, double positive; SP, single positive. (h) Clonality analysis by expression of Tcrb chains in Tlx1-induced tumors. Polyclonal expression of TCRB in normal thymocytes is shown as control (upper row).
Fig. 2.
Molecular signatures associated with \( TLX1 \)-induced transformation. (a) Heat map diagram of the 50 top ranking differentially expressed genes by \( t \)-test in mouse \( TLX1 \)-induced leukemias. (b) GSEA analysis of differentially expressed genes associated with \( TLX1 \)-induced transformation in mice demonstrates enrichment of this signature in human \( TLX1/TLX3 \) expressing T-ALLs. Gene set: Human orthologs of mouse \( TLX1 \) signature genes. Data set: \( TLX1/TLX3 \) positive vs. \( TLX1/TLX3 \) negative human leukemias. Enrichment plots (left) and heat map representations of the 50 top ranking genes in the leading edge (right) are shown. Genes in heat maps are shown in rows, each individual sample is shown in one column. The scale bar shows color coded differential expression from the mean in standard deviation units with red indicating higher levels and blue lower levels of expression.
Fig 3. Developmental defects in thymocyte development in TLX1-transgenic mice

(a) Preleukemic TLX1 transgenic mice at 6 weeks of age showed decreased thymus weight and cellularity compared with littermate controls. Scale bar 10 mm. (b) Cell cycle analysis of control and preleukemic TLX1-transgenic thymocytes via PI staining of DNA content analyzed by flow cytometry. (c) Flow cytometry analysis of T-cell development in preleukemic TLX1-transgenic animals. Accumulation of CD44+CD25+ cells shows a differentiation block at the DN2 stage of thymocyte development. (d) Apoptosis analysis of control and TLX1-transgenic preleukemic thymocytes via annexinV/PI staining. (e) TUNEL staining on thymus tissue sections. Scale bars represent 100 μm. (f-h) Transgenic expression of BCL2 inhibits apoptosis (f) and reverses thymic weight (g) and cellularity (h) in preleukemic TLX1+BCL2 double transgenic mice.
Fig. 4.
Numerical and structural chromosomal alterations in TLX1-induced mouse T-ALLs. (a) Mouse chromosomal ideogram showing the areas of genetic gain and loss identified by aCGH in TLX1 thymic tumors. Red bars represent areas of gain. Green bars represent areas of copy number loss. (b) Schematic representation of the chromosome 12q commonly deleted region encompassing the Bcl11b locus in mouse TLX1-induced tumors. (c) Array CGH plot showing a focal deletion of the Bcl11b gene in a mouse TLX1-induced T-ALL. (d) Schematic representation of Bcl11b mutations identified in mouse TLX1-induced T-ALLs.
(e) DNA sequence chromatograms corresponding to \( Bcl11b \) mutations identified in mouse \( TLX1 \)-induced T-ALLs.
Fig. 5.
BCL11B is a TLX1 target gene mutated in human T-ALL. (a) Array CGH plots showing focal deletions in chromosomal band 14q32.2 encompassing the BCL11B locus in human T-ALL. (b) Schematic representation of BCL11B mutations identified in human T-ALL. (c) DNA sequence analysis of BCL11B in diagnostic and remission T-ALL samples. (d) ChIP analysis of TLX1 binding to BCL11B regulatory sequences in ALL-SIL cells. (e) Western blot analysis of TLX1 and RT-PCR analysis of BCL11B expression in ALL-SIL cells electroporated with control or TLX1 siRNAs.
Fig. 6. Numerical chromosomal alterations and defects in the mitotic checkpoint in TLX-transgenic mice. (a) SKY analysis of a mouse TLX1-induced tumor with trisomy 15. (b) Distribution of numerical chromosomal aberrations found by SKY and aCGH analysis in mouse TLX1-induced leukemias. (c) Cell cycle analysis of vehicle only, taxol and nocodazole treated mouse T-ALLs showing defective activation of the mitotic checkpoint in mouse TLX1-positive leukemia cells. (d) GSEA analysis of mitotic regulators identified as TLX1 direct target genes by ChIP-chip in sorted thymocytes (DN2 cells) from wild type and preleukemic TLX1 transgenic mice. Gene set: TLX1 direct targets in mitotic cell cycle (GO:0000278). Data set: TLX1 transgenic preleukemic cells vs. wild type. The enrichment plot (left) and heat map representation of the top 25 mitotic genes in the rank of transcripts differentially expressed in TLX1-preleukemic cells (right) are shown. The scale bar at the bottom shows color coded differential expression from the mean in standard deviation units with red indicating higher levels and blue lower levels of expression. (e) RT-PCR analysis of Chek1 expression in thymocytes isolated from wild type or TLX1-transgenic mice. (f) ChIP analysis of TLX1 binding to CHEK1 regulatory sequences in ALL-SIL cells. (g) RT-PCR analysis of TLX1 and CHEK1 expression in ALL-SIL cells electroporated with control or TLX1 siRNAs. (h) Cell cycle analysis of vehicle only and taxol treated mouse thymocytes from

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