

The *C-MYB* locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children

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The *C-Myb* transcription factor is essential for hematopoiesis, including in the T-cell lineage. The *C-Myb* locus is a common site of retroviral insertional mutagenesis, however no recurrent genomic involvement has been reported in human malignancies. Here, we identified 2 types of genomic alterations involving the *C-MYB* locus at 6q23 in human T-cell acute leukemia (T-ALL). First, we found a reciprocal translocation, t(6;7)(q23;q34), that juxtaposed the *TCRB* and *C-MYB* loci (*n* = 6 cases). Second, a genome-wide

copy-number analysis by array-based comparative genomic hybridization (array-CGH) identified short somatic duplications that include *C-MYB* (*MYB*^{dup}, *n* = 13 cases of 84 T-ALL, 15%). Expression analysis, including allele-specific approaches, showed stronger *C-MYB* expression in the *MYB*-rearranged cases compared with other T-ALLs, and a dramatically skewed *C-MYB* allele expression in the *TCRB-MYB* cases, which suggests that a translocation-driven deregulated expression may overcome a cellular attempt to down-

regulate *C-MYB*. Strikingly, profiling of the T-ALLs by clinical, genomic, and large-scale gene expression analyses shows that the *TCRB-MYB* translocation defines a new T-ALL subtype associated with a very young age for T-cell leukemia (median, 2.2 years) and with a proliferation/mitosis expression signature. By contrast, the *MYB*^{dup} alteration was associated with the previously defined T-ALL subtypes. (Blood. 2007;110:1251-1261)

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Introduction

C-Myb is a leucine zipper transcription factor, the expression of which is associated with immature and proliferative cellular stages and turned off during the maturation of the hematopoietic lineage.^{1,2} Silencing strategies in mice have shown that *c-Myb* plays a major role in hematopoiesis, including lineage commitment, proliferation, and differentiation.³⁻⁶ In the T-cell lineage, *c-Myb* is involved at several key steps throughout the maturation process.⁷⁻¹¹ The *c-Myb* gene was first identified as the cellular homologue of the transforming *v-Myb* gene of 2 avian retroviruses that induce leukemia, AMV and E26.¹²⁻¹⁵ Transgenic expression of *v-Myb*, which is a truncated and mutated form of mammalian *c-Myb*, induces lymphoid or myeloid tumors in mice.^{16,17} The murine *c-Myb* locus is also a common site of retroviral insertion in lymphoid and myeloid leukemia.¹⁸⁻²³ In humans, the *C-MYB* gene is located at chromosomal band 6q23.3, and interestingly the chromosome 6q is frequently involved in chromosomal abnormalities in human cancer, including hematologic malignancies.²⁴ In addition, *C-MYB* is frequently expressed in human cancer.^{14,25-27} However, despite intensive studies in a large range of human neoplasias including leukemia, no clear recurrent involvement of

the *C-MYB* locus in genomic abnormalities has been reported to date.^{23,28-31}

T-cell acute lymphoblastic leukemias (T-ALLs) are highly malignant tumors that derive from T-cell progenitors.³²⁻³⁴ Immunophenotypic and gene expression analyses of leukemic cells have revealed heterogeneity that partially reflects distinct stages of T-cell maturation arrest.³⁵⁻³⁸ Correlations between oncogene expression, immunophenotype, and large-scale expression profiles have allowed the definition of distinct oncogenic T-ALL subtypes.^{37,38} Genetic studies have pointed out an increasing number of oncogenes in T-ALLs, further demonstrating the complexity of T-ALL oncogenesis and the requirement for several cooperative oncogenic events.^{33,34,38} T-ALL oncogenes include aberrantly activated transcription factor, namely bHLH (*TAL1*, *TAL2*, *LYL1*, and *BHLHB1*), LIM-only genes (*LMO1* and *LMO2*), homeobox genes (*HOXA*, *TLX1/HOX11*, *TLX3/HOX11L2*, *NKX2-5*), and the *CALM-AF10* and *MLL* fusion genes.^{33,34,38-42} In addition, the tumor suppressor locus *CDKN2A/p16/ARF* is inactivated in most T-ALL cases,^{43,44} and *NOTCH1* is activated by mutations in half the cases.^{45,46} The Cyclin D2 gene and the *NUP214-ABL1* fusion can also be involved

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in multistep oncogenesis.^{47,48} Notably, most T-ALL oncogenes were identified initially by their involvement in recurrent genomic abnormalities. It is expected that the identification of genetic lesions underlying each subtype of T-ALL will help to adapt therapy, including the development of specific agents against oncogenic pathways—so-called targeted therapy.⁴⁹

Using combined cytogenetic, genomic, and molecular tools, we demonstrate for the first time a clear recurrent genomic involvement of the *C-MYB* gene locus at 6q23.3 in a human cancer, namely T-ALL, by 2 distinct types of somatic alteration: *TCRB*-related translocation and cryptic duplication of a short genome region. Expression analysis suggested that a deregulated *C-MYB* expression may be oncogenic in T-ALL. Moreover, the t(6;7) *TCRB-MYB* translocation defines a new T-ALL subtype that is associated with a very young age and a proliferation/mitosis expression signature.

Patients, materials, and methods

Patient samples and molecular annotations

A series of 92 T-ALL patients (56 children, 36 adults) were diagnosed and treated at Saint-Louis Hospital, Paris, France. The patients' age at diagnosis ranged from 13 months to 66 years (median age, 15 years). Informed consent was obtained from the patients or relatives in accordance with the Declaration of Helsinki. The study was approved by the Hôpital Saint-Louis and Institut Universitaire d'Hématologie Institutional Review Board. Four T-cell lines, CCRF-CEM, HSB-2, Jurkat, and MOLT-4, were included in the analysis. This series was previously characterized for oncogene expression and classified for oncogenic groups according to combined immunophenotypic data, oncogene expression, and global gene expression analysis using Affymetrix (Santa Clara, CA) data.³⁸ Three additional T-ALL cases were added to this series, one from Saint-Louis Hospital (TL93) and 2 that were analyzed by W.A.D. and A.W.L. (T142), and B.M. and B.N. (UPN5846). Large-scale expression data were obtained for case TL93 and were used after normalization using previously described methods³⁸ for a new global analysis. The array-based comparative genomic hybridization (array-CGH) study was performed on 84 samples with available material of the 92 initial cases (80 T-ALL cases and the 4 T-cell lines), as part of the CIT National French program. Constitutional genomic DNA was obtained from follow-up samples of 6 patients in complete remission; these DNA samples were used as paired controls in array-CGH experiments to rule out copy-number polymorphisms. Additional T-ALL oncogenic annotations were obtained as follows: *CDKN2A/p16/ARF* genomic data using array-CGH analysis and/or locus-specific fluorescent in situ hybridization (FISH), and *NOTCH1* mutations by sequencing polymerase chain reaction (PCR)-amplified genomic fragments of exons 26, 27, and 34 (encoding the heterodimerization domain [HD]), and the PEST domain.⁴⁵

Cytogenetic and molecular analyses

Interphasic fluorescence in situ hybridization (FISH) analyses were performed on cryopreserved leukemic cells. All FISH probes were prepared from bacterial artificial chromosome (BAC) or P1-derived artificial chromosome (PAC) clones obtained from the BAC/PAC Resource Center at Children's Hospital Oakland Research Institute Oakland, CA (<http://bacpac.chori.org>). Whole chromosome painting FISH was performed using WCP6 and WCP7 from Abbott Laboratories (<http://www.vysis.com>). The *TCRB*-flanking FISH probes have been described previously.³⁸ The *C-MYB* locus FISH probes were RP11-845K5, RP11-184J4, RP11-104D9, RP11-141K5, RP11-55H4, and RP11-166A21. For molecular combing analysis (fiber-FISH), DNA was combed using the Molecular Combing System (Genomic Vision, Paris, France) as described.⁵⁰ All FISH micrograph images were performed with an upright epifluorescence microscope (Leica DM6000 B LT, Rueil-Malmaison, France) equipped with appropriate filter blocks for fluorescence analysis (fluorescein isothiocyanate [FITC], Texas Red, and 4',6-diamidino-2-phenylindole [DAPI]). The images were cap-

tured through a PlanApo 63×/1.32 oil immersion objective (0.132 mm/pixel; Leica) with a CCD camera (3 × 1/2") JAI M300 (<http://www.jai.com>) and Isis FISH analysis software (Metasystems, Altlußheim, Germany). Images shown in the figures are at original magnification, ×630. Southern blot analysis for the *C-MYB* locus was performed using *EcoRI*, *HindIII*, and *XbaI* digestions and a panel of PCR-amplified probes from BAC RP3-388E23 DNA. Molecular cloning of the *TCRB* translocation breakpoint sequences was performed using ligation-mediated PCR methods as previously reported.³⁸

Genome wide array-CGH analysis

A 4K BAC-PAC array-CGH (Curie Institute–French Ligue Contre le Cancer, Paris, France) was used on 84 T-ALL samples. This array contained 3922 BAC and PAC DNAs, as previously described.⁵¹ This array was designed to regularly cover the genome and also included additional clones for putative oncogenes such as *C-MYB*, or for regions known to be commonly rearranged in cancer. Hybridization, scanning, normalization, and data analysis were performed using standard procedures.⁵¹

High-density 244K oligonucleotide arrays (Agilent Technologies, <http://www.agilent.com>) were used to map the *C-MYB* duplication precisely, and to confirm somatic gain in paired tumor and constitutional genomic DNA from the same patients in cases TL29 and TL59.

Genomic databases and bioinformatics tools

The following databases and tools were used: the UCSC Genome Browser (<http://genome.ucsc.edu>), Ensembl (<http://www.ensembl.org/index.html>), the Immunogenetics (IMGT) repertoire for immunoglobulins and T-cell receptors (<http://imgt.cines.fr/textes/IMGTrepertoire>), the NCBI blast tools (<http://ncbi.nlm.nih.gov/blast>), the Mitelman Database of Chromosome Aberrations in Cancer (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>), the Database of Genomic Variants (<http://projects.tcag.ca/variation>), the Mouse Retrovirus Tagged Cancer Gene Database (<http://rtcgdb.ncicfcrf.gov>), the Entrez databases (<http://www.ncbi.nlm.nih.gov/>), and the Gene Ontology project database (<http://www.geneontology.org>).

Array-CGH data were analyzed using the Curie Institute VAMP tools⁵² (http://www.curie.fr/recherche/themes/detail_equipe.cfm/lang/_gb/id_equipe/303.htm) and the CGH Analytics 3.2 software (Agilent Technologies, <http://www.agilent.com>).

Large-scale gene expression data were normalized, and the analysis was performed using dChip (<http://www.dchip.org>), as previously described.³⁸

C-MYB expression and sequence analyses

RNA samples were reverse transcribed and cDNAs were analyzed for *C-MYB* transcripts by real-time quantitative PCR (RQ-PCR) using Taqman methods (Applied Biosystems, <http://www.appliedbiosystems.com/>). Results were normalized on expression of the housekeeping gene *TBP*, according to the formula: level of *C-MYB* = $2^{(C_{TBP} - C_{MYB})}$. RQ-PCR system for analysis of MYB expression was as follows: MYB-F: 5'-CTATTACCACATTCTGAAGCACAAAA-3', MYB-R: 5'-GCTGAGG-GACATTGACTATATTTACATG-3', and MYB probe: 5'-6-FAM-CTC-CAGTCATGTTCCATACCCTGTAGCGTT-TAMRA-3'; *TBP* housekeeping gene: TBP-F: 5'-CACGAACCACGGCACTGATT-3', TBP-R: 5'-TTTCTTGCTGCCAGTCTGGAC-3', and TBP probe: 5'-6-FAM-TGTGACAG-GAGCCAAGAGTGAAGA-TAMRA-3'. Additional PCR systems for the analysis of alternative transcripts and differential use of promoters are shown in the Figure S4 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). In selected cases, the entire *C-MYB* open reading frame was sequenced on both strands from leukemic cDNA after PCR of cDNA from leukemic cells; primers sequences are available in Figure S4. A polymorphic poly-T (T8/9) microsatellite found in the 3'-UTR region was used for analysis of *C-MYB* allelic expression. Fragment size analysis of a fluorescent-labeled PCR fragment using Genescan methods (Applied Biosystems) allowed discrimination of the T8/T9 alleles. PCR primers were as follows: S217F-5'-6-FAM-AGGTAATGAATTGTAGCCAG-3' and S218R-5'-AAATACTGATCTGTTGGATCC-3'. Leukemic genomic DNA samples were first analyzed to determine the heterozygous cases, and then

leukemic cDNAs from informative cases were analyzed to determine *C-MYB* allele expression.

Results

Identification of a recurrent translocation t(6;7)(q23;q34) in T-ALL involving the *TCRB* and *C-MYB* loci

In the course of conventional cytogenetic analysis at diagnosis of acute leukemia patients in Saint-Louis Hospital, we identified a chromosomal translocation t(6;7)(q23;q34) in 2 pediatric T-ALL cases (TL34 and TL92, Figure 1A,B). Dual-color FISH using *TCRB* flanking probes demonstrated involvement of the *TCRB* locus at 7q34 in both cases (Figure 1B). By using inverse PCR from the *TCRB* sequence, we amplified and characterized the breakpoints' derivative sequences in case TL34 (Figure 1C). The breakpoint on chromosome 6q23.3 was mapped in the vicinity of the *C-MYB* oncogene. Probes were derived from the *C-MYB* locus and demonstrated in FISH and Southern blot experiments that the same locus was involved in case TL92 (Figure 1D). The t(6;7)(q23;q34) involves telomeric regions in both chromosomes 6 and 7, hence we assumed that this translocation could have been missed in systematic T-ALL karyotype series. We therefore used *C-MYB* flanking FISH probes that allowed us to identify 2 additional cases with translocations involving the *C-MYB* and *TCRB* loci (TL33 and TL93, Figure 1E) from a series of 84 T-ALL (80 T-ALL patients

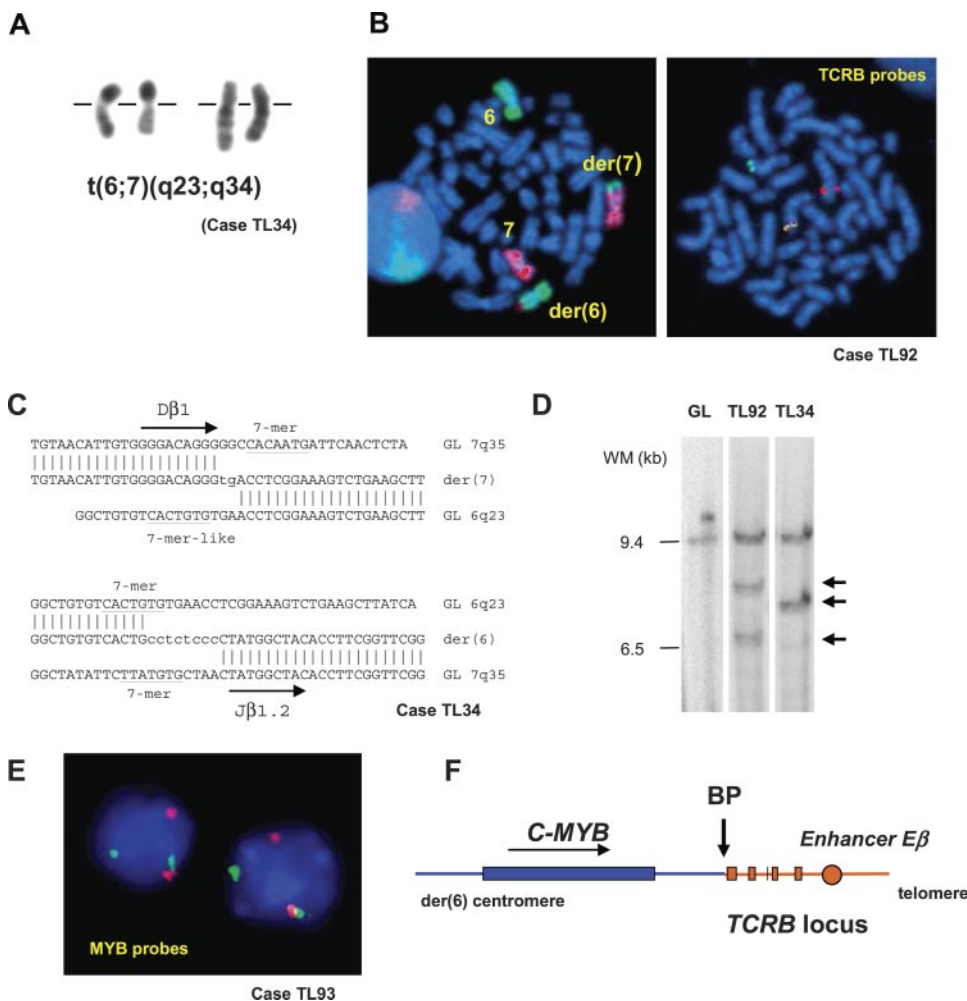
and 4 T-cell lines). Finally, 2 isolated cases with a *TCRB-MYB* translocation (UPN5846 and T142) were detected from independent T-ALL series by systematic molecular analysis of oncogenic *TCRB* genes rearrangements, as performed by B.M. and B.N., and W.A.D. and A.W.L. These cases were added to the present study, giving a total of 6 *TCRB-MYB* cases, including 3 cases fully characterized at the molecular level on both derivative chromosomes (Figure 1C; Figure S1). Sequence analysis of the *TCRB* derivative sequences was suggestive of a specific mechanism of V(D)J-mediated translocation typically seen at the *TCRB* locus.^{53,54} These translocations were reciprocal and balanced, and led to the juxtaposition of the *C-MYB* proto-oncogene near to the *TCRB* regulatory sequence (Figure 1F), which suggested deregulated expression.

A genome-wide array-CGH screen in T-ALL identified recurrent somatic genomic duplications at the *C-MYB* locus

A genome-wide analysis of genomic copy number was performed in 84 of the 92 T-ALL cases (80 primary samples and 4 T-cell lines), in order to detect genomic imbalances in T-ALL. Leukemic DNA samples and a normal DNA XY control (GYPT) were cohybridized on a 4K BAC/PAC array ("Patients, materials, and methods"). Remarkably, examination of the data for chromosome 6 revealed a DNA copy gain of a 6q23.3 region in 13 cases of 84 T-ALL (11 T-ALL patients and 2 T-cell lines, Figure 2A). In 2 cases, the DNA copy gain consisted of a large

Figure 1. A recurrent translocation t(6;7)(q23;q34) in T-ALL involves the *TCRB* and *C-MYB* loci.

(A) Partial R-banded karyotype with translocation t(6;7)(q23;q34). (B, left panel) FISH whole chromosome painting of chromosomes 6 (green) and 7 (red) on metaphases from leukemic cells confirmed the reciprocal translocation t(6;7). (B, right panel) Dual-color FISH analysis of the *TCRB* locus using *TCRB*-flanking probes (centromeric, CTD-3092H9 labeled in red; telomeric, RP11-168115, labeled in green). Dissociation of the probes in leukemic cells with t(6;7) demonstrated the involvement of the *TCRB* locus as partner of the translocation. (C) Derivative sequences of t(6;7)(q23;q34) breakpoints in case TL34 and corresponding germ-line sequences. Recognition sequence signal (RSS) heptamer and putative heptamer-like sequence are indicated according to consensus⁵³; untemplated nucleotides (N-diversity) are typed in lowercase. GL indicates germ line; der, derivative chromosomes. Breakpoint sequences for cases T142 and UPN5846 are shown in Figure S1. (D) Southern blot mapping of the translocation breakpoint in case TL92 using a 6q23.3 probe derived from BAC RP11-388E23. Germ-line (GL) and TL34 (*TCRB-MYB*) DNAs are shown as negative and positive controls, respectively. *EcoRI* rearranged bands are shown by arrows. (E) Interphase FISH screening of T-ALL using flanking *C-MYB* locus probes RP11-845K5 (green) and RP11-184J4 (red) identified additional MYB-translocated cases TL33 and TL93, further shown to juxtapose the *TCRB* and *C-MYB* loci using combinations of FISH probes. (F) Schematic representation of the der(6) genomic region of t(6;7), according to data from the IMGT database and the UCSC Genome Bioinformatics site, and to the DNA derivative sequence of the breakpoint region in case T142. An arrow indicates the breakpoint (BP). See "Patients, materials, and methods; Cytogenetic and molecular analyses" for details about FISH image acquisition and manipulation.



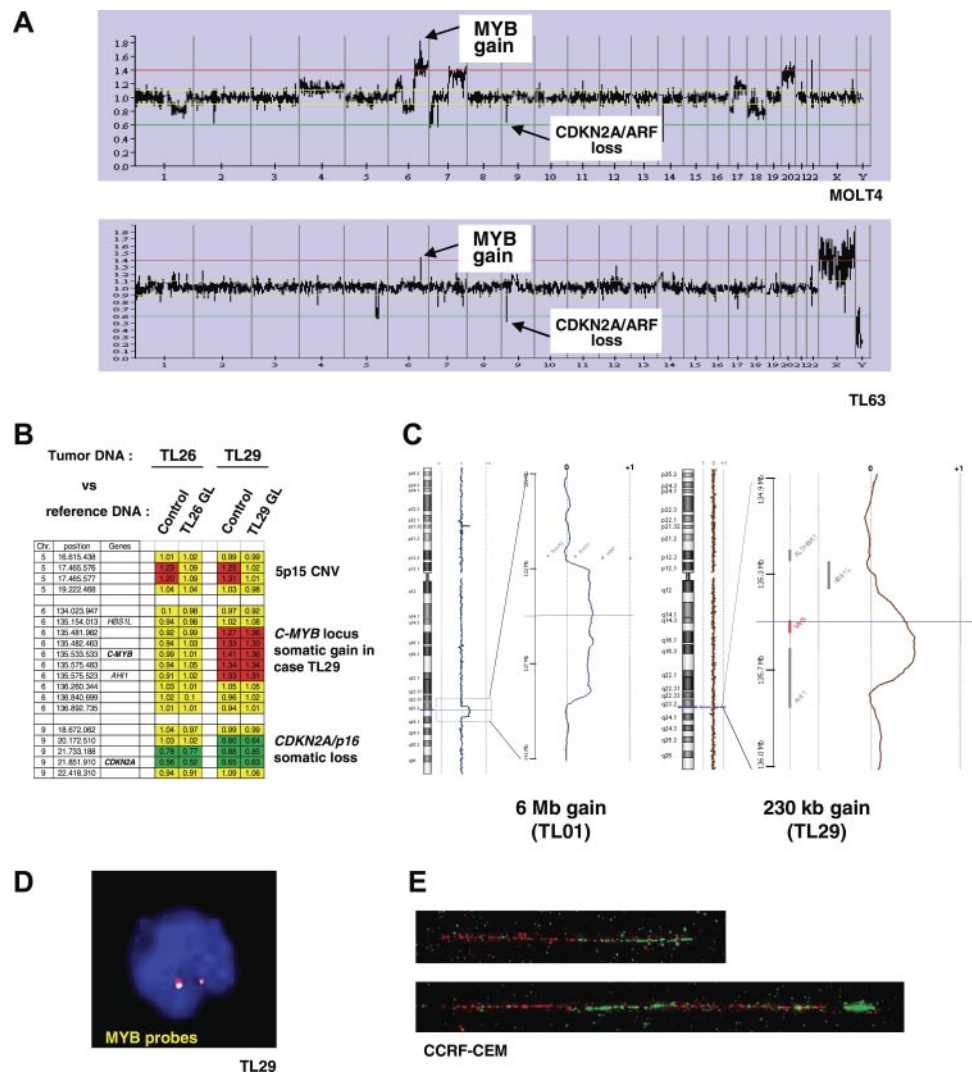
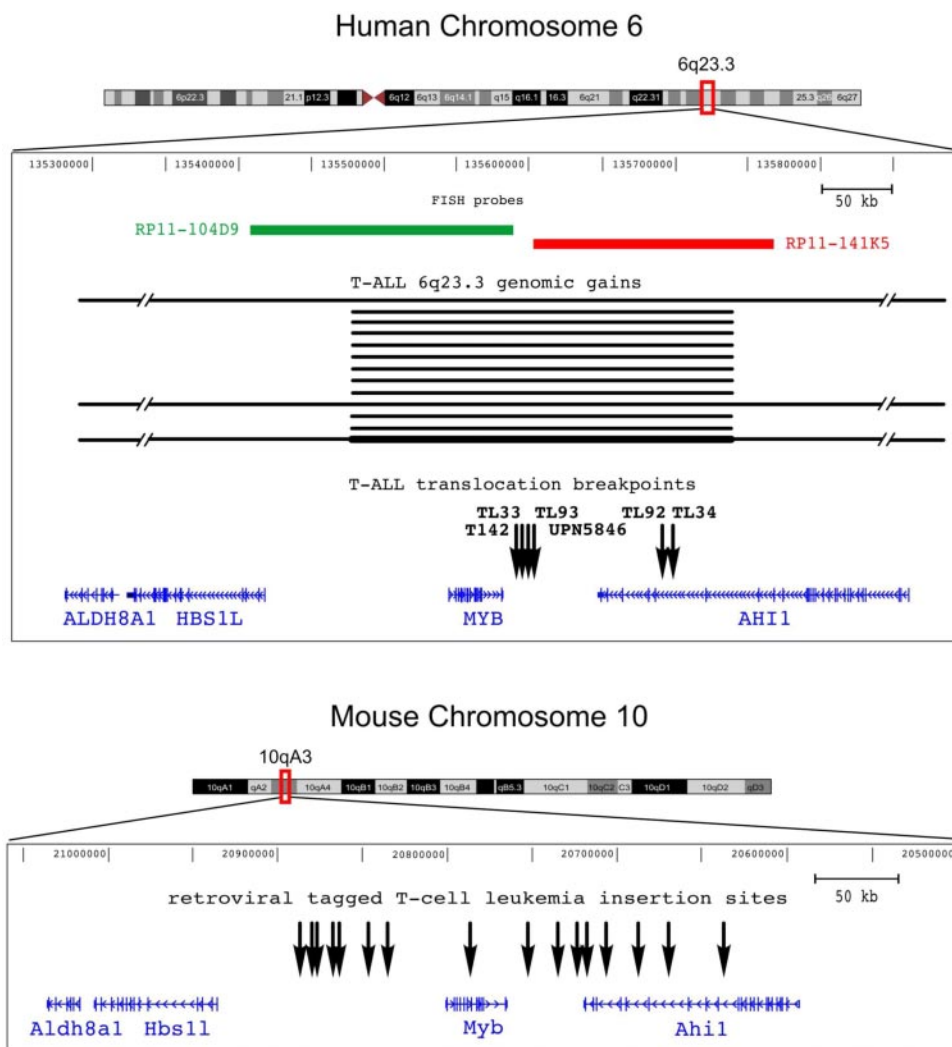


Figure 2. Identification of recurrent somatic genomic duplications at the *C-MYB* locus in T-ALL. (A) Global representation of large-scale analysis of genomic copy number using a 4K array-CGH in MOLT4 cell line (upper panel) and TL63 primary T-ALL case (lower panel). DNA copy gain at the *C-MYB* locus at 6q23 is indicated, as well as the DNA copy loss at the *CDKN2A/p16/ARF* locus at 9p21 as an example. (B) Compared analysis of array hybridization using as normal DNA either a healthy subject DNA (control) or germ-line DNA (GL) of the same patient as DNA reference. This analysis allowed us to distinguish copy-number variations (CNVs) and somatic genomic imbalances. As an example, a CNV at 5p15 was resolved in the TL26 and TL29 cases (a gain was observed when the leukemic DNA was cohybridized with an unrelated control DNA, but it disappeared using paired leukemic and GL controls). A somatic loss of *CDKN2A/p16/ARF* was evidenced in both the TL26 and TL29 cases, because the imbalance persisted after cohybridization with paired control; similarly the somatic gain at the 6q23/*C-MYB* locus was confirmed in case TL29. For each BAC/PAC of the array, gains were represented in red; losses, in green; and balanced signals, in yellow. (C) Copy-number analysis using a very high-density oligonucleotide array (Agilent), focused on the *C-MYB* region, enabled the minimal region of genomic gain to be mapped to approximately 230 kb. (C, right panel) A 6-Mb-sized copy gain region was found in case TL01; (left panel) a short minimal genomic gain including the *C-MYB* gene was evidenced in case TL29 (array-CGH performed with paired leukemic and GL DNAs). The genomic region of gain of chromosome 6 is magnified, with the horizontal cursor (blue) pointing out the *C-MYB* gene (shown in red in the right panel). The so-called “moving average” ratio between leukemic and GL DNA appears as a blue line in case TL01, and a brown line in case TL29. Two copies (alleles) of the locus appear as a moving average close to 0, whereas a DNA copy gain of an allele shifts the line close to ratio + 0.5 along the corresponding genomic region. (D) Interphasic *C-MYB* FISH using the RP1-32B1 (green) and RP3-388E23 (red) probes in the *MYB*^{del} cases showed no extra signal (except in the complex MOLT4 pseudotetraploid cell line, not shown), which suggests local duplication. (E) Molecular combing analysis using *C-MYB* locus probes RP11-55H4 (red) and RP11-166A21 (green) demonstrated a local duplication (bottom); the normal allele is shown on the top. See “Patients, materials, and methods; Cytogenetic and molecular analyses” for details about FISH image acquisition and manipulation.

chromosomal gain (6.0 Mb in TL01, and 22.4 Mb in TL76), while in the other cases the amplified region was restricted to a region less than 2 Mb including the *C-MYB* locus. The MOLT4 cell line showed a more complex profile with a gain of a large region of chromosome 6q in a pseudotetraploid karyotype reinforced by higher level of amplification in a region less than 2 Mb at the *C-MYB* locus (Figure 2A top panel). We then investigated whether the short DNA copy gain of the *C-MYB* locus was a somatic rearrangement that originated in leukemic cells, or whether it could be a constitutional large-size copy-number variation (CNV) as was recently reported in the human genome.⁵⁵⁻⁵⁷ Notably, no CNV was reported in this region in the Database of Genomic Variants (Figure S2). Then we compared our data to those from 2 independent array-CGH studies on breast

cancer samples that were performed using the same array and the same control DNA (GYPT, a unique XY healthy subject whose DNA was used in several studies); no recurrent genomic imbalance in the *C-MYB* locus was observed in these other studies, which suggested that there was no frequent CNV in this region and that the *C-MYB* gain was associated with T-ALL (data not shown). Finally, we were able to demonstrate definitively the somatic origin of the *C-MYB* gain by cohybridizing on the same array paired leukemic and constitutional DNAs from the same patient in 2 T-ALL cases with available constitutional material (Figure 2B). Thereafter, we defined a minimal region of DNA copy gain using high-density 244K oligonucleotide array-CGHs (Agilent) to a consistent region of length approximately 230 kb including the

Figure 3. Genomic rearrangements target the *C-MYB* locus in both human and mouse T-cell leukemias. Representation of the *C-MYB* locus in both the human and mouse genomes according to the UCSC database; note that the orientation of the mouse locus was inverted in this figure in order to maintain the orientation of the human locus and facilitate comparison. The *ALDH8A1*, *HSB1L*, *C-MYB*, and *AHI1* genes are shown according to annotations in UCSC. The genomic rearrangements we described in this work in human T-ALL are indicated: breakpoints (vertical black arrows) of the 6 *TCRB-MYB* cases, and duplicated genomic regions (horizontal black bars) in the 13 T-ALL cases (TL01, TL29, TL38, TL40, TL49, TL59, TL61, TL63, TL66, TL76, TL77, CCRF-CEM, and MOLT4). The darker horizontal bottom line refers to a *MYB*^{dup} in addition to a larger 6q gain in MOLT4, leading to extra copies of the locus. Two FISH probes that were used for breakpoint mapping are shown. On the mouse genome (bottom panel), all the reported retroviral insertion sites of T-cell leukemias are indicated (vertical black arrows), based on data from the Retrovirus Tagged Cancer Gene Database.



C-MYB gene (Figure 2C). A panel of probes covering the *C-MYB* locus detected no extra signal in metaphases or interphase nuclei in cases with *C-MYB* copy gain (Figure 2D), which suggests a local duplication. That was demonstrated by molecular combing analysis showing a direct tandem duplication of the *C-MYB* locus (Figure 2E). Cases with *C-MYB* locus duplication are further referred to in the study reported herein as *MYB*^{dup} cases.

Genomic location of the *C-MYB* locus rearrangements in human and mouse T-cell leukemias

In the present study 2 types of recurrent somatic rearrangement were identified in T-ALL, namely reciprocal chromosomal translocation *TCRB-MYB*, and short genomic duplications *MYB*^{dup}, which target a unique 6q23.3 region that includes the *C-MYB* proto-oncogene. Molecular mapping of the 6 t(6;7)(q23; q34) chromosomal breakpoints, which included complete breakpoint sequencing in 3 cases (TL34, T142, and UPN5846), showed 2 discrete breakpoint clusters at 6q23.3 (Figure 3 top panel): one located 5 kb telomeric, 3' of the *C-MYB* gene (4 cases: TL33, TL93, T142, and UPN5846), and the other 50 kb more telomeric (2 cases: TL34 and TL92). In all cases, the translocation placed the *C-MYB* proto-oncogene in the vicinity of the *TCRB* regulatory sequence, which suggests that abnormal

regulation of *C-MYB* expression could confer oncogenic properties. The 6q23 genomic duplication was mapped to a short region of approximately 230 kb that encompassed the entire *C-MYB* gene in all duplicated cases (Figure 3 top panel), which reinforced the view of a targeting of this gene by oncogenic somatic events in T-ALL. Another gene, known as *AHI1*, was located in the vicinity of the t(6;7) breakpoints and was disrupted in 2 of 6 cases with the t(6;7), but not in the other 4 cases. It was also partially included in the minimal region of duplication.

Reminiscent of the 6q23 rearrangements that we identified in human T-ALL, the *C-Myb* locus is known to be a frequent insertion site in retrovirally induced leukemias. The lower panel of Figure 3 shows the insertion sites in murine T-cell leukemia as reported in the Retrovirus Tagged Cancer Gene Database.^{19,21-23} These sites are distributed around the *c-Myb* gene, within a region that extends from approximately 100 kb upstream to 150 kb downstream of the gene. Importantly, insertion sites flank both sides of *c-Myb*, which suggests that this gene is an oncogenic target in this chromosomal region.

Therefore the combined genomic data from human (present study) and murine T-cell leukemias (from previous reports) demonstrate that this locus is recurrently targeted in T-cell oncogenesis

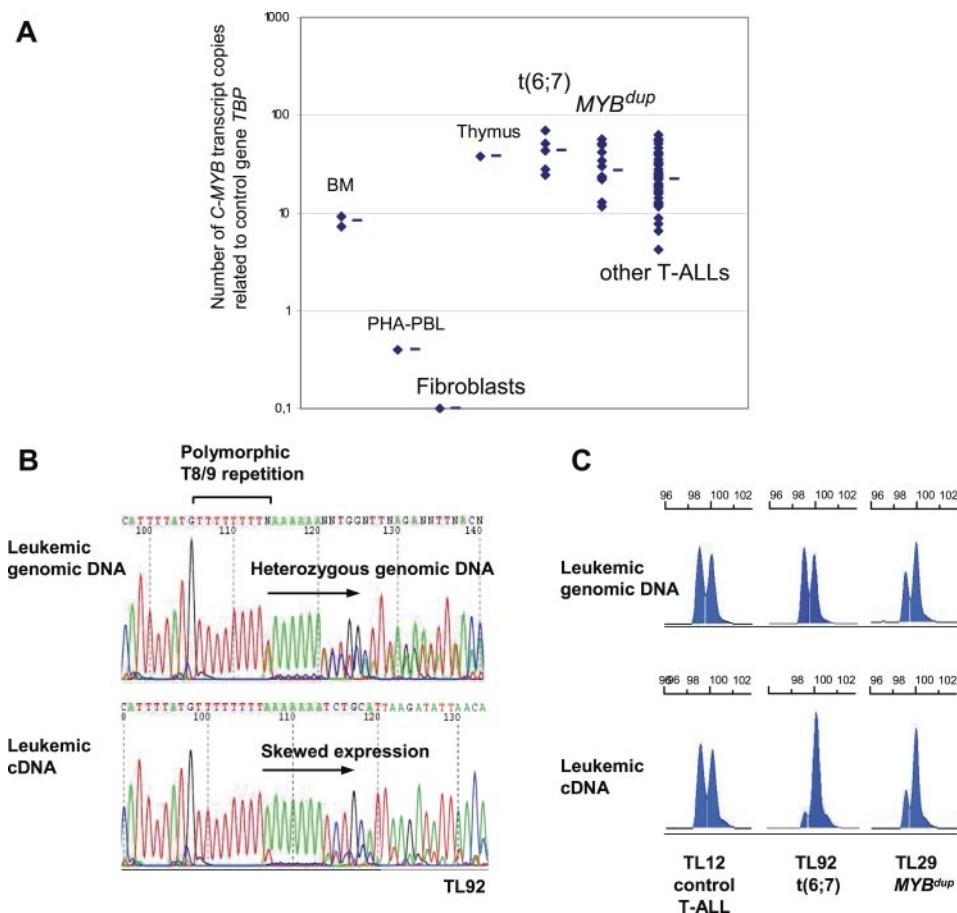


Figure 4. Expression analysis of the *C-MYB* gene in T-ALL. (A) *C-MYB* transcript levels were quantified by RQ-PCR in a series of samples: normal bone marrow (BM, $n = 2$), PHA-stimulated PBL (PHA-PBL, $n = 1$), growing fibroblasts ($n = 1$), thymus ($n = 1$), t(6;7) *TCRB-MYB* cases ($n = 5$, one case not available), *MYB*^{dup} ($n = 13$), other T-ALL ($n = 54$) as indicated. Median value is indicated by a horizontal bar for each group. (B) Sequencing of *C-MYB* in TL92 tumor genomic DNA showed heterozygosity for a polymorphic T8/9 repetition in the 3'UTR region; sequencing of the TL92 leukemic cDNA in this *TCRB-MYB* case demonstrated a skewed allelic expression. (C) Analysis of the T8/9 polymorphic microsatellite using fragment size analysis. Heterozygous cases without *C-MYB* rearrangement ($n = 27$) showed balanced expression; heterozygous cases with t(6;7) translocation ($n = 3$; TL33, TL34, and TL92) showed a skewed expression of one *C-MYB* allele; and heterozygous cases with *MYB*^{dup} ($n = 7$) showed an imbalanced profile of both leukemic genomic DNA and leukemic cDNA.

and point to the *C-MYB* gene as a strong candidate oncogene in these T-ALL cases.

C-MYB expression analysis in human T-ALLs

The murine *c-Myb* gene has been shown to be strongly expressed in normal thymus, and it is necessary for progression through several stages of T-cell differentiation.⁷⁻¹¹ To gain insights in the putative oncogenic role of *C-MYB* rearrangements in human T-ALL, we analyzed *C-MYB* expression using real-time quantitative PCR (RQ-PCR) in the *MYB*-rearranged T-ALL cases in comparison with other T-ALL cases and control cells. We found strong *C-MYB* expression in the T-ALL cases (ie, not only in the rearranged cases), compared with normal PHA-stimulated peripheral blood leukocytes (PBLs), normal bone marrow, and growing fibroblasts (Figure 4A). In this high range, the median expression of *C-MYB* transcripts was stronger in the t(6;7) and *MYB*^{dup} cases than in other T-ALLs (Figure 4A). Consistent results were obtained by analyzing *C-MYB* expression from large-scale expression data, whereas no difference was found for the other genes of the region among the T-ALL groups (Figure S3). An analysis was performed using RQ-PCR systems that were designed to evaluate several alternative transcripts (Figure S4). Systems included the main *C-MYB* transcripts, long variant transcripts that include the so-called alternative exon 9A, the use of distinct promoters (an alternative transcription site has been reported just upstream of exon 2 in humans and mice), and the use of the 3'UTR region in exon 15. Also, standard RT-PCR was performed to search for additional small exons. This analysis

did not reveal changes in the differential pattern of alternative transcripts in the *MYB*-rearranged cases compared with other T-ALL cases and healthy controls, nor did it reveal additional exons (Figure S4). *C-MYB* immunoblot analysis of a number of T-ALL primary cases and cell lines did not reveal aberration of *C-MYB* levels or size (Figure S4). Then we sequenced the *C-MYB* open reading frame from leukemic cDNA in 2 t(6;7) cases (TL34, TL92) and in the 4 T-cell lines including the 2 *MYB*^{dup} cell lines (CCRF-CEM and MOLT4), and no mutation was found. At this point, we investigated *C-MYB* allelic expression in the T-ALL leukemic cells, taking advantage of a T8/T9 polymorphic repetition in the 3'UTR of the *C-MYB* gene (Figure 4B). Forty-one cases of 84 T-ALLs showed a heterozygous T8/T9 polymorphism in leukemic genomic DNA and were therefore informative, including 3 *TCRB-MYB* cases (TL33, TL34, and TL92) and 7 *MYB*^{dup} cases (TL01, TL29, TL38, TL49, TL61, TL76, and MOLT4). Strikingly, analysis at the RNA level demonstrated a dramatically imbalanced *C-MYB* expression in the 3 *TCRB-MYB* cases, suggesting a skewed *TCRB*-driven expression and, importantly, a low expression of the other allele (Figure 4B-C). In the 7 informative *MYB*^{dup} cases, allelic expression of *C-MYB* was imbalanced in the same way as genomic DNA copy number, or even more in some cases (Figure 4C). By contrast, a balanced *C-MYB* expression was found in all nonrearranged T-ALL cases ($n = 27$ informative cases, Figure 4C). In all, our data suggest that the expression of *C-MYB* transcripts can be sustained inappropriately at strong levels due to the translocation in the *TCRB-MYB* cases, and reinforced due to copy-number increase in the *MYB*^{dup} cases.

Table 1. Patient and molecular data on C-MYB rearranged T-ALL cases

Case no.	Sex	Age, y	Oncogenic transcripts*	CDKN2A/p16/ARF deletion†	NOTCH1 mutations‡
t(6;7) TCRB-MYB cases					
TL92	M	1.1	Neg	+	HD-N
TL93	F	1.3	Neg	+	wt
TL34	M	1.8	Neg	—	wt
T142	F	2.5	Neg	+	PEST
UPN5846	F	2.9	Neg	+	HD-N
TL33	F	10	Neg	+	PEST
MYB^{dup} cases					
TL38	M	5	MLL-ENL	—	wt
TL29	F	6	SIL-TAL	+	HD-N
TL77	F	8	Neg	+	wt
TL61	M	10	TLX3	+	PEST
TL66	M	16	TLX3	+	PEST
TL40	M	17	CALM-AF10	+	HD-N + PEST
TL76	M	24	Neg	—	HD-N
TL49	M	40	TLX1	+	HD-C
TL59	M	45	TLX1, NUP214-ABL1	+	wt
TL01	F	50	Neg	+	wt
TL63	F	52	TLX3	+	HD-N + PEST
CCRF-CEM	na	na	SIL-TAL	+	HD-N
MOLT4	na	na	Neg	+	HD-N + PEST

Cases are ordered by increasing age in the *TCRB-MYB* and *MYB^{dup}* cases.

M indicates male; F, female; +, deletion detected; —, no deletion detected; wt, wild type; and na, not applicable.

*Oncogenic transcripts searched for were as follows: SIL-TAL, TLX1, TLX3, HOXA, CALM-AF10, MLL, and NUP214-ABL1 fusions transcripts.

†CDKN2A/p16/ARF locus analysis.

‡NOTCH1 mutations were searched in exons 26, 27, and 34, encoding N-terminal part of heterodimerization domain (HD-N), C-terminal part of heterodimerization domain (HD-C), and PEST domain, respectively. Immunophenotypic data of the 6 *TCRB-MYB* cases are shown in Figure S6.

The TCRB-MYB translocation defines a new distinct T-ALL oncogenic subtype, associated with very young age and a proliferation/mitosis signature by microarray large-scale expression analysis, whereas the MYB^{dup} is found in other T-ALL subtypes

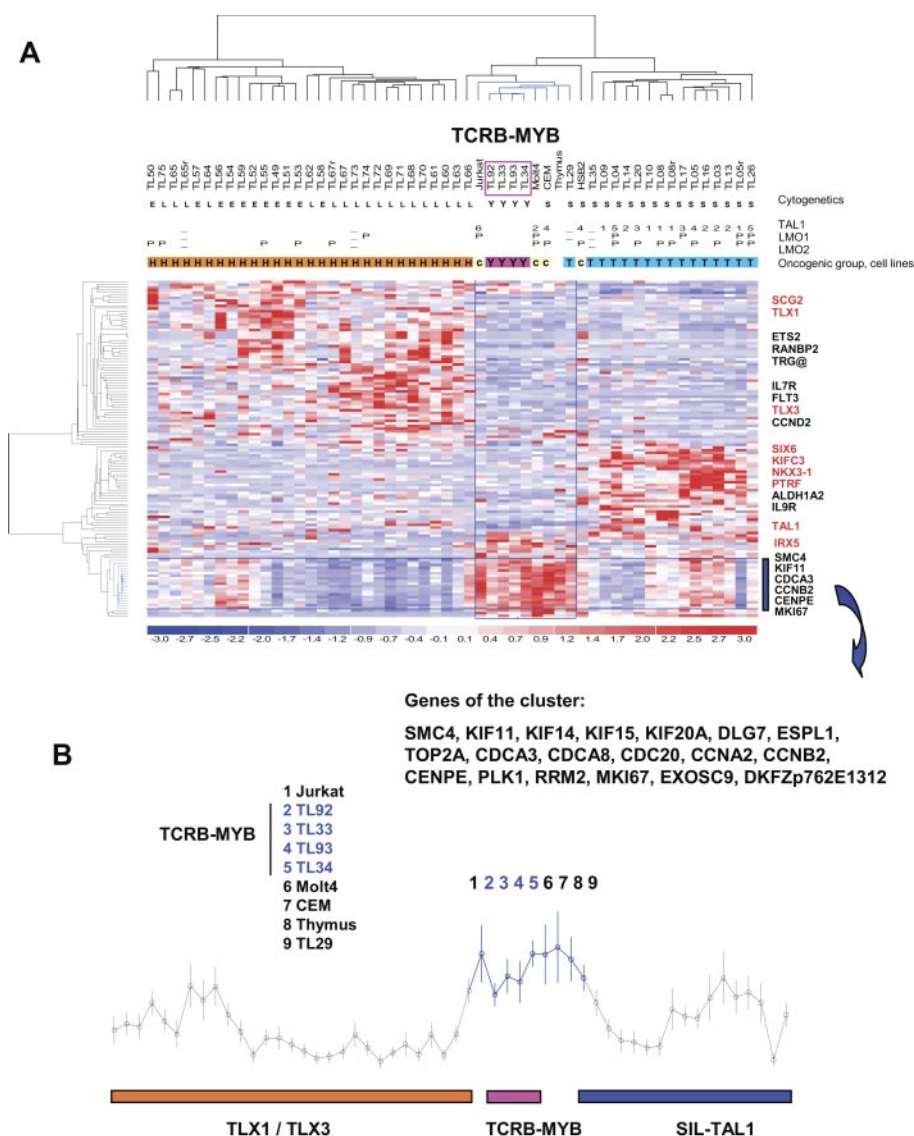
Clinical and biologic data were collected for patients with *C-MYB* rearrangements (Table 1). Strikingly, 5 of the 6 cases with the translocation t(6;7) were diagnosed in very young children (1.1, 1.3, 1.8, 2.5, and 2.9 years old [Table 1]; median age of the *TCB-MYB* patients, 2.2 years). This clustered age range is very unusual in T-ALL where patients are generally older than in precursor B-ALL. For comparison, the median age in pediatric T-ALL was 9.4 years (range, 1 to 19.5 years) in the French randomized multicentric pediatric trials FRALLE 93 and FRALLE 2000 (n = 355 pediatric T-ALL patients), and only 26 (7.3%) of 355 children were younger than 3 years ($P < .001$). Such a unifying feature associated with the *TCRB-MYB* translocation suggests strongly that it defines a new distinct entity in T-ALL. Consistently, the common oncogenic abnormalities defining other T-ALL subtypes were not found in the *TCRB-MYB* patients (ie, *SIL-TAL*, *TLX3/HOX11L2*, *TLX1/HOX11*, *HOXA*, *MLL*, and *CALM-AF10*; Table 1). As in other oncogenic subtypes, the *TCRB-MYB* cases frequently displayed the common additional genomic events, *CDKN2A/p16/ARF* deletion and *NOTCH1* mutations; this demonstrates genetic multievent oncogenesis in this new T-ALL subtype (Table 1).

The situation was clearly different in T-ALL patients with genomic *C-MYB* duplication. Patients had various ages (median, 17 years; range, 5 to 52 years; Table 1). The *MYB^{dup}* leukemic cells frequently expressed various other oncogenic transcripts: *SIL-TAL* (cases TL29 and CCRF-CEM), *TLX1* (TL49, TL59), *TLX3* (TL61, TL63, TL66), *MLL-ENL* (TL38), and *CALM-AF10* (TL40), and 2 cases were classified as immature (TL76 and TL77). Additional genomic events were also found in the *MYB^{dup}* cases: *CDKN2A/p16/*

ARF deletion, *NOTCH1* mutations, and *NUP214-ABL1* amplification (Table 1).

In order to characterize further the *MYB*-rearranged T-ALL, we analyzed large-scale gene expression data. Three cases with the *TCRB-MYB* rearrangement (TL33, TL34, and TL92) and all *MYB^{dup}* cases have been previously included in a series of large-scale gene expression analysis experiments.³⁸ A fourth case (TL93) was also tested and included in a new global analysis. The *MYB*-rearranged cases, as other T-ALLs, displayed high expression of cyclin D3⁵⁸ and of genes belonging to a global T-ALL signature⁵⁹ (data not shown). An unsupervised classification was performed demonstrating that the 4 *TCRB-MYB* cases clustered closely (Figure S5). These cases were then compared by *t* test analysis to the 3 major T-ALL subtypes as defined by the molecular abnormalities *SIL-TAL1*, *TLX1*, and *TLX3*. A large number of differentially expressed probe sets was obtained in each comparison (median *P* value, .001; median, FDR < 10%). A merged list of genes was built (Table S1), and hierarchical clustering analysis was performed. The 4 *TCRB-MYB* cases stably coclustered with 3 cell lines, namely Jurkat, CCRF-CEM, and MOLT4, the thymus sample, and the *SIL-TAL* case TL29 (Figure 5A). This cluster was included in a large branch that contains all cases with a *SIL-TAL1* rearrangement. The cluster was characterized by high expression of 19 genes (Figure 5B), 18 of them linked by Gene Ontology (GO) terms to cell cycle, mitosis, and cell proliferation (Table S2). Interestingly, no expression of the ectopic genes previously associated with the *SIL-TAL1*, *TLX1*, or *TLX3* subtypes was found in any of the 4 cases. By contrast, the *MYB^{dup}* cases were distributed in the clusters according to oncogene expression (*SIL-TAL1*, *TLX1*, *TLX3*, *CALM-AF10*, *MLL*, immature), and they did not cluster together (Figure S5).

These data show that the *TCRB-MYB* translocation defines a new T-ALL subtype, associated with very young age, no association to the other common oncogenic transcripts, and a proliferation/



high-density oligonucleotide array-CGH. Local tandem duplication was demonstrated using molecular combing. These data highlight the strength of high-density array-CGH to identify cryptic copy-number abnormalities in leukemia, as was shown recently for the short *LMO2* activating deletions in T-ALL.⁵⁹ Importantly, we demonstrated that the duplication was somatic and not due to one of the constitutional copy-number variations (CNVs) that was recently discovered to be relatively common in the human genome.⁵⁵⁻⁵⁷ For this purpose, the availability of samples from the same patients in remission, which allowed paired constitutional and tumor analysis, was invaluable (Figure 2B). Interestingly, the *MYB*^{dup} abnormality was frequently found in association with the known classifying oncogene transcripts (SIL-TAL, CALM-AF10, MLL-ENL, TLX1, TLX3),^{37,38} which suggests that *MYB*^{dup} could be an additional oncogenic event.

In contrast with the *MYB*^{dup} abnormality, clinical and biologic data suggest that the *TCRB-MYB* translocation defines a new T-ALL subtype. Strikingly, patients with this translocation were very young (no adult; median age, 2.2 years) compared with the age range of T-ALL patients. Moreover, the ectopic transcripts that have been associated previously with the principal T-ALL subtypes^{37,38} were not found in these cases, which supports further the view that this rearrangement is associated with distinct oncogenic pathways. Coclustering of these cases with T-cell lines and normal thymus sample in a subgroup that is characterized by a high expression of cell cycle, cell proliferation, and mitosis genes suggests a specific related biology (Figure 5, Figure S5, and Table S2).

The *c-Myb* locus is known as a frequent insertion site in retrovirus-mediated oncogenesis in animals.^{19,21-23} A detailed map of the mouse insertion sites based on the RCG database and comparison with human data shows that the *c-Myb* insertions mimic the genomic abnormalities here described in human leukemia (Figure 3), and provides new validation of the power of these experimental approaches in animals to pinpoint human oncogenes. In all, genomic data in mice and humans involve a genomic region encompassing the *c-Myb* gene, with retroviral sites at both sides of the *c-Myb* gene in mice, which suggests that this gene is a major oncogenic target in this chromosomal region. Three other genes, namely *AHI1* (*Abelson Helper Integration*), *ALDH8A1* (*aldehyde dehydrogenase 8 family, member A1*), and *HBS1L* (*HBS1-like, S cerevisiae*), are located by this genomic region. The *AHI1* gene was disrupted in 2 of 6 t(6;7) T-ALL cases. *AHI1* is a WD40 and SH3-containing protein that has a complex pattern of isoforms.⁶³ It was found to be mutated in a congenital brain malformation syndrome known as Joubert syndrome,⁶⁴ and was investigated as a potential oncogene at retroviral insertions *Ahi1* in *Abl*⁺ mice, and in human leukemic cell lines and Ph⁺ samples.^{63,65} Although we found no deregulation of *AHI-1* gene expression, or of the other genes of the region, in the t(6;7) or in the *MYB*^{dup} T-ALL cases (Figure S3), the possibility remains that *AHI-1* gene could play a role as an oncogenic cofactor, in a "1 hit, 2 targets" genomic event. Comprehensive *c-Myb* and *Ahi1* deregulation in cellular and animal models should be useful to investigate this issue.

The molecular characterization of the 6 *TCRB-MYB* cases that was performed in the present work showed that this translocation juxtaposed the C β enhancer in the vicinity of *C-MYB* and suggested transcriptional deregulation. In *MYB*^{dup} cases, copy-number gain could be associated with reinforced *C-MYB* expression. Notably, the transcriptional consequences of the *C-MYB* genomic abnormalities appear unusual considering classical models of clearly ectopic oncogene expression, as seen in TLX1 and

TLX3 cases for instance. Considering that *C-MYB* is expressed at high levels in normal thymus, it is likely that it is a strong and sustained, rather than ectopic, expression that may be oncogenic. It has been previously reported in mice that *c-Myb* levels are tightly regulated throughout hematopoietic differentiation, including during T-cell differentiation, with brief up and down level changes that regulate transition between differentiation stages and a final down-regulation.^{4,6-11} Abrogation of subtle levels of *C-MYB* into a stable massive expression due to *TCRB* regulatory sequences is likely to disturb progression through T-cell differentiation. This view is strongly supported by our finding that *C-MYB* expression was skewed massively toward single allele expression in the *TCRB-MYB* translocated cases, which suggests a cellular attempt to down-regulate *C-MYB* in these cases (Figure 4C). In the *MYB*^{dup} cases, an increase of *C-MYB* expression related to copy-number gain may also reinforce oncogenic pathways. For both types of abnormality, in vitro and in vivo models should be useful to investigate the role of *c-Myb* in T-cell oncogenesis.

A number of published reports have shown that *C-MYB* knockdown by antisense inhibit the in vitro growth of leukemic cells, including T-cell lines.⁶⁶⁻⁶⁸ Although these experiments have to be confirmed in primary T-ALL samples using new silencing strategies, they suggest that *C-MYB* expression is necessary for the growth of leukemic T cells. Interestingly, *C-MYB* expression was recently involved in an oncogenic *MLL* \rightarrow *HOXA* \rightarrow *C-MYB* transcriptional pathway, which suggests that indirect biallelic stimulation of *C-MYB* could also be involved in oncogenic pathways.⁶⁹

In conclusion, we report for the first time a recurrent somatic involvement of the *C-MYB* locus in human leukemia due to 2 distinct genomic events, *TCRB*-associated translocation and cryptic duplication of a short genome region, and leading to deregulated *C-MYB* expression. Moreover, a new T-ALL clinicobiologic entity has been here identified that associates a very young age, the t(6;7) *TCRB-MYB* translocation, no association with other common oncogenic transcripts, and a proliferation/mitosis expression signature. These results suggest that *C-MYB* could play an oncogenic role in T-ALL, and point to this gene as a potential target for therapeutic intervention in human malignancies.

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Authorship

Contribution: E.C. performed experiments, analyzed the data, and wrote the paper; W.C. performed the array-CGH experiments; A.K. and A.C. performed experiments; J.-M.C. contributed to the molecular annotations of T-ALL cases; W.A.D., A.W.L., B.M., and B.N. characterized additional t(6;7) T-ALL cases; P.W. performed molecular combing experiments; O.D. and A.A. developed the 4K array; H.D., T.L., and A.B. managed the patients and conducted the

French national trials GRAALL and FRALLE; A.M.G. contributed to data analysis; F.S. led the T-ALL project at Saint-Louis Hospital and performed the gene expression profiling analysis; J.S. designed the study, analyzed the data, and wrote the paper.

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