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MicroRNA-193b-3p acts as a tumor suppressor by targeting the *MYB* oncogene in T-cell acute lymphoblastic leukemia

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Abstract

The *MYB* oncogene is a leucine zipper transcription factor essential for normal and malignant hematopoiesis. In T-cell acute lymphoblastic leukemia (T-ALL), elevated *MYB* levels can arise directly through T-cell receptor-mediated *MYB* translocations, genomic *MYB* duplications or enhanced TAL1 complex binding at the *MYB* locus or indirectly through the TAL1/miR-223/FBXW7 regulatory axis. In this study, we used an unbiased *MYB* 3'untranslated region–microRNA (miRNA) library screen and identified 33 putative *MYB*-targeting miRNAs. Subsequently, transcriptome data from two independent T-ALL cohorts and different subsets of normal T-cells were used to select miRNAs with relevance in the context of normal and malignant T-cell transformation. Hereby, miR-193b-3p was identified as a novel *bona fide* tumor-suppressor miRNA that targets *MYB* during malignant T-cell transformation thereby offering an entry point for efficient *MYB* targeting-oriented therapies for human T-ALL.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy affecting children, adolescents and adults. In T-ALL development, immature thymocytes undergo oncogenic transformation caused by cooperative genetic lesions that affect proliferation, survival and differentiation.¹ Constitutive activation of NOTCH1 signaling provoked by activating *NOTCH1* mutations² or inactivating *FBXW7* aberrations³ is the most prominent oncogenic pathway in T-ALL pathogenesis. In addition, T-ALL patients often display recurrent translocations, duplications and interstitial deletions resulting in aberrant expression of several transcription factor oncogenes, including *MYB*. Illegitimate expression of these transcriptional regulators drives specific gene expression programs that catalog T-ALL patients in different molecular genetic subgroups.⁴⁻⁶

The *MYB* proto-oncogene is a leucine zipper transcription factor that is aberrantly activated in a subset of T-ALL patients through a recurrent T-cell-receptor (TCR)-mediated translocation t(6;7)(q23; q34) leading to *TCRβ*-mediated *MYB* overexpression in 7% of cases or somatic duplications in 10–15% of cases.^{7,8} Furthermore, additional mechanisms of *MYB* activation include direct *MYB* upregulation by the oncogenic TAL1/GATA3/RUNX1 complex⁹ or indirect through the TAL1/miR-223/FBXW7 regulatory axis.^{10,11} In line with this notion, T-ALL patients that show aberrant expression of the *TAL1* oncogene (60%)⁴ are characterized by high levels of *MYB* expression.⁹ During normal hematopoiesis, *MYB* has a major role in directing proliferation, lineage commitment and differentiation.^{12,13} Interestingly, T-cell-specific ectopic expression of v-Myb, a truncated and rearranged form of *MYB*, in transgenic mice gives rise to high-grade T-cell lymphomas, underscoring the driver role of *MYB* in normal and malignant T-cell development.¹⁴ Finally, *MYB* is an essential downstream target of homeobox (*HOX*) genes, which serve as critical regulators of normal and malignant hematopoiesis.¹⁵

In the past decades, microRNAs (miRNAs) were identified as a group of small non-coding RNA molecules that negatively regulate gene expression. Sequence complementarity of the seed of the miRNA and the 3' untranslated region (UTR) of the target mRNA initiates the formation of the RNA-induced silencing complex leading to mRNA degradation or inhibition of translation.¹⁶ Deregulated miRNA expression is a common feature of human cancers and can be caused by specific chromosomal abnormalities, including somatic deletions (for example, miR-15a/miR-16-1 deletions in chronic lymphoblastic leukemia¹⁷) or translocations (for example, *TCRβ*-miR-17-92 translocation in T-ALL¹⁸). Importantly, some of these small RNA molecules have emerged as important players in the control of driver genes implicated in T-ALL^{10,11,18-20} and might serve as excellent candidates for miRNA-based therapies in the treatment of T-ALL.²¹

Thus far, the miRNA regulatory network controlling T-ALL oncogenes has not been extensively studied. Here we selected the *MYB* proto-oncogene as the first candidate for an in-depth exploration of this miRNA network through an unbiased, high-throughput 3'UTR-miRNA library screen and subsequent analyses of miRNA-mRNA correlation data during normal T-cell differentiation and in primary T-ALL patient samples. Using this unique

integrative approach and subsequent *in vitro* and *in vivo* validation, we identified miR-193b-3p as a *bona fide* T-ALL tumor-suppressor miRNA, targeting MYB.

Materials and Methods

MiRNA profiling of T-ALL patient samples and normal T-cell subsets

MiRNA profiling (430 miRNAs) was performed on a T-ALL patient cohort of 50 primary patient samples (15 *TAL/LMO*, 15 *TLX1/TLX3*, 12 *HOXA* and 8 cases could not be categorized) and on 5 normal thymocyte subsets (CD34+, CD4+CD8+CD3−, CD4+CD8+CD3+, CD4+, CD8+). Human thymus tissue was derived from pediatric patients undergoing cardiac surgery and was obtained and used according to the guidelines of the Medical Ethical Commission of the Ghent University Hospital (Belgium). Thymus tissue was immediately processed to generate a single-cell suspension that was subsequently used to isolate distinct developmental stages. CD34+ thymocytes were purified using magnetic-activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD4+CD8+CD3−, CD4+ CD8+CD3+, CD4+CD8−CD3+ and CD4−CD8+CD3+ subsets were purified using FACS (fluorescence-activated cell sorter)-mediated cells' sorting following labeling of total thymocytes with CD4, CD8 and CD3 antibodies. Purity for all subsets was at least 98%. MACS-enriched CD34+ thymocytes are the immediate T-cell progenitors as illustrated by the co-expression of CD7 by > 98% of the cells.²² This miRNA data set was reported before, and for a detailed description of the miRNA profiling protocol, we referred to Mavrakis *et al.*¹⁹ This study (2008/531) was approved by the Medical Ethical Commission of Ghent University Hospital (Belgium, registration B67020084745).¹⁹

In addition, we performed miRNA profiling (755 miRNAs) of a second T-ALL patient cohort of 64 primary patient samples (15 immature, 25 *TAL/LMO*, 17 *TLX1/TLX3* and 7 *HOXA*). These patient samples were previously investigated in a study of Clappier *et al.*²³ The samples were collected with informed consent according to the declaration of Helsinki from Saint-Louis Hospital (Paris, France), and the study was approved by the Institut Universitaire d'Hématologie Institutional Review Board.

Transcriptome profiling of T-ALL patient samples

On the cohort of 64 primary T-ALL patient samples, gene expression analysis was performed. Briefly, RNA quality was evaluated on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Next, transcriptome profiling was performed on GeneChip Human Genome U133 2.0 Plus arrays (Affymetrix, Santa Clara, CA, USA) according to the standardized procedures at the IGBMC (www.igbmc.fr, Strasbourg, France). Normalization of the gene expression profiles was done using the variance stabilization and calibration (VSN) R package.²⁴ Correlation analysis was performed using the Spearman's rank correlation analysis in R statistical programming environment (version 3.0.1). Microarray data files are available from ArrayExpress under accession no. E-MTAB-604.²³

Transcriptome profiling of mouse T-ALL samples

RNA was isolated from mouse leukemia samples with the miRNeasy mini kit with DNA digestion on-column (Qiagen, Venlo, The Netherlands) and concentration measured using the NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). RNA quality was assessed using the Experion Automated Electrophoresis System according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, gene expression analysis of the RNA samples of the mouse leukemias was done using the SurePrint G3 Mouse 8×60K Microarrays according to the manufacturer's instructions (Agilent Technologies). The gene expression data were normalized by quantile normalization using R and background correction using the dark corner probe group of the Agilent slides.

MYB 3'UTR-miRNA library screen

Twenty-four hours after seeding, HEK-293 T cells (10 000 cells/well in 96-well plates) were transfected with a pRL-TK control vector (20 ng) containing *Renilla* luciferase (Promega, Madison, WI, USA) and a reporter vector (100 ng) containing wild-type 3'UTR of *MYB* downstream of firefly luciferase (SwitchGear Genomics, Menlo Park, CA, USA) together with a library of 470 miRNA mimics (2.5 pmol) (Ambion's Pre-miR miRNA Precursor Library (Foster City, CA, USA)—Human V3, design based on miRBase release 9.2 with exclusion of hsa-miR-122a, Life Technologies, Carlsbad, CA, USA) by use of Dharmafect (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, luciferase activities were measured using the Dual-Luciferase Reporter assay (Promega) on a Fluostar Optima (BMG Labtech, Ortenberg, Germany). For each miRNA a robust *Z*-score was calculated, which represents the ratio between firefly/*Renilla* luciferase activities of a specific miRNA subtracted with the median firefly/*Renilla* value of the 96-well plate. An interaction score was then calculated, which represents the robust *Z*-score of a miRNA subtracted with the median robust *Z*-score of this miRNA over all the screens that were performed. This screen is part of a large screening effort of 17 known cancer genes (Van Peer *et al.*, manuscript in preparation). Notably, the lower the interaction score, the more likely a miRNA will interact with the 3'UTR of interest. Two criteria were used to determine the cutoff with the highest accuracy using a receiver operator characteristic curve analysis (cutoff < -1.93 , specificity = 99%, sensitivity = 51%) to separate interactions from non-interactions. To perform this receiver operator characteristic curve analysis, interaction scores were taken into account of a set of selected miRNA interactions that were validated in literature as well as interaction scores of miRNAs derived from an empty 3'UTR vector miRNA library screen. Our *MYB* 3'UTR-miRNA library screen was performed in duplicate to determine the reproducibility of our screening method.

Single 3'UTR-miRNA reporter gene assays and rescue experiments

To produce a *MYB* 3'UTR mutant vector in which the two 6-mer target sites for miR-193b-3p were mutated, the Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) was used according to the manufacturer's guidelines.

Primer sequences for mutagenesis were: 6-mer1 F, 5'-GGTAATGAATTGTATCACTTTGTTAATATCTTAATGCAG-3'; 6-mer1 R, 5'-CTGCATTAAGATATTAACAAAGTGATACAATTCATTACC-3'; 6-mer2 F, 5'-

CTGTGGTTGATAGTCACTTCACTGCCTTAAG-3'; and 6-mer2 R, 5'-CTTAAGGCAGTGAAGTGACTATCAACCACAG-3'. The wild-type or mutant 3'UTR vector of *MYB* (SwitchGear Genomics, Carlsbad, CA, USA) and a pRL-TK control vector (Promega) were co-transfected in HEK-293 T cells with either a miRNA mimic for miR-193b-3p (PM12383, Ambion) or a scrambled, non-targeting control miRNA mimic (AM17111, Ambion), followed by luciferase measurements as described above.

Electroporation of miRNA mimics in T-ALL cell lines

First, 64×10^6 cells from T-ALL cell lines were suspended in 2 ml RPMI-1640 medium together with the miRNA mimic or non-targeting control (final concentration: 400 nM). Next, 500 μ l of each cell suspension was transferred to electroporation tubes followed by electroporation (0.300 kV and 1 mF) with the Gene Pulser II (Bio-Rad Laboratories). The cells from one electroporation were divided over four wells of a 12-well plate and diluted to a final cell suspension of 1.6×10^6 /ml. Finally, cells were collected for RNA and protein isolation after 24, 48 and 72 h.

NOTCH1-induced T-ALL mouse model

The institutional and national guide for the care and use of laboratory animals was followed. At embryonic day 13–14, we isolated fetal liver cells with hematopoietic progenitor cells (HPCs) from pregnant mice. Next, we transduced the HPCs with ICN1 (active NOTCH1, co-expression of mCherry) and a vector encoding antagomiR (sequence=5'-AGCGGGACTTTGAGGGCCAGTT-3') against miR-193b-3p or an empty vector control (co-expressing green fluorescent protein). After irradiation of mouse recipients, the transduced HPCs were injected using tail vein injection followed by monitoring of leukemia onset. Various tissues were collected at time of leukemia formation and fixed in formaldehyde for immunohistochemistry. Spleen and thymus cells were mashed to single-cell suspensions and frozen in 10% dimethyl sulfoxide. RNA and protein lysates were generated from the mouse samples and used for quantitative PCR and western blotting analysis. Leukemia onset data were analyzed using the Kaplan–Meier method and the log-rank test for statistical significance.

Statistical analyses

We identified all differential expressed miRNAs between T-ALL patient samples and normal thymocyte subsets by use of the standard *t*-test combined with Benjamini–Hochberg multiple testing correction ($P < 0.05$). We applied the same *t*-test to identify all miRNAs that are differentially expressed between the TAL subgroup and other T-ALL subgroups ($P < 0.05$) and to identify all differentially expressed genes between the antagomiR-193b-3p and NOTCH1-only control mouse samples ($P < 0.05$). Linear regression analysis was performed using the GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

MiRNA-193b-3p nomenclature

Official nomenclature and sequence annotation for miR-193b-3p by the miRBase database (<http://www.mirbase.org/>) changed a few times during the course of this study. During the

time of the 3'UTR high-throughput screening effort and the miRNA profiling of cohort 1, miR-193b was annotated in miRbase with the following mature sequence: 5'-AACUGGCCCUCAAAGUCCCGCUUU-3'. Before profiling of cohort 2, the sequence of this miRNA changed to 5'-AACUGGCCCUCAAAGUCCCGCU-3', but the nomenclature remained unchanged. As the sequence change occurred at the 3' end of the miRNA's sequence, the seed sequence remained unchanged, which likely does not affect the function of miR-193b. At the start of our validation studies, a new miRbase release was published and the nomenclature for miR-193b changed to miR-193b-3p. In this manuscript, we therefore refer to this miRNA as miR-193b-3p according to the latest miRBase release.

Results

Identification of miRNAs targeting MYB using an unbiased *MYB* 3'UTR-miRNA library screen

Given the oncogenic role of MYB in T-ALL and the relevance of perturbed miRNA signaling in T-cell transformation, we conducted a miRNAome-wide screen to identify novel miRNAs that target the *MYB* proto-oncogene. More specifically, we performed a high-throughput dual luciferase-based *MYB* 3'UTR-miRNA library screen to explore possible interactions between the 3'UTR of *MYB* in HEK-293 T cells and 470 miRNA mimics (includes all annotated miRNAs based on release 9.2 of the miRBase reference database). After co-transfection of the miRNA mimics together with the *MYB* 3'UTR and control vector, we measured the luciferase activities and calculated an miRNA interaction score for each miRNA mimic (see Materials and Methods) (Figure 1a). We conducted two independent *MYB* 3'UTR library screens to evaluate the reproducibility of our screening method and ensure the reliable identification of positive miRNA hits (Figure 1b, Pearson's correlation coefficient $r = 0.85$). Next, we determined positive hits based upon interaction scores < -1.93 , a cutoff that was established based on 3'UTR library screens for 17 known cancer genes (see Materials and methods; Van Peer *et al.*, manuscript in preparation). As such, this *MYB* 3'UTR screening analysis yielded a total of 33 miRNAs that could potentially target *MYB* (Figure 1c and Supplementary Table S1). Eleven out of these 33 hits were not predicted by various target prediction programs (TargetScan, PicTar, miRanda, MirTarget2, DianamicroT) emphasizing the strength of our screening method as an unbiased tool for the identification of novel non-predicted miRNA-target gene interactions. The fact that the number of experimentally identified hits through the 3'UTR library screen (33 miRNAs) is significantly lower as those generated by online target prediction databases (for example, 74 predicted miRNAs by TargetScan or 159 predicted miRNAs using DianamicroT) underscores the significant level of false positives generated by computational prediction programs. In addition, our analysis identified five out of the eight (63%) known miRNA-MYB interactions described in literature, including miR-150,²⁵ miR-200b,²⁶ miR-424,²⁷ miR-429²⁸ and miR-126.²⁹ The three remaining miRNAs (miR-155,²⁷ miR-15a³⁰ and miR-200c³¹) could represent false negatives in our screen.

MiRNA-193b-3p potentially targets MYB in normal and malignant T-cell development and shows low expression in the *TAL*-rearranged T-ALLs

Following the unbiased identification of 33 positive miRNA hits in our *MYB* 3'UTR–miRNA library screen, we explored the miRNA expression profiles of normal T-cell subsets representing major stages of thymocyte maturation³² and two genetically well-characterized T-ALL patient cohorts to identify relevant miRNAs in the context of normal and malignant T-cell development. First, we evaluated the expression of 430 miRNAs in a primary T-ALL cohort consisting of 50 T-ALL patient cases as well as 5 normal T-cell subsets (CD34+, CD4+CD8+CD3 – (DP3 –), CD4+CD8+CD3+ (DP3+), CD4+ (SP4+) and CD8+ (SP8+)).¹⁹ Next, we explored both the miRNAome (755 miRNAs) (Supplementary Table S2) and transcriptome²³ of a second independent T-ALL patient cohort of 64 primary T-ALL cases to identify putative miRNA–mRNA interactions based on integrative miRNA–mRNA correlation analysis.

By performing differential miRNA expression analysis, we first identified 43 putative tumor-suppressor miRNAs with significant reduced levels of expression in T-ALL patients as compared with normal T-cell subsets ($P < 0.05$; fold change < -1.50 Supplementary Table S3). Cross-comparison of the 43 putative tumor-suppressor miRNAs with the 33 hits identified in the *MYB* 3' UTR screen retained 5 miRNAs shared in both analyses (Figure 2a). Interestingly, two miRNAs out of these five common miRNAs, namely miR-195 and miR-193b-3p, showed strong opposite expression patterns with *MYB* expression during normal T-cell differentiation (Pearson's correlation coefficient $r = -0.75$ for miR-193b-3p and $r = -0.66$ for miR-195, Figures 2b–d, Supplementary Figures S1a–d). However, only miR-193b-3p was negatively correlated with *MYB* expression in primary T-ALLs (Spearman's rank correlation with correlation factor < -0.5 ; Supplementary Table S4), suggesting that *MYB* regulation by miR-193b-3p might be functional in both normal and malignant T-cell context (Figures 2e–g). Of importance, miR-193b-3p clearly demonstrates a reduction in expression levels in the vast majority of the T-ALL patient samples (cohort 1) compared with the normal T-cell subsets (Supplementary Figure S1e). Finally, we evaluated the miR-193b-3p expression in molecular genetic subtypes of human T-ALL. Significantly lower expression levels of miR-193b-3p were identified in *TAL*-rearranged T-ALLs (Supplementary Tables S5 and S6, Figures 2e–f), which corresponds to the previously reported higher expression levels of *MYB* in *TAL*-rearranged leukemias.⁹

In vitro validation of the interaction between *MYB* and miR-193b-3p in T-ALL cells

Following the selection of miR-193b-3p as putatively functionally relevant miRNA in the context of normal T-cell maturation and T-ALL oncogenesis, we decided to validate the direct interaction between miR-193b-3p and the 3'UTR of *MYB* using single luciferase-based reporter assays to confirm the 3'UTR–miRNA library screen results. This assay showed a substantial downregulatory effect of miR-193b-3p on luciferase activity as compared with a non-targeting miRNA control (Figure 3a). We identified two potential 6-mer-binding sites for miR-193b-3p (Figure 3b), and mutation of both target sites abolished the downregulatory effect of miR-193b-3p on luciferase activity, demonstrating the seed-specific interaction between miR-193b-3p and the 3'UTR of *MYB* (Figure 3c).

Next, we investigated whether miR-193b-3p regulates MYB expression levels in the T-ALL cell line RPMI-8402, which contains a genomic *MYB* duplication and in the T-ALL cell line LOUCY, which harbors high MYB protein expression levels as compared with other T-ALL cell lines (Supplementary Figure S2a).⁸ In both T-ALL cell lines, overexpression of miR-193b-3p induced downregulation of MYB expression levels compared with a non-targeting miRNA control at different time points (Figures 3d and e, Supplementary Figures S2b and d). Previous studies identified MCL1 as a miR-193b-3p target in other cancer types.^{33,34} Importantly, MCL1 was also shown to contribute to T-ALL formation.¹⁹ Therefore we tested the effect of overexpression of miR-193b-3p on MCL1 levels and observed that miR-193b-3p induced downregulation of MCL1 expression levels in both T-ALL cell lines (Figures 3d and e, Supplementary Figures S2c and e). Hence, the putative tumor-suppressor miR-193b-3p interacts with both *MYB* and *MCL1* by site-specific complementarity and affects the MYB and MCL1 protein levels in T-ALL, thus further broadening the miR-193b-3p network in T-ALL.

***In vivo* downregulation of miR-193b-3p accelerates T-ALL onset**

Next, we evaluated the possible oncogenic effect of loss of the putative T-ALL tumor-suppressor miR-193b-3p in a NOTCH1-induced T-ALL mouse model.³⁵ In brief, fetal liver cells were retrovirally transduced with an ICN1 (NOTCH1) construct (co-expressing mCherry) as well as a construct encoding antagomiR against miR-193b-3p (co-expressing green fluorescent protein) or an empty vector construct. Next, the transduced

HPCs were tail vein injected into lethally irradiated recipient mice, and T-ALL onset was monitored (Figure 4a). We observed accelerated leukemia onset in mice injected with HPCs containing the antagomiR against miR-193b-3p ($n = 15$, mean latency = 43.5 days, log-rank test $P < 0.05$) compared with NOTCH1-only controls ($n = 9$, mean latency = 54.8 days) (Figure 4b). Notably, the T-cell lymphoblasts derived from the murine leukemias displayed a clear green fluorescent protein enrichment at the time of disease onset (data not shown). Furthermore, an increase in Myb protein levels was observed in the leukemias with reduced miR-193b-3p compared with controls (Figure 4c). Moreover, gene expression analysis between four antagomiR-193b-3p samples and four NOTCH1-only control samples identified a set of 465 genes that are upregulated or downregulated after miR-193b-3p knockdown (fold change > 2 and $P < 0.05$) (Supplementary Table S7). Immunophenotypic analysis of the murine leukemias using CD3 immunohistochemistry (Figure 4d) and flow cytometric analysis of CD3, CD4, CD8 and TCR gamma delta (TCR $\gamma\delta$) (Figure 4e, Supplementary Table S8) shows that the mouse leukemias represent typical T-cell leukemias characterized by the presence of CD3, CD4 and CD8 on the cell surface of the T-cell blasts. Finally, immunohistochemical analysis of the murine leukemias showed an aggressive leukemia phenotype with infiltration of highly proliferative T-cell lymphoblasts in the liver, kidney, spleen and lungs in both miR-193b-3p loss and NOTCH1-only control samples (Supplementary Figure S3). No difference in haematoxylin and eosin or Ki67 levels between the miR-193b-3p loss and NOTCH1-only control samples was observed (Supplementary Figure S3).

Discussion

The *MYB* oncogene was discovered > 30 years ago as a viral-transforming gene in hematopoietic cells.³⁶ More recently, two independent research groups described *MYB* duplications (10–15%) and translocations (7%) in primary T-ALL patient samples,^{7,8} linking oncogenic *MYB* activity to T-ALL pathogenesis. Furthermore, T-ALL patient samples marked with aberrant *TAL1* expression display high *MYB* expression levels directly provoked by *TAL1* complex binding.⁹ Therapeutic approaches targeting *MYB* include direct strategies like the antisense *MYB* oligodeoxynucleotides currently tested in clinical trials³⁷ as well as indirect strategies like histone deacetylase inhibitors.^{38,39} Therapeutic miRNA delivery might represent an alternative approach for targeting *MYB*.⁴⁰

Hence, we designed and performed a miRNAome-wide *MYB* 3'UTR–miRNA library screen in HEK-293 T cells in which 33 miRNAs (positive hits) were identified that putatively target *MYB*. In our screen, one-third of the positive miRNA hits were not predicted by five different miRNA target prediction programs, underlining the power of this unbiased strategy in the identification of non-predicted miRNA–target gene interactions. Alternatively, comparison of the 33 hits from the screen to the extensive list of miRNAs predicted by computational prediction programs suggests that online tools predict many false positives. Literature data on other genes that were tested in our library screens also show that many *bona fide* targets remain undetected using prediction tools and likewise only a small fraction of the predicted miRNAs were identified as hits in these library screens.^{41,42} To our knowledge, this is the first unbiased 3'UTR library screen that was conducted for the identification of *MYB*-targeting miRNAs. Hence, we provide a catalog of *MYB*-regulating miRNAs to guide prioritization for further validation and functional characterization in different cancer types.

Integration of these *MYB* 3'UTR–miRNA library screen results with miRNA and mRNA expression profiles of primary T-ALL patient samples as well as normal thymocytes allowed us to identify miR-193b-3p as the top candidate putative tumor-suppressive miRNA targeting *MYB* in normal and malignant T-cell development. In our sorted normal T-cell subsets, we observed and validated that *MYB* expression levels drastically decrease during T-cell differentiation.^{43,44} *MYB* expression levels are tightly regulated in this developmental process, whereby *MYB* essentially contributes to three specific T-cell developmental stages.^{44,45} Next to *MYB*, miRNAs are also essential contributors to correct T-cell development as deletion of *Dicer*, an essential component in the miRNA biogenesis pathway, in mice impedes the survival of $\alpha\beta$ T-cells in early T-cell stages.⁴⁶ In our data, miR-193b-3p was shown to be strongly anti-correlated with *MYB* expression during normal T-cell differentiation, and miR-193b-3p controlled regulation of *MYB* levels through a seed-specific interaction. Taken together, we can propose miR-193b-3p as an important regulator of *MYB* expression during normal T-cell development.

Based on T-ALL patient data and miR-193b-3p perturbation studies in both T-ALL cell lines as well as the NOTCH1 T-ALL mouse model, we could demonstrate that miR-193b-3p acts as *bona fide* tumor-suppressor gene targeting *MYB* in T-ALL. The oncogene *MCL1* has previously been described as a miR-193b-3p target in liver cancer and melanoma,^{33,34} which

we could confirm in our *in vitro* model system in T-ALL. Of interest, TAL1 knockdown in T-ALL cell lines¹⁰ or enforced expression of miR-92a and miR-223 in NOTCH1-driven mouse T-ALLs also influences MCL1 protein levels in T-ALL. Mechanistically, MCL1 is a downstream target of the TAL1/miR-223/FBXW7¹⁰ or miR-92a/FBXW7 regulatory axes.^{19,47} Hence, in our study we identified the novel tumor-suppressor miR-193b-3p that can directly regulate MYB and MCL1 levels in T-ALL. Importantly, this indicates that the *in vivo* tumor-suppressor activity of miR-193b-3p will be mediated by the cooperative and simultaneous repression of multiple oncogenes, a notion that has been postulated for multiple other miRNAs.¹⁹

In line with previous studies,⁹ MYB expression levels were elevated in TAL-positive T-ALLs in both T-ALL patient cohorts that we analyzed. Furthermore, we found miR-193b-3p to be significantly lower expressed in TAL-rearranged T-ALLs, which corresponds with the unraveled miR-193b-3p–MYB interaction shown in our study. In the paper of Correia *et al.*,¹¹ miR-193b-3p is downregulated after TAL1 overexpression in the P12-ICHI T-ALL cell line, indicating that the TAL1 complex could indirectly repress miR-193b-3p expression.

Finally, using the mouse model, we could show for the first time that elevated MYB levels, caused by introduction of an antagomiR against miR-193b-3p, cooperate *in vivo* with ectopic expression of NOTCH1. Accordingly, a strong synergistic inhibitory effect on proliferation and viability was already demonstrated *in vitro* after combined knockdown of MYB and NOTCH1 in GSI-sensitive T-ALL cell lines.⁸ Hence, we conclude that the novel tumor-suppressor miR-193b-3p can cooperate with NOTCH1 in T-ALL pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. *J Clin Invest.* 2012; 122:3398–3406. [PubMed: 23023710]

2. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004; 306:269–271. [PubMed: 15472075]
3. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, et al. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med*. 2007; 204:1813–1824. [PubMed: 17646409]
4. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002; 1:75–87. [PubMed: 12086890]
5. Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood*. 2005; 106:274–286. [PubMed: 15774621]
6. Homminga I, Pieters R, Langerak AW, de Rooi JJ, Stubbs A, Verstegen M, et al. Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2011; 19:484–497. [PubMed: 21481790]
7. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, et al. 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. *Blood*. 2007; 110:1251–1261. [PubMed: 17452517]
8. Lahortiga I, De Keersmaecker K, Van Vlierberghe P, Graux C, Cauwelier B, Lambert F, et al. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. *Nat Genet*. 2007; 39:593–595. [PubMed: 17435759]
9. Sanda T, Lawton LN, Barrasa MI, Fan ZP, Kohlhammer H, Gutierrez A, et al. Core transcriptional regulatory circuit controlled by the TAL1 complex in human T cell acute lymphoblastic leukemia. *Cancer Cell*. 2012; 22:209–221. [PubMed: 22897851]
10. Mansour MR, Sanda T, Lawton LN, Li X, Kreslavsky T, Novina CD, et al. The TAL1 complex targets the FBXW7 tumor suppressor by activating miR-223 in human T cell acute lymphoblastic leukemia. *J Exp Med*. 2013; 210:1545–1557. [PubMed: 23857984]
11. Correia NC, Durinck K, Leite AP, Ongenaert M, Rondou P, Speleman F, et al. Novel TAL1 targets beyond protein-coding genes: identification of TAL1-regulated microRNAs in T-cell acute lymphoblastic leukemia. *Leukemia*. 2013; 27:1603–1606. [PubMed: 23448994]
12. Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *EMBO J*. 2003; 22:4478–4488. [PubMed: 12941699]
13. Sakamoto H, Dai G, Tsujino K, Hashimoto K, Huang X, Fujimoto T, et al. Proper levels of c-Myb are discretely defined at distinct steps of hematopoietic cell development. *Blood*. 2006; 108:896–903. [PubMed: 16597594]
14. Badiani PA, Kioussis D, Swirsky DM, Lampert IA, Weston K. T-cell lymphomas in v-Myb transgenic mice. *Oncogene*. 1996; 13:2205–2212. [PubMed: 8950988]
15. Hess JL, Bittner CB, Zeisig DT, Bach C, Fuchs U, Borkhardt A, et al. c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. *Blood*. 2006; 108:297–304. [PubMed: 16507773]
16. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009; 136:215–233. [PubMed: 19167326]
17. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002; 99:15524–15529. [PubMed: 12434020]
18. Mavrikis KJ, Wolfe AL, Oricchio E, Palomero T, de Keersmaecker K, McJunkin K, et al. Genome-wide RNA-mediated interference screen identifies miR-19 targets in Notch-induced T-cell acute lymphoblastic leukaemia. *Nat Cell Biol*. 2010; 12:372–379. [PubMed: 20190740]
19. Mavrikis KJ, Van Der Meulen J, Wolfe AL, Liu X, Mets E, Taghon T, et al. A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). *Nat Genet*. 2011; 43:673–678. [PubMed: 21642990]

20. Li X, Sanda T, Look AT, Novina CD, von Boehmer H. Repression of tumor suppressor miR-451 is essential for NOTCH1-induced oncogenesis in T-ALL. *J Exp Med*. 2011; 208:663–675. [PubMed: 21464222]
21. Jackson AL, Levin AA. Developing microRNA therapeutics: approaching the unique complexities. *Nucleic Acid Ther*. 2012; 22:213–225. [PubMed: 22913594]
22. Taghon T, Waegemans E, Van de Walle I. Notch signaling during human T cell development. *Curr Top Microbiol Immunol*. 2012; 360:75–97. [PubMed: 22692833]
23. Clappier E, Gerby B, Sigaux F, Delord M, Touzri F, Hernandez L, et al. Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. *J Exp Med*. 2011; 208:653–661. [PubMed: 21464223]
24. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002; 18(Suppl 1):S96–104. [PubMed: 12169536]
25. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007; 131:146–159. [PubMed: 17923094]
26. Cesi V, Casciati A, Sesti F, Tanno B, Calabretta B, Raschella G. TGFbeta-induced c-Myb affects the expression of EMT-associated genes and promotes invasion of ER+ breast cancer cells. *Cell Cycle*. 2011; 10:4149–4161. [PubMed: 22101269]
27. Imig J, Motsch N, Zhu JY, Barth S, Okoniewski M, Reineke T, et al. microRNA profiling in Epstein-Barr virus-associated B-cell lymphoma. *Nucleic Acids Res*. 2011; 39:1880–1893. [PubMed: 21062812]
28. Luo Z, Zhang L, Li Z, Li X, Li G, Yu H, et al. An in silico analysis of dynamic changes in microRNA expression profiles in stepwise development of nasopharyngeal carcinoma. *BMC Med Genomics*. 2012; 5:3. [PubMed: 22260379]
29. Grabher C, Payne EM, Johnston AB, Bolli N, Lechman E, Dick JE, et al. Zebrafish microRNA-126 determines hematopoietic cell fate through c-Myb. *Leukemia*. 2011; 25:506–514. [PubMed: 21079614]
30. Chung EY, Dews M, Cozma D, Yu D, Wentzel EA, Chang TC, et al. c-Myb onco-protein is an essential target of the dleu2 tumor suppressor microRNA cluster. *Cancer Biol Ther*. 2008; 7:1758–1764. [PubMed: 18708755]
31. Siebzehnrbu FA, Silver DJ, Tugertimur B, Deleyrolle LP, Siebzehnrbu D, Sarkisian MR, et al. The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. *EMBO Mol Med*. 2013; 5:1196–1212. [PubMed: 23818228]
32. Taghon, T.; Waegemans, E.; Van de Walle, I. Notch signaling during human T cell development. In: Radtke, F., editor. *Notch Regulation of the Immune System*. Springer; Berlin, Heidelberg, Germany: 2012. p. 75-97.
33. Braconi C, Valeri N, Gasparini P, Huang N, Taccioli C, Nuovo G, et al. Hepatitis C virus proteins modulate microRNA expression and chemosensitivity in malignant hepatocytes. *Clin Cancer Res*. 2010; 16:957–966. [PubMed: 20103677]
34. Chen J, Zhang X, Lentz C, Abi-Daoud M, Pare GC, Yang X, et al. miR-193b regulates Mcl-1 in melanoma. *Am J Pathol*. 2011; 179:2162–2168. [PubMed: 21893020]
35. Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med*. 1996; 183:2283–2291. [PubMed: 8642337]
36. Beug H, von Kirchbach A, Doderlein G, Conscience JF, Graf T. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell*. 1979; 18:375–390. [PubMed: 227607]
37. Gewirtz AM. Myb targeted therapeutics for the treatment of human malignancies. *Oncogene*. 1999; 18:3056–3062. [PubMed: 10378701]
38. Amaru Calzada A, Todoerti K, Donadoni L, Pelliccioli A, Tuana G, Gatta R, et al. The HDAC inhibitor Givinostat modulates the hematopoietic transcription factors NFE2 and C-MYB in JAK2(V617F) myeloproliferative neoplasm cells. *Exp Hematol*. 2012; 40:634–645. e610. [PubMed: 22579713]

39. Chambers AE, Banerjee S, Chaplin T, Dunne J, Debernardi S, Joel SP, et al. Histone acetylation-mediated regulation of genes in leukaemic cells. *Eur J Cancer*. 2003; 39:1165–1175. [PubMed: 12736119]
40. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. 2009; 137:1005–1017. [PubMed: 19524505]
41. Cheng J, Guo S, Chen S, Mastriano SJ, Liu C, D'Alessio AC, et al. An extensive network of TET2-targeting microRNAs regulates malignant hematopoiesis. *Cell Rep*. 2013; 5:471–481. [PubMed: 24120864]
42. Zhou P, Xu W, Peng X, Luo Z, Xing Q, Chen X, et al. Large-scale screens of miRNA-mRNA interactions unveiled that the 3'UTR of a gene is targeted by multiple miRNAs. *PLoS One*. 2013; 8:e68204. [PubMed: 23874542]
43. Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. *Nat Rev Cancer*. 2008; 8:523–534. [PubMed: 18574464]
44. Bender TP, Kremer CS, Kraus M, Buch T, Rajewsky K. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat Immunol*. 2004; 5:721–729. [PubMed: 15195090]
45. Allen RD 3rd, Bender TP, Siu G. c-Myb is essential for early T cell development. *Genes Dev*. 1999; 13:1073–1078. [PubMed: 10323859]
46. Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med*. 2005; 201:1367–1373. [PubMed: 15867090]
47. Inuzuka H, Shaik S, Onoyama I, Gao D, Tseng A, Maser RS, et al. SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature*. 2011; 471:104–109. [PubMed: 21368833]

Figure 1.

An unbiased high-throughput *MYB* 3'UTR-miRNA library screen identifies 33 candidate miRNAs targeting *MYB*. **(a)** HEK-293 T cells were transfected with a reporter vector containing the wild-type 3'UTR of *MYB* downstream of a firefly luciferase gene and a control vector containing a *Renilla* luciferase gene together with a library of 470 miRNA mimics. Forty-eight hours later, firefly and *Renilla* luciferase activities were measured. After normalization, inter-plate calibration and correction for systematic error, a miRNA interaction score was calculated for each miRNA in the screen. The lower the interaction score, the more likely a miRNA will interact with the 3'UTR of *MYB*. **(b)** The plot of the interaction scores for each miRNA from two independent *MYB* 3'UTR library screens demonstrates the high reproducibility of our assay (Pearson's correlation coefficient $r=0.85$). **(c)** Our screens reveal 33 candidate miRNAs (Supplementary Table S1) targeting the 3'UTR of *MYB* (interaction score < -1.93). The average interaction score of the two screens is plotted on the y axis for each miRNA that was included in the screen (x axis).

Figure 2.

Selection of miR-193b-3p as a top candidate *MYB* targeting miRNA in T-ALL. **(a)** Selection criteria to identify top candidate miRNAs targeting *MYB* in T-ALL: (1) positive hits from the *MYB* 3'UTR-miRNA library screen, (2) miRNAs that are significantly lower expressed in T-ALL versus normal T-cell subsets (*t*-test, $P < 0.05$), (3) miRNAs that display anti-correlation with *MYB* expression in primary human T-ALL patient samples (Spearman's rank correlation factor < -0.5). **(b)** *MYB* is highly expressed in the immature CD34+ stage and thereafter decreases during T-cell development. Bar graphs represent the mean ($\pm$ s.e.m.) of two technical replicates. **(c)** MicroRNA-193b-3p exhibits lower expression in the immature CD34+ stage followed by a strong upregulation in the more mature stages. Bar graphs represent the mean ($\pm$ s.e.m.) of two technical replicates. **(d)** Correlation graph of the miR-193b-3p and *MYB* expression levels in the normal T-cell subsets ($r = -0.75$ and linear regression curve $y = -0.701x - 2.251$). **(e)** *MYB* is highly expressed in the TAL-positive T-ALL subgroup. **(f)** miR-193b-3p is significantly lower expressed in the TAL rearranged patients samples compared with other T-ALL subgroups (*t*-test, $P < 0.05$). **(g)** Correlation graph of the miR-193b-3p and *MYB* expression levels in the T-ALL patient samples of cohort 2 ($r = -0.503$ and linear regression curve $y = -0.085x + 8.446$).

Figure 3.

MicroRNA-193b-3p directly targets the 3'UTR of *MYB* and downregulates *MYB* expression in T-ALL cell lines. (a) Transfection of miR-193b-3p reduces the luciferase activity of the wild-type *MYB* 3'UTR reporter construct in HEK-293 T cells. Bar graphs represent the mean ($\pm$ s.d.) of four independent experiments relative to the non-targeting miRNA transfection control (NTC). (b) The human *MYB* 3'UTR contains two 6-mer sites for miR-193b-3p. The sequences for the wild-type (WT) vector, the mutated (MUT) vector and miR-193b-3p are depicted. (c) Mutation of the two 6-mer target sites of miR-193b-3p in the 3'UTR *MYB* vector completely abolishes the downregulatory effect of miR-193b-3p on luciferase activity. Bar graphs represent the mean ($\pm$ s.d.) of three independent experiments relative to NTC. (d, e) MicroRNA-193b-3p overexpression downregulates the *MYB* and *MCL1* levels in LOUCY (d) and RPMI-8402 (e) T-ALL cell lines. The results shown from one experiment are representative for two independent biological replicates. *MYB* and *Tubulin* protein levels were quantified by the use of Image J software. Subsequently, *MYB* levels were normalized to *Tubulin* levels and compared with the relative *MYB* levels of the NTC samples.

Figure 4.

Downregulation of miR-193b-3p accelerates T-ALL onset *in vivo*. **(a)** Schematic representation of the NOTCH1-induced T-ALL mouse model. Fetal liver cells derived from pregnant mice were partially transduced with a vector encoding antagomiR against miR-193b-3p or an empty vector control (labeled in green fluorescent protein), together with the NOTCH1 (ICN1) construct (co-expressing mCherry). Next, the transduced cells were tail vein injected in irradiated mouse recipients followed by monitoring of leukemia formation. **(b)** Survival plot (Kaplan–Meier) depicting accelerated leukemia onset in the mouse recipients with reduced miR-193b-3p levels ($n = 15$, $P < 0.05$ log-rank test, red) compared with NOTCH1-only controls ($n = 9$, black). **(c)** Western blotting analysis of Myb protein levels in mouse tumor samples from both groups. Myb protein levels are quantified by Image J, normalized to tubulin levels and compared with NOTCH1-only vector controls (t -test: $P = 0.0002$). **(d)** CD3 Immunohistochemistry of lymphoblasts in the kidney, liver, lung and spleen tissue of a representative antagomiR-193b-3p and control mouse leukemia sample (black scale bar 100 μm). **(e)** Immunophenotypical flow cytometric analysis of CD4, CD8, CD3 and TCR $\gamma\delta$ T-cell markers in an antagomiR-193b-3p and an empty vector control mouse leukemia sample. A representative example of each subtype is depicted.