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## Regulation of PI3K signaling in T cell acute lymphoblastic leukemia: a novel PTEN/Ikaros/miR-26b mechanism reveals a critical targetable role for PIK3CD

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## Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy, and T-ALL patients are prone to early disease relapse and suffer from poor outcomes. The PTEN, PI3K/AKT, and Notch pathways are frequently altered in T-ALL. PTEN is a tumor suppressor that inactivates the PI3K pathway. We profiled miRNAs in *Pten*-deficient mouse T-ALL and identified miR-26b as a potentially dysregulated gene. We validated decreased expression levels of miR-26b in mouse and human T-ALL cells. In addition, expression of exogenous miR-26b reduced proliferation and promoted apoptosis of T-ALL cells *in vitro*, and hindered progression of T-ALL *in vivo*. Furthermore, miR-26b inhibited the PI3K/AKT pathway by directly targeting *PIK3CD*, the gene encoding PI3K $\delta$ , in human T-ALL cell lines. ShRNA for *PIK3CD* and CAL-101, a PIK3CD inhibitor, reduced the growth and increased apoptosis of T-ALL cells. Finally, we showed that PTEN induced miR-26b expression by regulating the differential expression of Ikaros isoforms that are transcriptional regulators of miR-26b. These results suggest that miR-26b functions as a tumor suppressor in the development of T-ALL. Further characterization of targets and regulators of miR-26b may be promising for the development of novel therapies.

## Keywords

miR-26b; T-ALL; PTEN; Ikaros; PI3K/AKT; PIK3CD

## Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a clinically aggressive hematologic malignancy that accounts for 15% of pediatric and 25% of adult ALL cases.<sup>1</sup> Limited targeting therapies are available for patients with T-ALL because of an insufficient understanding of T-ALL genetics and biology. Despite current therapies, including chemotherapy and bone marrow transplant, 25–50% of T-ALL patients relapse, resulting in a poor outcome with a 5-year overall survival rate of approximately 45%.<sup>2–4</sup>

Class I phosphoinositide-3 kinases (PI3Ks) are a group of signaling transduction enzymes involved in the production of intracellular second messenger lipid signals. The class I PI3Ks are sub-divided into two groups based on their structures: class IA PI3Ks are heterodimers consisting of one regulatory subunit and one catalytic subunit (p110 $\alpha$ , p110 $\beta$ , or p110 $\delta$ ). Unlike p110 $\alpha$  and p110 $\beta$ , which are ubiquitously expressed, p110 $\delta$  is highly enriched in leukocytes. PI3K p110 $\delta$  (PI3K $\delta$ ) is encoded by *PIK3CD*. The PI3K pathway is activated in 92% of T-ALL cell lines and 81% of primary T-ALL samples, respectively.<sup>5</sup> PTEN, a tumor

suppressor, is the most important negative regulator of the PI3K signaling pathway. In addition to its canonical, PI3K inhibition-dependent functions, PTEN also can function as a tumor suppressor in a PI3K-independent manner. PTEN loss of function due to gene mutations or deletions occurs in 22% of T-ALL patients, leading to hyperactivation of PI3K and its downstream AKT signaling pathway.<sup>6</sup>

Ikaros is a zinc finger transcriptional factor encoded by *IKZF1* and is a key regulator and tumor suppressor in hematopoiesis.<sup>7</sup> Loss of Ikaros function is common in human T-ALL.<sup>7</sup> Ikaros isoforms share a common structure of two functional domains composed of zinc-fingers. The first four zinc-fingers represent a DNA-binding domain (DBD) whereas the last two zinc-fingers are components of a dimerization domain; the latter allows competitive binding between isoforms.<sup>8</sup> These domains are encoded by seven different exons, and differential splicing generates different isoforms. Ikaros isoforms that display at least three zinc-fingers in the DBD are considered dominant positive (DP, IK1-3), whereas Ikaros isoforms with less than three zinc-fingers in the DBD are considered dominant negative (DN, IK4-9). DN isoforms are not only defective typically due to decreased/no DNA binding capacity but also may interfere with the activity of functional isoforms. Mice with the heterozygous loss of Ikaros rapidly develop T-cell leukemia.<sup>9, 10</sup>

microRNAs (miRs) are short noncoding RNAs of 20–22 nucleotides that function to regulate gene expression at the posttranscriptional level. miRs play fundamental roles in the regulation of cellular proliferation, differentiation, and apoptosis. miRs are dysregulated in many types of cancer, including T-ALL. miRs can function as oncogenes, favoring the initiation and progression of cancers, or as tumor suppressors, preventing tumorigenesis.<sup>11–29</sup> The biological functions of miRs in T-ALL are largely unknown. To better understand T-ALL pathogenesis and identify new therapeutic targets in T-ALL, we previously developed a *Pten* knockout T-ALL mouse model.<sup>30</sup> In this study, we profiled the miRs in the *Pten* deficient mouse T-ALL. miR-26b was shown to be aberrantly expressed. Recent studies have implicated aberrant expression of miR-26b in several types of non-hematopoietic cancer.<sup>31–33</sup> However, the expression level of miR-26b and its role in T-ALL is unknown. In this study, we investigated the expression level of miR-26b in T-ALL, showed its aberrant expression, and studied the effects of its altered expression on human T-ALL cells.

## Materials and Methods

### Patient samples

We obtained 27 bone marrow samples from newly diagnosed T-ALL patients, from 2009 to 2013, accessioned at the Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, PR China. The median patient age was 26 years old (range 18–66). The median percentage of blasts in bone marrow was 92% (range, 80%–98%). The diagnosis of T-ALL in all cases was established on the basis of morphologic findings, and immunophenotypic, cytogenetic, and molecular data according to the World Health Organization (WHO) classification and the National Comprehensive Cancer Network (NCCN) guidelines. Mononuclear bone marrow cells were separated using Ficoll-Hypaque density gradient centrifugation and stored in liquid nitrogen. This study was approved by the Institutional Review Boards of the Institute of Hematology

and Blood Disease Hospital and informed consent was obtained from each patient according to the revised Declaration of Helsinki.

### Cell lines and thymocytes

The human T-ALL cell lines CCRF-CEM, KOPT-K1, MOLT4, JURKAT, LOUCY, SUPT1 and the 293T cell line were purchased from American Type Culture Collection (Manassas, VA, USA) and recently identified by DNA fingerprint. Two human postnatal normal thymocyte samples were provided by Dr. Andrew Weng (Terry Fox Laboratory, Canada). The mouse T-ALL cell lines (LPN248, LPN236, LPN228) were generated from mouse *Pten* knock-out T-ALL models and LPN211 was generated from *Ink4a/Arf* knock-out mice.<sup>30</sup> The CCRF-CEM-FFluc cell line was obtained from Dr. Malcolm K. Brenner and was described previously.<sup>34</sup> The cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 mM L-glutamine. 293T cells were cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% FBS. Cells were kept at 37°C in 5% CO<sub>2</sub> and tested without cytoplasm contamination.

### miRNA expression profiling

RNA labeling and hybridization on miRNA microarray chips were performed as described.<sup>35, 36</sup> Briefly, 5 µg of total RNA from each sample was biotin-labeled by reverse transcription using 5' biotin end-labeled random octomer oligo primers. Hybridization of biotin-labeled cDNA was carried out on a miRNA microarray chip (MD Anderson miRNA expression Bioarray Version 5), which contains 2300 miRNA probes, including 1400 human and 900 mouse miRNA genes, in duplicate. Hybridization signals were detected by biotin binding of a streptavidin–Alexa647 conjugate b using Axon Scanner 4000B (Axon Instruments, Union City, CA). The images were quantified by GENEPIX 6.0 software (Axon Instruments).

### Murine xenograft model

10 female 8 week-old Non-obese diabetic/severe combined immune deficient gamma (NSG) mice (Jackson Laboratory, Sacramento, CA, USA) were randomly divided into 2 groups and transplanted intravenously with CCRF-CEM-FFluc cells (5 mice in control group: pCDH empty vector lentivirus infected; 5 mice in miR-26b overexpressed group: pCDH-miR-26b lentivirus infected). The engraftment of CCRF-CEM-FFluc was monitored by flow cytometry (anti-human CD45-APC antibody (BD Biosciences, San Jose, CA, USA) and GFP on mouse retro-orbital bleeding samples. Phospho-AKT was measured by intracellular staining flow cytometry (Cell Signaling; #11962) on mixed mouse retro-orbital bleeding samples. Transplanted mice underwent *in vivo* bioluminescence imaging at various time points. The mice in each group were followed for survival. 2 groups of the mice were treated and experimented under the same condition. This study was approved by Institutional Review Board (IRB) the University of Texas MD Anderson Cancer Center.

### Statistical analysis

Statistical analyses were performed and figures created using Graphpad Prism software 6.0 (Graphpad software Inc, La Jolla, CA, USA). Each data point represents the mean and

standard deviation (SD) of experiments performed at least in triplicate. The group data comparisons were performed using Student *t* test, Two-way ANOVA and  $\chi^2$  test. Linear regression was carried out to test the correlation between miR-26b and *PTEN* expression level. The Kaplan Meier method was used to generate survival curve. The P-values were two-sided and  $P < 0.05$  was considered significant.

Additional methods are presented in the supplementary data.

## Results

### Expression of miR-26b is decreased in mouse T-ALL cells null for *Pten* and in human T-ALL cell lines

We generated a mouse T-ALL model with deletion of the *Pten* tumor suppressor gene and profiled the miRs from this model.<sup>30</sup> A miR array identified a subset of miRs that show aberrant expression levels in *Pten* deficient mouse T-ALL cells (ArrayExpress database E-MTAB-5053) (Figure 1A). We selected miR-26b for this study as its expression level was significantly lower (−3.32 fold) in the *Pten* deficient mouse T-ALL samples (n=4) as compared with mouse wild-type (WT) thymocytes (n=2). In addition, aberrant expression of miR-26b has been identified in several types of non-hematopoietic cancer.<sup>31–33</sup> To further validate that miR-26b is down-regulated in the *Pten* deficient mouse T-ALL, a quantitative RT-PCR (qRT-PCR) was performed. Indeed, the expression level of miR-26b in the *Pten* deficient mouse T-ALL cells was markedly lower in comparison to that of WT thymocytes and *Ink4a/Arf* knock-out (KO) mouse T-ALL cells (LPN211) (Figure 1B). The decreased expression level of miR-26b in *Pten* deficient mouse T-ALL along with its potential role in the pathogenesis of human T-ALL led us to explore if miR-26b expression levels are also altered in human T-ALL. We first performed quantitative RT-PCR on a panel of human T-ALL cell lines. Indeed, the expression level of miR-26b is significantly lower (6 to 8 fold) in human T-ALL cell lines, including the PTEN wild type cell lines SUP-T1 and KOPT-K1, and PTEN null cell lines CCRF-CEM, LOUCY, JURKAT and MOLT4, when compared with that of normal human thymocytes (Figure 1C). miR-26b expression level in human primary T-ALL were also determined by Quantitative RT-PCR. We selected 27 primary bone marrow T-ALL samples. miR-26b was found to be down-regulated in the primary T-ALL cells compared with normal human thymocytes ( $P < 0.0001$ , Figure 1D), and miR-26b expression levels correlated with PTEN expression level in primary human T-ALL samples (Figure 1E,  $r = 0.3987$ ,  $P = 0.039$ ). These data indicate that miR-26b expression level is consistently decreased in mouse and human T-ALL cells and is associated with *PTEN* level.

### miR-26b inhibits proliferation and promotes apoptosis in T-ALL cells *in vitro*

To functionally assess whether miR-26b plays a role in the growth of human T-ALL cells, we expressed exogenous miR-26b in a panel of human T-ALL cell lines (Figure 2A). In contrast with the expression levels of miR-26b in empty vector infected T-ALL cells, T-ALL cells infected with lentivirus expressing exogenous miR-26b showed modestly elevated mRNA levels of miR-26b ( $P < 0.05$ ) in CCRF-CEM cells (2.2 fold higher), and Koptk1 and Molt4 T-ALL cells (approximately 5 fold higher). Exogenous expression of miR26b significantly reduced the proliferation of T-ALL cells ( $P < 0.05$ ) with the most significant

inhibition in Molt4 cells (Figure 2B). In addition, the effects of exogenous miR-26b on apoptosis of T-ALL cells were evaluated by flow cytometry using Annexin-V and 7-AAD stains. Indeed, the expression of miR-26b significantly increased the apoptosis of all of the three human T-ALL cell lines (Figure 2C,  $P < 0.05$ ). CCRF-CEM, with expression of miR-26b, showed 49.49% apoptosis (42.6% early apoptosis in Q3 and 6.89% late apoptosis in Q2). In contrast, CCRF-CEM with only vector showed 3.48% of apoptosis (2.5% early apoptosis in Q3 and 0.98% late apoptosis in Q2). Similarly, expression of exogenous miR-26b in KOPT-K1 and MOLT4 cells showed increased apoptosis, 51% apoptosis (15.9% early apoptosis in Q3 and 35.1% late apoptosis in Q2) and 56.3% apoptosis (18.8% early apoptosis in Q3 and 37.5% late apoptosis in Q2), respectively. Vector infected KOPT-K1 and MOLT4 exhibited much lower apoptosis, 14.98% (1.88% early apoptosis in Q3 and 13.1% late apoptosis in Q2) and 19.4% (7.8% early apoptosis in Q3 and 11.6% late apoptosis in Q2), respectively. These results suggest that miR-26b has tumor suppressor activities and may be important in T-ALL pathogenesis.

### miR-26b inhibits the growth of T-ALL cells *in vivo*

To evaluate the effect of miR-26b on the growth of T-ALL *in vivo*, we established a T-ALL cell line xenograft model by injecting vector-control and miR-26b overexpressed CCRF-CEM-FFluc cells into NSG mice. Bioluminescence imaging showed that the growth of miR-26b overexpressing T-ALL cells was significantly slower than that of control cells (Figure 3A). The mice with vector treated T-ALL cells demised by 18 days and showed extensive T-ALL involving peripheral blood (Figure 3B and C,  $P < 0.05$ ). In contrast, mice with the expression of exogenous miR-26b had a prolonged overall survival (Figure 3C,  $P = 0.0031$ ). The miR-26b overexpressing mice also exhibited lower phospho-AKT levels in the CCRF-CEM-FFluc T-ALL cells, as detected by flow cytometry (Figure 3B,  $P < 0.05$ ). These data indicate that miR-26b suppresses the growth of T-ALL cells *in vivo*.

### miR-26b directly targets *PIK3CD* and inhibits PI3K/AKT signaling

Because miRs exert their functions through targeting gene expression, we performed bioinformatic analysis and screening to identify possible miR-26b targets (<http://www.microrna.org>). One of the potential target genes of miR-26b is *PIK3CD* that encodes PI3K p110 $\delta$ . *PIK3CD* was selected as a miR-26b target gene because of the well matched 3'-UTR binding site by miR-26b and its potential role in hematologic malignancies.<sup>37</sup> To confirm if miR-26b binds to the 3'-UTR of *PIK3CD*, we cloned the 3'-UTR of *PIK3CD* into a dual luciferase vector. The dual-luciferase assay showed that miR-26b inhibited luciferase activity with wt-*PIK3CD*-3'-UTR co-transfection compared with vector ( $P < 0.001$ ), but did not influence luciferase activity with Mut-*PIK3CD*-3'-UTR or Null-*PIK3CD*-3'-UTR co-transfection ( $P > 0.05$ ) (Figure 4A–B). The inhibitory effect of miR-26b on *PIK3CD*-3'-UTR was also confirmed by detecting PIK3CD protein (PI3K p110 $\delta$ ) level after overexpressing miR-26b in T-ALL cell lines. Binding of miR-26b to the 3'-UTR of *PIK3CD* mRNA inhibited protein translation of the PI3K p110 $\delta$  in CCRF-CEM, KOPTK1 and MOLT4 T-ALL cells (Figure 4C). To assess the effect of the decreased PI3K p110 $\delta$  on PI3K/AKT pathway, Western blot analysis was used to determine p-AKT expression levels. Phosphorylated-AKT was reduced in T-ALL cells with miR-26b overexpression compared with vector control (Figure 4C). Additionally, co-expression of exogenous miR-26b and

PIK3CD can significantly rescue the miR-26b effect on apoptosis in CCRF-CEM cells (Supplemental Figure 1). These results suggest that *PIK3CD* is a direct target of miR-26b.

### Inhibition of PIK3CD by CAL-101 or shRNAs reduces the growth of T-ALL cells

Because the PI3K pathway is frequently altered in T-ALL, we hypothesized that decreased expression of PI3K p110 $\delta$  affects the growth and survival of T-ALL cells. T-ALL cells, including CCRF-CEM, KOPT-K1 and MOLT4, were treated with various concentrations of CAL-101 (Idelalisib, GS-1101), a selective p110 $\delta$  inhibitor.<sup>38</sup> CAL-101 demonstrated a dose-dependent inhibition of T-ALL viability, indicating that inhibition of p110 $\delta$  reduces the growth in T-ALL cells (Figure 5A). Next we confirmed these findings by reducing the expression level of PIK3CD with two *PIK3CD* shRNAs. The shRNAs significantly dampened the expression level of the PI3K p110 $\delta$  in CCRF-CEM and KOPT-K1 T-ALL cells as shown by Western blotting (Figure 5B). Compared with vector treated cells, T-ALL cells infected with the two shRNAs showed markedly decreased proliferation and increased apoptosis (Figure 5C). Thus, PIK3CD is important for the growth and survival of T-ALL cells, suggesting that miR-26b-mediated downregulation of PIK3CD may have a direct impact on T-ALL pathogenesis.

### miR-26b is regulated by the PTEN/PI3K pathway via the transcriptional factor IKZF1

miR-26b is frequently down-regulated in T-ALL, and this downregulation is important for the growth and survival of T-ALL cells. Therefore, we were interested in determining how miR-26b is regulated. Since miR-26b is downregulated in *Pten* deficient mouse T-ALL cell lines and human T-ALL cell lines, we hypothesized that the PTEN/PI3K pathway affects the expression level of miR-26b. Interestingly, restoration of PTEN expression in PTEN<sup>null</sup> human T-ALL cell lines (CCRF-CEM and MOLT4) that lack endogenous PTEN expression significantly elevated the expression level of miR-26b (Figure 6A,  $P < 0.05$ ). Expression of exogenous PTEN in the KOPT-K1 that has endogenous PTEN expression had no or minimal impact on miR-26b expression (Figure 6A,  $P > 0.05$ ). In contrast, knocking-down PTEN in KOPT-K1 cells by shRNAs down-regulated miR-26b levels (Figure 6B,  $P < 0.05$ ). Those data indicate that PTEN/PI3K pathway regulates miR-26b.

Recent studies have shown that lack of PTEN, which negatively regulates PI3K signaling, alters the expression of different isoforms of the tumor suppressor Ikaros,<sup>39</sup> leading us to hypothesize that the PTEN/PI3K pathway may modulate miR-26b through modulation of Ikaros activity. Ikaros protein has several isoforms that are divided into two groups: dominant positive ones (IK-DP, including IK1-IK3) and dominant negative ones (IK-DN, including IK4-9). In this study, we identified two predominant Ikaros isoforms in the CCRF-CEM, MOLT4 and KOPT-K1 cell lines (Figure 6C). We cloned and sequenced the two isoforms. One was IK1 that is the longest IK-DP isoform (Figure 6C). The other one is a novel Ikaros isoform that retained the DBD, lost most of the C-terminal zinc finger domain for polymerization, and showed barely detectable expression levels in normal thymocytes (Figure 6C and Supplemental Figure 2A–B). Additionally, the novel isoform also had an in-frame deletion of 30 nucleotides that encode 10 amino acids right before the C-terminal zinc finger domain (Supplemental Figure 2A–B). The other Ikaros DP or DN isoforms were not detectable by Western blotting (data not shown).

To assess whether Ikaros, the IKZF1 gene coding protein, binds to the promoter of miR-26b, we performed a search for a binding site(s). Bioinformatic analysis by PROMO 3.0 ([http://algggen.lsi.upc.es/recerca/menu\\_recerca.html](http://algggen.lsi.upc.es/recerca/menu_recerca.html)) predicted that Ikaros binds to the miR-26b promoter region as a transcriptional factor. There are five potential IK1 binding sites that have a core GGGAA sequence, according to the Ikaros ChIP-seq report.<sup>40</sup> To confirm that IK1 specifically binds to the site(s) in the miR-26b promoter region, we overexpressed a Flag-tagged version of the Ikaros isoform IK1 in CCRF-CEM, MOLT4, and KOPT-K1 cells (Figure 6D). The expression of exogenous IK1 significantly elevated miR-26b expression levels. In addition, a ChIP assay was performed in the CCRF-CEM cell line, and the ChIP PCR primers recovered two GGGAA cores on the promoter region. Ikaros-antibody, which can bind to all Ikaros isoforms, was used to precipitate crosslinked DNA from CCRF-CEM cells that were infected by empty lentivirus vector or PTEN overexpression lentivirus. Flag antibody was used to precipitate crosslinked DNA from CCRF-CEM cells that were infected with lentivirus overexpressing Flag-tagged IK1 (IK1-Flag). We found that more Ikaros protein was bound to the miR-26b promoter region in CCRF-CEM cells when PTEN or IK1 was overexpressed in CCRF-CEM cells. In contrast, there was very weak or no binding of Ikaros protein in empty vector lentivirus infected CCRF-CEM (control) cells (Figure 6E).

To further confirm that the transcriptional factor IK1 binds to the miR-26b promoter as a transcription factor, we generated a pGL3 luciferase reporter vector with an inserted miR-26b promoter region. This construct was co-transfected with IK1 expression plasmid (designated as Ikaros-DP) or vector control into 293T cells. We found that luciferase activity was significantly higher in 293T cells with the miR-26b promoter region than in those with the empty vector ( $p < 0.01$ , Figure 6F). As the novel isoform of Ikaros (IK-DN) had an in-frame deletion and loss of most of the C-terminal zinc finger, we hypothesized that it is a dominant negative isoform. We generated a construct that expresses Ikaros-DN. Interestingly, co-transfection of IK-DN with IK1 expression plasmid significantly diminished the IK1 dependent luciferase activity in 293-T cells ( $p < 0.05$ , Figure 6F). Since PTEN expression elevates miR-26b expression, we hypothesized that PTEN expression differentially modulates the expression of IK-DP and IK-DN in T-ALL cells. To this end, the three T-ALL cell lines (CCRF-CEM, MOLT4 and KOPT-K1) were infected with PTEN expression lentivirus. Expression of PTEN markedly decreased the levels of IK-DN isoform in CCRF-CEM and MOLT4, two T-ALL cell lines that have no endogenous PTEN expression (Figure 6G). Exogenous expression of PTEN had no significant impact on the IK-DN isoform expression in KOPT-K1, a T-ALL cell line with endogenous PTEN expression. However, knocking-down PTEN by shRNAs increased the level of IK-DN isoform in the KOPT-K1 cell line (Figure 6H). Interestingly, expression of exogenous PTEN or knocked-down PTEN has no significant effect on the expression level of IK-DP isoform in any of the T-ALL cell lines, regardless of the presence of endogenous PTEN expression (Figure 6G and H). Taken together, these data suggest that miR-26b is regulated by the Pten/PI3K pathways through altered splicing isoforms of Ikaros.

## Discussion

Activation of the PI3K/AKT pathway occurs frequently in T-ALL and has emerged as a potential therapeutic target.<sup>41, 42, 43</sup> Our study showed that miR-26b directly targets

*PIK3CD*, the gene encoding the PI3K p110 $\delta$  isoform, and inhibits T-ALL cell proliferation by inactivating the PI3K/AKT pathway *in vitro* and *in vivo*. Furthermore, we showed that CAL-101, a selective PI3K p110 $\delta$  inhibitor, reduces proliferation and promotes apoptosis in T-ALL cell lines. The T-ALL cell lines we studied have constitutively activated PI3K activity. Two of the T-ALL cell lines (CCRF-CEM and MOLT4) do not express PTEN and the third T-ALL cell line (KOPT-K1) expresses PTEN, suggesting that the presence of PI3K p110 $\delta$  is not totally dependent on PTEN and may be activated by other mechanism(s).

Since PTEN is not a transcription factor, but likely affects the expression of miR-26 via transcription factor(s), we screened for transcriptional factors that regulate miR-26b expression. Sequencing analysis revealed several potential Ikaros core binding sites in the promoter region of miR-26b. Using a polyclonal antibody, we found IK1 and a novel Ikaros isoform (IK-DN). The IK-DN retains the four N-terminal zinc-finger DNA binding domain, but lacks 10 amino acids encoded by exon 7 and most of the 3'-end C-terminal zinc-finger polymerization domain. Previously, others have shown that PTEN loss activates the PI3K/AKT pathway and aberrant Ikaros dominant negative isoforms are expressed in p53 mouse T-ALL cells.<sup>44</sup> PI3K/AKT signaling mediates inappropriate Ikaros mRNA splicing by downregulation of FoxO1 in PTEN<sup>-/-</sup> pro-B cells.<sup>39</sup> Exogenous PTEN expression led to increased expression of FoxO1 in T-ALL cell lines with null endogenous PTEN (CCRF-CEM and MOLT4). Thus, this could be one of the mechanisms of Ikaros regulation. In contrast, FoxO1 expression level was not changed in the PTEN wt T-ALL cell line KOPT-K1 upon the expression of exogenous PTEN. Future research on this mechanism might be fruitful (data not shown). As a transcriptional factor, Ikaros binds DNA as dimeric and multimeric complexes and specific Ikaros isoforms influence their functions.<sup>45, 46</sup> Dominant negative Ikaros isoforms modulate Ikaros transcriptional complexes during transcription initiation, elongation or termination.<sup>47-49</sup> Co-expression of dominant positive and dominant negative Ikaros in a transcriptional complex could alter its affinity for pericentromeric heterochromatin, leading to chromatin remodeling and activation of target genes.<sup>50</sup>

The ChIP assay confirmed the presence of two Ikaros core binding sites that are adjacent to each other (Figure 6D and Supplement Figure 3). Overexpression of IK1, the strongest functional isoform of Ikaros, significantly induced miR-26b expression in CCRF-CEM, MOLT4, and KOPT-K1 cell lines. The IK-DN retains the 4 N-terminal zinc-finger DNA binding domain, which explains its remaining binding activity to the miR-26b promoter (Figure 6E). However, this binding is much weaker than that of IK1 (Figure 6E). Moreover, the novel Ikaros isoform functions similarly to the dominant negative isoforms of Ikaros as its presence strongly diminished the binding activities of IK1 to the miR-26 promoter. This observation suggests there is potentially competitive binding of the novel IK-DN and IK1 to the miR-26b promoter. The finding of a differential impact of PTEN on the expression of IK1 and the novel isoform is intriguing (Figure 6G). The finding that the novel isoform of IK-DN, but not IK1, is regulated after exogenous PTEN expression or knocking-down along with the fact that IK1 expression enhances the binding of Ikaros to the miR-26b promoter region indicate that Ikaros may regulate miR-26b in both a dose- and isoform-dependent manner in human T-ALL cells. Previous studies have shown that Ikaros isoforms competitively form dimer or polymer complexes to affect gene transcription. Reduced

expression of the novel Ikaros isoform may relatively increase dominant-positive IK1, or vice versa, therefore regulating miR-26b expression (Figure 6F and Figure 7).

*NOTCH1* is frequently mutated and thought to be oncogenic in T-ALL.<sup>51</sup> We hypothesized that miR-26b may have an effect on *NOTCH1*. Interestingly, expression of exogenous miR-26b with lentivirus diminished the levels of *NOTCH1* and its downstream target *HES-1* in the T-ALL cell lines--CCRF-CEM, KOPT-K1, and MOLT4 (Supplementary Figure 4). However, there is no consensus miR-26b binding site in the 3'UTR of *NOTCH1* (data not shown), suggesting that miR-26b may downregulate the Notch pathway indirectly. T-ALL cells often display chronic activation of PI3K/AKT associated with Notch activation.<sup>52</sup> Inhibition of both the PI3K and Notch1 pathways may be beneficial for T-ALL treatment and further characterization of miR-26b and its regulation of target genes may lead to targeted T-ALL therapy.

In summary, miR-26b is involved in the pathogenesis of T-ALL by targeting *PIK3CD* of the PI3K/AKT pathway and indirectly regulating the Notch pathway. The tumor suppressor *PTEN* inhibits the PI3K pathway which alters isoforms of Ikaros, and is one of the up-regulators of miR-26b in T-ALL (Figure 7). In this circle of PI3K modulation, a therapeutic PI3K inhibitor may be helpful in treating patients with T-ALL. Further studies of the target genes and regulators of miR-26b may pave the road to the development of improved therapy for T-ALL patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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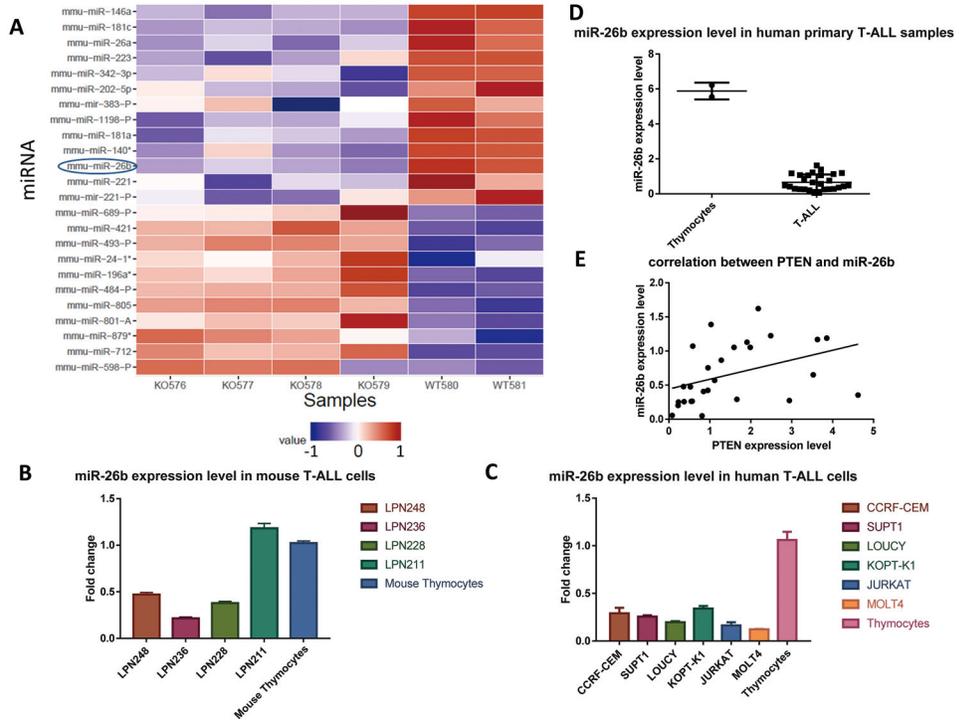
## References

1. 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 Feb; 1(1):75–87. [PubMed: 12086890]
2. Marks DI, Paietta EM, Moorman AV, Richards SM, Buck G, DeWald G, et al. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). *Blood*. 2009 Dec 10; 114(25):5136–5145. [PubMed: 19828704]
3. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. *Lancet*. 2008 Mar 22; 371(9617):1030–1043. [PubMed: 18358930]

4. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006 Jan 12; 354(2):166–178. [PubMed: 16407512]
5. Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M, et al. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest*. 2008 Nov; 118(11):3762–3774. [PubMed: 18830414]
6. Jenkinson S, Kirkwood AA, Goulden N, Vora A, Linch DC, Gale RE. Impact of PTEN abnormalities on outcome in pediatric patients with T-cell acute lymphoblastic leukemia treated on the MRC UKALL2003 trial. *Leukemia*. 2016 Jan; 30(1):39–47. [PubMed: 26220040]
7. Kastner P, Chan S. Role of Ikaros in T-cell acute lymphoblastic leukemia. *World journal of biological chemistry*. 2011 Jun 26; 2(6):108–114. [PubMed: 21765975]
8. Orozco CA, Acevedo A, Cortina L, Cuellar GE, Duarte M, Martin L, et al. The combined expression patterns of Ikaros isoforms characterize different hematological tumor subtypes. *PloS one*. 2013; 8(12):e82411. [PubMed: 24324784]
9. Geimer Le Lay AS, Oravec A, Mastio J, Jung C, Marchal P, Ebel C, et al. The tumor suppressor Ikaros shapes the repertoire of notch target genes in T cells. *Sci Signal*. 2014 Mar 18.7(317):ra28. [PubMed: 24643801]
10. Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell*. 1995 Oct 20; 83(2):289–299. [PubMed: 7585946]
11. Miao MH, Ji XQ, Zhang H, Xu J, Zhu H, Shao XJ. miR-590 promotes cell proliferation and invasion in T-cell acute lymphoblastic leukaemia by inhibiting RB1. *Oncotarget*. 2016 Mar 28.
12. Fan SJ, Li HB, Cui G, Kong XL, Sun LL, Zhao YQ, et al. miRNA-149\* promotes cell proliferation and suppresses apoptosis by mediating JunB in T-cell acute lymphoblastic leukemia. *Leukemia research*. 2016 Feb.41:62–70. [PubMed: 26725775]
13. Yin JJ, Liang B, Zhan XR. MicroRNA-204 inhibits cell proliferation in T-cell acute lymphoblastic leukemia by down-regulating SOX4. *International journal of clinical and experimental pathology*. 2015; 8(8):9189–9195. [PubMed: 26464665]
14. Oliveira LH, Schiavinato JL, Fraguas MS, Lucena-Araujo AR, Haddad R, Araujo AG, et al. Potential roles of microRNA-29a in the molecular pathophysiology of T-cell acute lymphoblastic leukemia. *Cancer science*. 2015 Oct; 106(10):1264–1277. [PubMed: 26251039]
15. Xi Y, Li J, Zhang P, Bai W, Gao N, Bai W, et al. Upregulation of miRNA-17 and miRNA-19 is associated with unfavorable prognosis in patients with T-cell lymphoblastic lymphoma. *Experimental and molecular pathology*. 2015 Oct; 99(2):297–302. [PubMed: 26231295]
16. Verduci L, Azzalin G, Gioiosa S, Carissimi C, Laudadio I, Fulci V, et al. microRNA-181a enhances cell proliferation in acute lymphoblastic leukemia by targeting EGR1. *Leukemia research*. 2015 Apr; 39(4):479–485. [PubMed: 25740602]
17. Zhu H, Miao MH, Ji XQ, Xue J, Shao XJ. miR-664 negatively regulates PLP2 and promotes cell proliferation and invasion in T-cell acute lymphoblastic leukaemia. *Biochemical and biophysical research communications*. 2015 Apr 3; 459(2):340–345. [PubMed: 25735976]
18. Sanghvi VR, Mavrikis KJ, Van der Meulen J, Boice M, Wolfe AL, Carty M, et al. Characterization of a set of tumor suppressor microRNAs in T cell acute lymphoblastic leukemia. *Science signaling*. 2014 Nov 18.7(352):ra111. [PubMed: 25406379]
19. Ortega M, Bhatnagar H, Lin AP, Wang L, Aster JC, Sill H, et al. A microRNA-mediated regulatory loop modulates NOTCH and MYC oncogenic signals in B- and T-cell malignancies. *Leukemia*. 2015 Apr; 29(4):968–976. [PubMed: 25311243]
20. Mets E, Van der Meulen J, Van Peer G, Boice M, Mestdagh P, Van de Walle I, et al. MicroRNA-193b-3p acts as a tumor suppressor by targeting the MYB oncogene in T-cell acute lymphoblastic leukemia. *Leukemia*. 2015 Apr; 29(4):798–806. [PubMed: 25231743]
21. Malik D, Kaul D, Chauhan N, Marwaha RK. miR-2909-mediated regulation of KLF4: a novel molecular mechanism for differentiating between B-cell and T-cell pediatric acute lymphoblastic leukemias. *Molecular cancer*. 2014; 13:175. [PubMed: 25037230]
22. Mets E, Van Peer G, Van der Meulen J, Boice M, Taghon T, Goossens S, et al. MicroRNA-128-3p is a novel oncomiR targeting PHF6 in T-cell acute lymphoblastic leukemia. *Haematologica*. 2014 Aug; 99(8):1326–1333. [PubMed: 24895337]

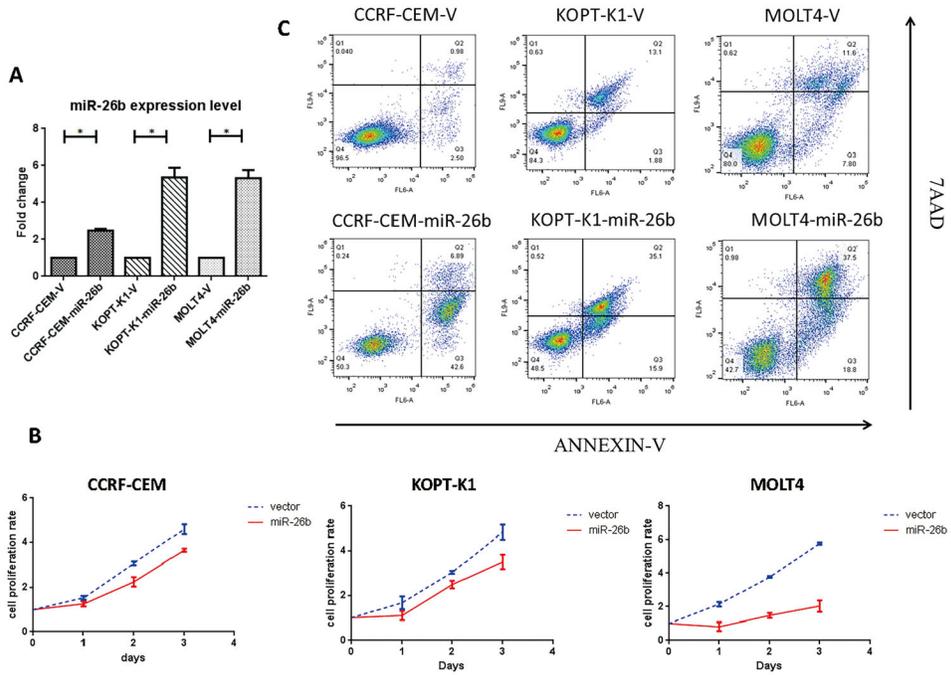
23. Xia H, Yamada S, Aoyama M, Sato F, Masaki A, Ge Y, et al. Prognostic impact of microRNA-145 down-regulation in adult T-cell leukemia/lymphoma. *Human pathology*. 2014 Jun; 45(6):1192–1198. [PubMed: 24745613]
24. Gimenes-Teixeira HL, Lucena-Araujo AR, Dos Santos GA, Zanette DL, Scheucher PS, Oliveira LC, et al. Increased expression of miR-221 is associated with shorter overall survival in T-cell acute lymphoid leukemia. *Experimental hematology & oncology*. 2013; 2(1):10. [PubMed: 23566596]
25. Ye H, Liu X, Lv M, Wu Y, Kuang S, Gong J, et al. MicroRNA and transcription factor co-regulatory network analysis reveals miR-19 inhibits CYLD in T-cell acute lymphoblastic leukemia. *Nucleic acids research*. 2012 Jul; 40(12):5201–5214. [PubMed: 22362744]
26. Mavrakis 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). *Nature genetics*. 2011 Jul; 43(7):673–678. [PubMed: 21642990]
27. Chiaretti S, Messina M, Tavaloro S, Zardo G, Elia L, Vitale A, et al. Gene expression profiling identifies a subset of adult T-cell acute lymphoblastic leukemia with myeloid-like gene features and over-expression of miR-223. *Haematologica*. 2010 Jul; 95(7):1114–1121. [PubMed: 20418243]
28. Schotte D, Chau JC, Sylvester G, Liu G, Chen C, van der Velden VH, et al. Identification of new microRNA genes and aberrant microRNA profiles in childhood acute lymphoblastic leukemia. *Leukemia*. 2009 Feb; 23(2):313–322. [PubMed: 18923441]
29. Spizzo R, Nicoloso MS, Croce CM, Calin GA. SnapShot: MicroRNAs in Cancer. *Cell*. 2009 May 1; 137(3):586–586. e581. [PubMed: 19410551]
30. Medyouf H, Gao X, Armstrong F, Gusscott S, Liu Q, Gedman AL, et al. Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss. *Blood*. 2010 Feb 11; 115(6):1175–1184. [PubMed: 20008304]
31. Zhang Z, Kim K, Li X, Moreno M, Sharp T, Goodheart MJ, et al. MicroRNA-26b represses colon cancer cell proliferation by inhibiting lymphoid enhancer factor 1 expression. *Molecular cancer therapeutics*. 2014 Jul; 13(7):1942–1951. [PubMed: 24785257]
32. Li D, Wei Y, Wang D, Gao H, Liu K. MicroRNA-26b suppresses the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-kappaB/MMP-9/VEGF pathways. *Biochem Biophys Res Commun*. 2016 Jan 28.
33. Zheng WD, Zhou FL, Lin N. MicroRNA-26b inhibits osteosarcoma cell migration and invasion by down-regulating PFKFB3 expression. *Genet Mol Res*. 2015; 14(4):16872–16879. [PubMed: 26681033]
34. Mamonkin M, Rouce RH, Tashiro H, Brenner MK. A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies. *Blood*. 2015 Aug 20; 126(8):983–992. [PubMed: 26056165]
35. Liu CG, Calin GA, Meloon B, Gamliel N, Seignani C, Ferracin M, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proceedings of the National Academy of Sciences of the United States of America*. 2004 Jun 29; 101(26):9740–9744. [PubMed: 15210942]
36. Liu CG, Calin GA, Volinia S, Croce CM. MicroRNA expression profiling using microarrays. *Nature protocols*. 2008; 3(4):563–578. [PubMed: 18388938]
37. Burger JA, Hoellenriegel J. Phosphoinositide 3'-kinase delta: turning off BCR signaling in Chronic Lymphocytic Leukemia. *Oncotarget*. 2011 Oct; 2(10):737–738. [PubMed: 22006556]
38. Nair KS, Cheson B. The role of idelalisib in the treatment of relapsed and refractory chronic lymphocytic leukemia. *Therapeutic advances in hematology*. 2016 Apr; 7(2):69–84. [PubMed: 27054023]
39. Alkhatib A, Werner M, Hug E, Herzog S, Eschbach C, Faraidun H, et al. FoxO1 induces Ikaros splicing to promote immunoglobulin gene recombination. *The Journal of experimental medicine*. 2012 Feb 13; 209(2):395–406. [PubMed: 22291095]
40. Schjerven H, McLaughlin J, Arenzana TL, Fritze S, Cheng D, Wadsworth SE, et al. Selective regulation of lymphopoiesis and leukemogenesis by individual zinc fingers of Ikaros. *Nature immunology*. 2013 Oct; 14(10):1073–1083. [PubMed: 24013668]

41. Gutierrez A, Sanda T, Grebliunaite R, Carracedo A, Salmena L, Ahn Y, et al. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood*. 2009 Jul 16; 114(3):647–650. [PubMed: 19458356]
42. Zhao WL. Targeted therapy in T-cell malignancies: dysregulation of the cellular signaling pathways. *Leukemia*. 2010 Jan; 24(1):13–21. [PubMed: 19865108]
43. Subramaniam PS, Whye DW, Efimenko E, Chen J, Tosello V, De Keersmaecker K, et al. Targeting nonclassical oncogenes for therapy in T-ALL. *Cancer cell*. 2012 Apr 17; 21(4):459–472. [PubMed: 22516257]
44. Levine AJ, Chan CS, Dudgeon C, Puzio-Kuter A, Hainaut P. The Evolution of Tumors in Mice and Humans with Germline p53 Mutations. *Cold Spring Harb Symp Quant Biol*. 2015 Dec 7.
45. Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J*. 1996 Oct 1; 15(19):5358–5369. [PubMed: 8895580]
46. Trinh LA, Ferrini R, Cobb BS, Weinmann AS, Hahm K, Ernst P, et al. Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. *Genes Dev*. 2001 Jul 15; 15(14):1817–1832. [PubMed: 11459831]
47. Bottardi S, Mavoungou L, Bourgoin V, Mashtalir N, Affar el B, Milot E. Direct protein interactions are responsible for Ikaros-GATA and Ikaros-Cdk9 cooperativeness in hematopoietic cells. *Molecular and cellular biology*. 2013 Aug; 33(16):3064–3076. [PubMed: 23732910]
48. Galy A, Christopherson I, Ferlazzo G, Liu G, Spits H, Georgopoulos K. Distinct signals control the hematopoiesis of lymphoid-related dendritic cells. *Blood*. 2000 Jan 1; 95(1):128–137. [PubMed: 10607695]
49. Tucker SN, Jessup HK, Fujii H, Wilson CB. Enforced expression of the Ikaros isoform IK5 decreases the numbers of extrathymic intraepithelial lymphocytes and natural killer 1.1+ T cells. *Blood*. 2002 Jan 15; 99(2):513–519. [PubMed: 11781232]
50. Ronni T, Payne KJ, Ho S, Bradley MN, Dorsam G, Dovat S. Human Ikaros function in activated T cells is regulated by coordinated expression of its largest isoforms. *J Biol Chem*. 2007 Jan 26; 282(4):2538–2547. [PubMed: 17135265]
51. 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 Oct 8; 306(5694):269–271. [PubMed: 15472075]
52. Hales EC, Taub JW, Matherly LH. New insights into Notch1 regulation of the PI3K-AKT-mTOR1 signaling axis: targeted therapy of gamma-secretase inhibitor resistant T-cell acute lymphoblastic leukemia. *Cellular signalling*. 2014 Jan; 26(1):149–161. [PubMed: 24140475]

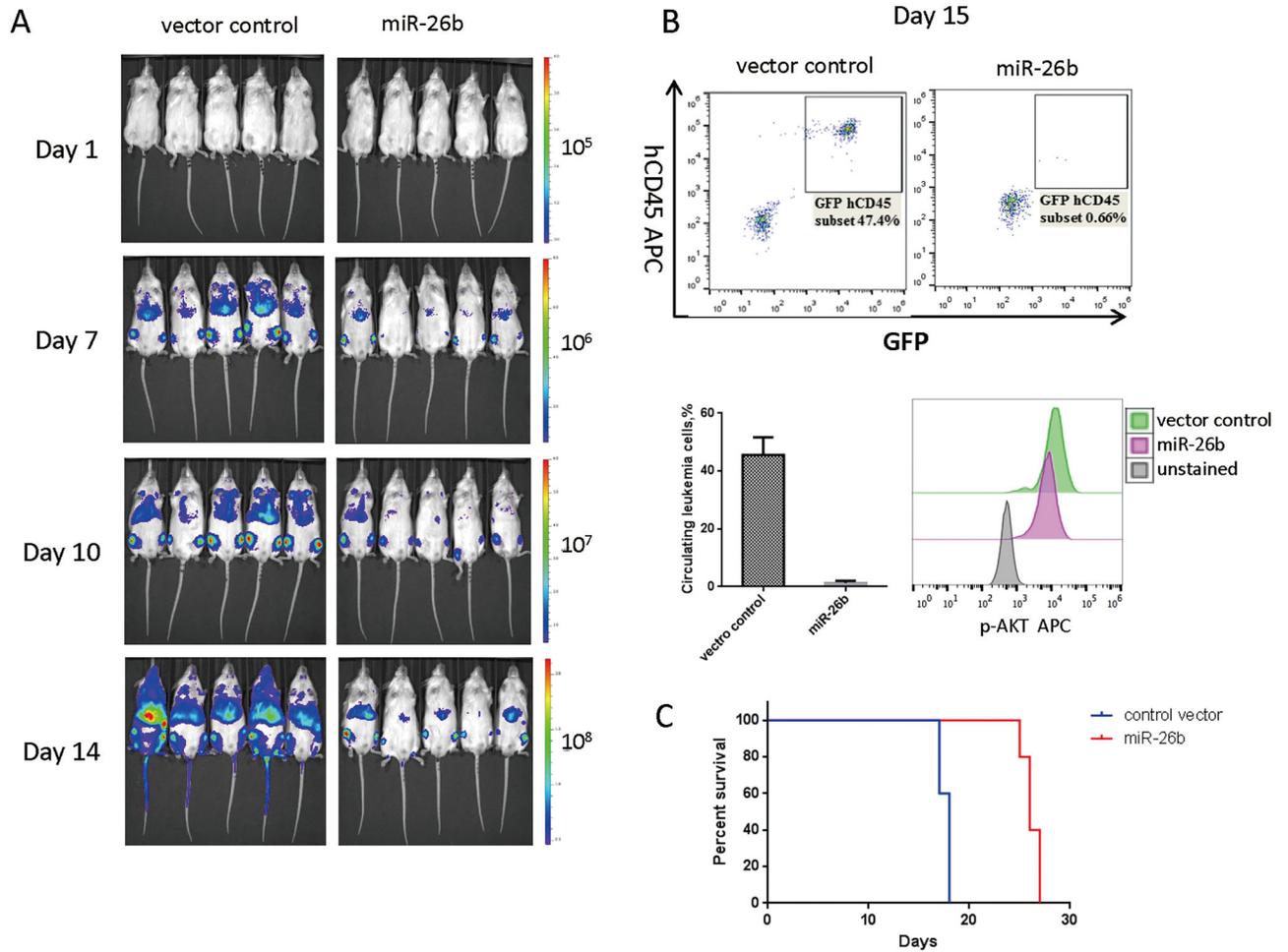


**Figure 1. Decreased expression of miR-26b in *Pten*-knockout mouse T-ALL cells and human T-ALL cells**

A: miRNA profile of Array data. KO576, KO577, KO578 and KO579 are *Pten*-knockout mouse T-ALL samples. WT580 and WT581 are wild-type mouse thymocytes. B: Decreased miR-26b expression level in mouse *Pten* deficient T-ALL cell lines (LPN248, LPN236 and LPN228) compared with mouse *Ink4a/Arf* knock-out T-ALL cell line (LPN211) and mouse wild type thymocytes ( $P < 0.001$ ). C: Decreased miR-26b in human T-ALL cell lines, CCRF-CEM, SUPT1, LOUCY, KOPT-K1, JURKAT and MOLT4 compared with postnatal normal human thymus ( $P < 0.001$ ). D: Decreased expression level of miR-26b in human primary T-ALL samples ( $P < 0.001$ ). E: Correlation of miR-26b expression level with *PTEN* level in human primary T-ALL samples ( $r = 0.3987$ ,  $P = 0.039$ ).

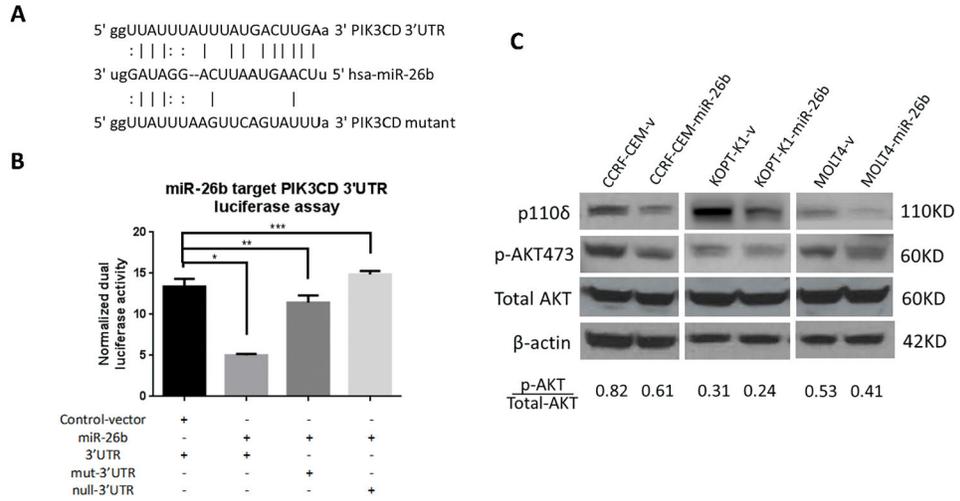


**Figure 2. miR-26b inhibits proliferation and promotes apoptosis in T-ALL cells *in vitro***  
 A: Exogenous miR-26b expression levels in T-ALL cell lines (\*P<0.05). B: Expression of exogenous miR-26b significantly reduced the proliferation of CCRF-CEM, KOPTK1, and MOLT4 cells (p<0.05). C: Expression of exogenous miR-26b significantly promoted apoptosis of CCRF-CEM, KOPT-K1, and MOLT4 cells (p<0.05). V: vector control.



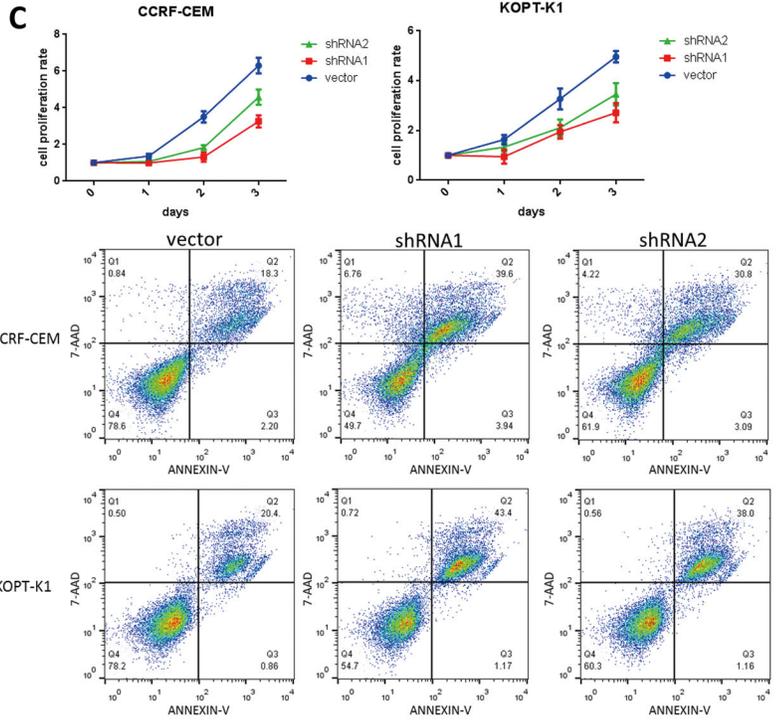
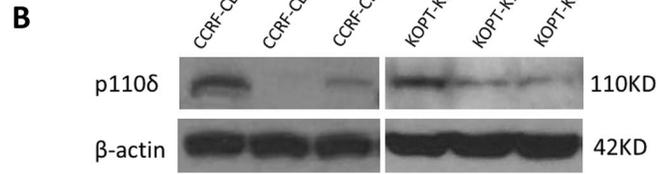
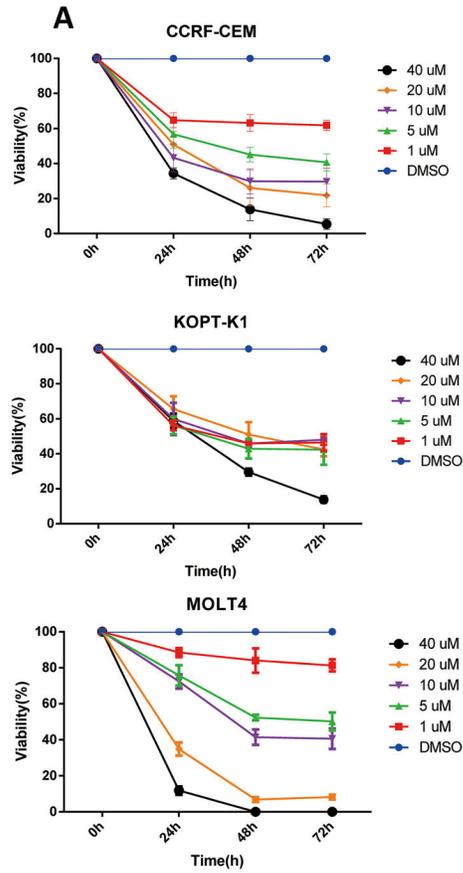
**Figure 3. miR-26b inhibits the growth of T-ALL cells *in vivo***

A: Tumor burden was monitored and assessed in a xenograft T-ALL mouse model by bioluminescence imaging at the indicated time points. B: Tumor burden of peripheral blood was detected by flow cytometry (GFP and hCD45 staining) and the phospho-AKT level of CCRF-CEM-FFluc cells was measured by intracellular staining flow cytometry on day 15. C: Kaplan-Meier survival curve (P=0.0031).

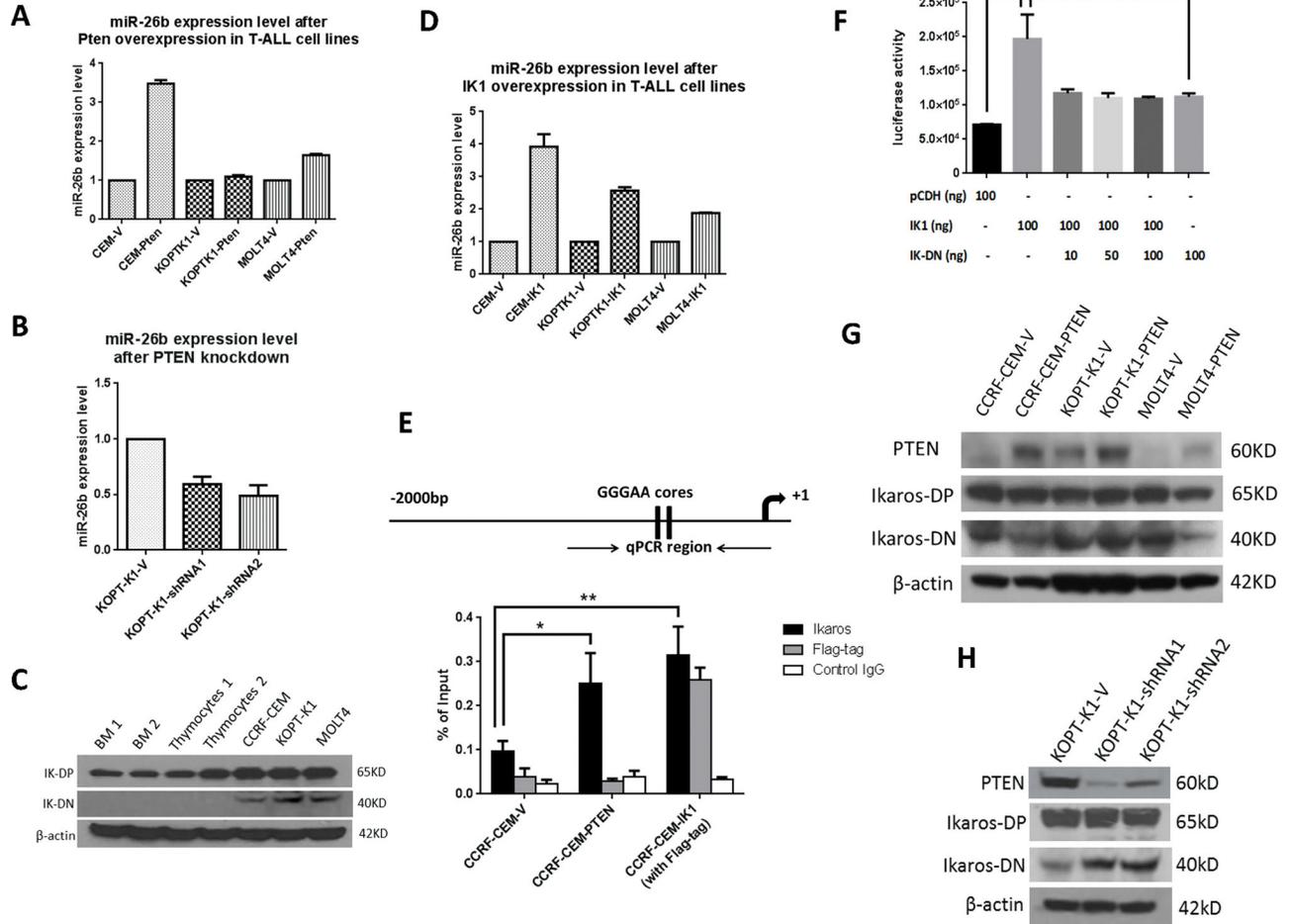


**Figure 4. PIK3CD is a miR-26b target gene**

A: miR-26b binding site on wild-type *PIK3CD*-3'UTR and mutant *PIK3CD*-3'UTR. *PIK3CD* mutant indicated the artificially mutated nucleotides and remaining wild type nucleotides of the 3'UTR of *PIK3CD*. B: Dual-luciferase assay analysis for miR-26b binding site; miR-26b inhibited the activity of luciferase containing wild-type 3'UTR (\* $P < 0.001$ ) but not that of luciferase containing mutant (mut) or null 3'UTR (\*\* $P > 0.05$ , \*\*\* $P > 0.05$ ). C: Protein levels of PIK3CD (p110δ), p-AKT and total AKT in T-ALL cells infected with control vector and overexpressing miR-26b. p-AKT expression levels were relatively decreased 25.6%, 22.6% and 22.6% in CCRF-CEM, KOPT-K1 and MOLT4 cells, respectively, after miR-26b overexpression. V: vector control.



**Figure 5. Inhibition of PIK3CD by CAL-101 or shRNAs reduced the growth of T-ALL cells**  
 A: T-ALL cell lines were treated with 1–40  $\mu$ M of CAL-101, and cell inhibition ratios were measured at several time points. B: PI3K p110 $\delta$  protein levels were assessed by Western blot in T-ALL cells infected with control vector or overexpressing shRNAs for *PIK3CD*. C: In CCRF-CEM and KOPT-K1 T-ALL cell lines, cell proliferation decreased and apoptosis increased after the knockdown of *PIK3CD* by shRNA1 or shRNA2.



**Figure 6. PTEN induces miR-26b expression by altering the expression patterns of Ikaros isoforms**

**A:** In CCRF-CEM and MOLT4 cells, miR-26b levels were increased after PTEN overexpression ( $P < 0.001$  and  $P < 0.01$ , respectively). In KOPT-K1 cells, there was no change in the miR-26b level ( $P > 0.05$ ). **B:** miR-26b levels were decreased after PTEN knocked-down by shRNAs in KOPT-K1 cells ( $P < 0.001$ ). **C:** Expression of Ikaros isoforms in normal bone marrow (1 and 2), normal thymocytes (1 and 2) and T-ALL cell lines by Western blotting. **D:** miR-26b levels were elevated after Ikaros dominant positive isoform IK1 overexpression in CCRF-CEM, MOLT4, and KOPT-K1 T-ALL cells ( $P < 0.001$ ). **E:** ChIP assay: Top panel indicates the position detected by real-time quantitative PCR. Bottom panel: ChIP analysis of CCRF-CEM cells with vector control, exogenous PTEN expression and IK1-Flag overexpression. DNA immune-precipitated by Ikaros antibody, Flag-tag antibody or immunoglobulin G (IgG negative control) was amplified by qPCR. The percentage of Input was significantly elevated in exogenous PTEN expression and IK1-Flag overexpression cells ( $*P < 0.05$ ,  $**P < 0.005$ ), compared with the vector control. **F:** IK1 significantly activated firefly luciferase expression by binding to the miR-26b promoter region on co-transfected pGL3 plasmid in 293T cells compared with empty vector and the Ikaros dominant negative isoform (IK-DN) ( $*P < 0.01$ ,  $**P < 0.05$ ). IK-DN (from 10 ng to 100 ng) also significantly

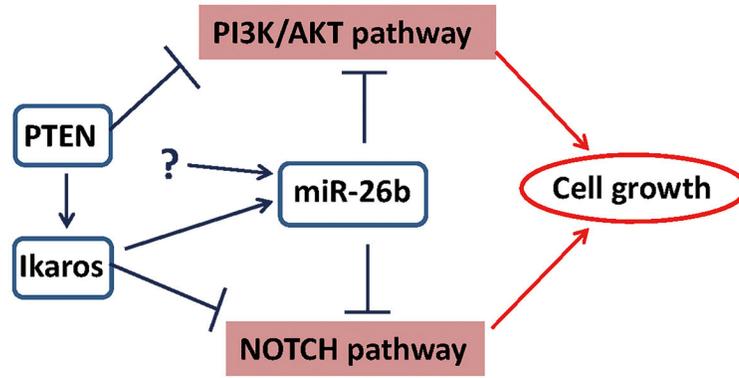
decreased the luciferase activity of IK1 co-transfected with pGL3 plasmid ( $P < 0.05$ ). G. Effect of exogenous expression on IK-DP and IK-DN. There was steady Ikaros dominant positive isoform and decreased Ikaros dominant negative isoform in CCRF-CEM and MOLT4 cells that have no endogenous PTEN expression. There was no change in the Ikaros dominant negative isoform in KOPT-K1 T-ALL cells, which have endogenous PTEN. H. Effect of knocking-down of PTEN on the expression levels of IK-DP and IK-DN. After PTEN knocked-down by shRNAs in KOPT-K1 cells, Ikaros-DN isoform was increased, but no changes of Ikaros-DP isoform. V: vector control.

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**Figure 7. miR-26b is involved in T-ALL pathogenesis as a tumor suppressor**  
 miR-26b directly inhibits *PIK3CD* in the PI3K/AKT pathway and indirectly inhibits the Notch pathway. Both pathways regulate T-ALL cell growth. Tumor suppressor PTEN inhibits the PI3K pathway and alters the splice forms of Ikaros, thus regulating the miR-26b expression level as one of its important regulators in T-ALL.