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***Hsa-mir-125b-2* is highly expressed in childhood *ETV6/RUNX1* (TEL/AML1) leukemias and confers survival advantage to growth inhibitory signals independent of p53**

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Abstract

MicroRNAs (miRNAs) regulate the expression of multiple proteins in a dose dependent manner. We hypothesized that increased expression of miRNAs encoded on chromosome 21 (chr 21) contribute to the leukemogenic role of trisomy 21. The levels of chr 21 miRNAs were quantified by qRT-PCR in four types of childhood ALL characterized by either numerical (trisomy or tetrasomy) or structural abnormalities of chr 21. Surprisingly high expression of the *hsa-mir-125b-2* cluster, consisting of three miRNAs, was identified in leukemias with the structural *ETV6/RUNX1* abnormality and not in ALLs with trisomy 21. Manipulation of *ETV6/RUNX1* expression and chromatin immunoprecipitation studies demonstrated that the high expression of the miRNA cluster is an event independent of the *ETV6/RUNX1* fusion protein. Overexpression of *hsa-mir-125b-2* conferred a survival advantage to Ba/F3 cells following IL-3 withdrawal or a broad spectrum of apoptotic stimuli through inhibition of caspase 3 activation. Conversely,

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Authors' contributions: SI, NG, VB and AB designed experiments. NG, VB, IL, MM, OW, MZ, JT, LE, NS, AN designed and performed experiments. LH analyzed data. NG, VB, AB, SI wrote the paper.

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knockdown of the *endogenous* miR-125b in the *ETV6/RUNX1* leukemia cell line REH increased apoptosis after Doxorubicin and Staurosporine treatments. P53 protein levels were not altered by miR-125b. Together these results suggest that the expression of *hsa-mir-125b-2* in *ETV6/RUNX1* ALL provides survival advantage to growth inhibitory signals in a p53 independent manner.

Keywords

Acute lymphoblastic leukemia (ALL); *ETV6/RUNX1*; TEL/AML1; microRNA

Introduction

Aberrations in chromosome 21 are commonly found in childhood ALL. The high-hyperdiploid ALL (HHD ALL)^{1, 2}, the most common type of childhood ALL with *numerical* chromosomal aberrations, is characterized by 3–4 copies of chr 21 and a variable presence of other specific chromosomal trisomies. The markedly increased incidence of ALL in children with Down Syndrome (DS)³ strongly suggests that trisomy 21 is leukemogenic, but the chr 21 genes involved in these leukemias are presently unknown. A newly discovered rare subtype of childhood ALL is characterized by an intrachromosomal amplification of a small region within the long arm of chr 21 (iAMP21) around the RUNX1 locus⁴. In contrast to *ETV6/RUNX1* and HHD ALL, it is associated with poor prognosis. The most common *structural* chromosomal aberration in childhood ALL occurring in approximately 25% of pediatric B-cell precursor ALL fuses the RUNX1 (AML1) gene on chr 21 with the ETV6 (TEL) gene on chr 12^{5, 6}. Although *ETV6/RUNX1* causes a prenatal preleukemic clonal expansion, additional genetic events are required for evolution of leukemia^{7, 8}.

MicroRNAs (miRNAs) are 20–25 nucleotide long non-coding RNAs that play a vital role in the regulation of protein expression⁹. Currently there are five validated mature miRNAs encoded on human chr 21 (<http://microrna.sanger.ac.uk> Figure 1A). *Hsa-mir-99a*, *hsa-let-7c* and *hsa-mir-125b-2* are clustered together in the same intron of the *C21ORF34* gene on 21q.21.1 (NC_000021.7) (*hsa-mir-125b-2* cluster). *Hsa-mir-125b-1*, the homologue of *C. elegans* lin-4, has been shown to be expressed in solid tumors and associated with enhanced cell proliferation and survival^{10–12}. An insertion of *hsa-mir-125b-1* into the IGH locus was described in a patient with B cell precursor ALL suggesting its involvement in the leukemic process¹³. It has been shown to be expressed in myeloid malignancies and to block myeloid differentiation¹⁴. Recently it was also reported to protect from apoptosis through negative regulation of p53 in zebrafish, human neuroblastoma cells and lung fibroblasts but not in mouse cells¹⁵. *Hsa-mir-155* has been linked to B cell development. We and others have shown its increased expression in lymphomas^{16–19}. Strikingly, transgenic expression of miR-155 induced pre-B cell leukemias and lymphomas in mice²⁰, thus miR-155 seems to be a potent oncogene.

The mechanisms of miRNA function suggest that their activity should be *correlated with their dosage* and that each miRNA may regulate multiple targets²¹. Thus we hypothesized that miRNAs contribute to the oncogenic effects of chromosomal trisomies, a situation in

which a small change ($\times 1.5$) in genomic dosage results in profound effects. This hypothesis is strengthened by our recent observations demonstrating a general increase in expression of multiple genes from the trisomic chromosomes²². Thus, we expected that miRNAs from chr 21 would be over-expressed in HHD and DS ALL (3–4 copies of chr 21) relative to *ETV6/RUNX1* and iAMP21 leukemias.

Here we report the surprising observations that unlike most of the genes encoded on chr 21, the expression of the *hsa-mir-125b-2* cluster does not correlate with gene copy number but rather is highly expressed in *ETV6/RUNX1* ALL. Additional studies suggest that the expression of the *hsa-mir-125b-2* cluster is an independent event in *ETV6/RUNX1* ALL, conferring survival advantage under growth inhibitory and apoptotic conditions in a p53 independent manner.

Materials and Methods

Patients

RNA was derived from diagnostic or remission bone marrow samples of childhood ALL patients obtained with informed consent. Diagnostic bone marrow samples contained at least 80% lymphoblasts. The samples were anonymized before shipping except for the information on the genetic subgroup. The study was approved by the IRB of the Israeli Health Ministry and Sheba Medical Center.

The rest of the material and methods are detailed in a supplementary file

Results

***Hsa-mir-125b-2* cluster is highly expressed in *ETV6/RUNX1* leukemias**

We measured the expression of the mature forms of four miRNAs encoded on chr 21 (*hsa-mir-99a*; *hsa-let-7c*; *hsa-mir-125b-2* and *hsa-mir-155*) by qRT-PCR in RNA derived from diagnostic bone marrow samples of four diverse subtypes of ALL with structural or numerical aberrations of chr 21: *ETV6/RUNX1* ALL (n=24), HHD ALL (n=10), DS ALL (n=10) and iAMP21 ALL (n=7). Surprisingly, extra copies of chr 21 did not contribute to the expression level of these miRNAs (Figure 1B). While *hsa-mir-155* is similarly expressed between the different ALLs, the other miRNAs *hsa-mir-99a*, *hsa-let-7c*, and *hsa-mir-125b-2* are up-regulated in the *ETV6/RUNX1* ALL, the subtype of leukemia with a *structural* aberration of chr 21. The expression was derived from the leukemic blasts as it was not observed in remission samples (Figure 1C). To ensure that the observed results were not due to the normalization to a single internal control (RNU 43), we have extended the miRNA profiling using TaqMan® Low Density miRNA arrays (TLDA, ABI) normalizing to two additional internal controls (RNU6 and RNU48). As can be seen in supplementary Figure 1, the TLDA results are similar to the results obtained with the singleplex miRNA expression profiling.

***Hsa-mir-125b-2* cluster is not a target of the *ETV6/RUNX1* fusion protein**

To test if *mir-125b-2* is a target of *ETV6/RUNX1* we performed overexpression experiments in two mouse hematopoietic progenitor cell systems: Ba/F3 cells and primary

mouse embryonic fetal liver progenitors It has been previously shown that ectopic expression of *ETV6/RUNX1* in these progenitors induces proliferation of B cell progenitors, similarly to the preleukemia observed in humans²³ (Supplementary Figure 3). In neither of these cells the expression level of the *mmu-mir-125b-2* cluster was affected by the fusion protein (Supplementary Figure 4). Since overexpression in mouse cells may not represent the relevant model, we have knocked down *ETV6/RUNX1* in the human REH ALL cell line with siRNA oligonucleotides (oligos) directed against the fusion part of this translocation (Supplementary Figure 5). Two third silencing efficiency of *ETV6/RUNX1* but not *RUNX1* was observed by qRT-PCR and by western blots 5 days after the first round of transfection (Supplementary Figure 6 and Figure 2A and 2B respectively). Figure 2C demonstrates that the expression of the *hsa-mir-125b-2* cluster was not influenced by the knockdown of the *ETV6/RUNX1* fusion protein. To further examine if *ETV6/RUNX1* protein binds to *RUNX1* sites on the vicinity of the *hsa-mir-125b-2* cluster we performed Chromatin Immunoprecipitation (ChIP) analysis. There are three potential *RUNX1* binding sites in the *hsa-mir-125b-2* cluster, one upstream of each miRNA (supplementary Figure 7). As a positive control we examined the promoter of granzyme B (GZMB), a *RUNX1* regulated protein²⁴. Figure 2D clearly shows that while the *RUNX1* binding site upstream granzyme B (GZMB) is occupied by the *ETV6/RUNX1* and *RUNX1* proteins, the putative *RUNX1* binding sites of the *hsa-mir-125b-2* cluster are not bound by the fusion protein.

Taken together these experiments suggest that the *hsa-mir-125b-2* cluster is *not* a direct target of *ETV6/RUNX1*, but rather an independent event occurring during leukemogenesis.

***Hsa-miR-125b-2* has a pro-survival effect under growth inhibitory conditions**

To test the hypothesis that the expression of the *hsa-mir-125b-2* cluster contributes to survival and growth of lymphoid progenitors we performed forced expression experiments in IL-3 dependent Ba/F3 cells. Transformation of these pro-B cells is commonly used to identify activating mutations of kinases²⁵. We decided to focus on *hsa-mir-125b-2* because of previous studies implicating a role of this miRNA in human cancers^{10, 14, 26, 27}. In addition the endogenous *mmu-miR-99a* and *let-7c* are expressed in Ba/F3 cells whereas *miR-125b* is not expressed. Furthermore, the sequence of the mature *miR-125b* is identical in human and mouse. We transduced Ba/F3 cells with an empty retroviral vector, a vector expressing *hsa-mir-125b-2* or a construct encoding *hsa-mir-125b-2* mutated in the seed region (see methods, supplementary Figure 8) and confirmed the expression of the mature miRNAs by northern blot analysis (Figure 3A). No differences in growth were observed under steady state conditions (not shown). However, Ba/F3 cells expressing *hsa-mir-125b-2* were highly resistant to a transient removal of IL-3. Upon reintroduction of IL-3 to the growth medium only *hsa-mir-125b2* transduced Ba/F3 cells, but not cells transduced with an empty vector or a mutated *hsa-mir-125b-2*, resumed their normal growth (Figure 3B). To examine the mechanism of resistance to IL-3 withdrawal we performed a cell cycle analysis of Ba/F3 cells transduced with the different constructs before and after IL-3 withdrawal. 24 hours after IL-3 deprivation most of the *hsa-mir-125b-2* transfected cells were arrested in G1 (71.7%) while most of the control Ba/F3 cells were apoptotic (73% in subG1 phase, Figure 4A). This relative protection from apoptosis conferred by *hsa-mir-125b-2* was evident at different time points during the first 24 hours of IL-3 deprivation (Figure 4B). The

anti-apoptotic activity of *hsa-mir-125b-2* was associated with a marked inhibition of caspase 3 activation and the cleavage of its substrate PARP (Figure 4C).

To examine how broad the anti-apoptotic phenotype observed in Ba/F3 cells over-expressing miR-125b is beyond the growth factor weaning, we exposed the cells to different apoptotic stimuli. Supplementary Figure 9 summarizes the percentage of living cells as been quantified by FACS analysis of Annexin negative and 7AAD negative staining of four different treatments. Since cytokine survival pathways involve activation of multiple kinases, we examined the effect of miR 125b on apoptosis induced by three kinase inhibitors with decreasing specificity: JAK Inhibitor I, AG490 and Staurosporine. Ba/F3 transduced with *hsa-mir-125b-2* were markedly resistant to each of these inhibitors in comparison with Ba/F3 cells transduced with mutated miR or with empty vector. Mild, but statistically significant resistance was also observed after treatment with Doxorubicin. Thus miR125b provides survival advantage in response to multiple pro-apoptotic stimuli.

To test if the survival advantage observed in Ba/F3 cells over-expressing miR-125b is relevant to *ETV6/RUNX1* leukemia, we used REH cells, a cell line derived from a patient with *ETV6/RUNX1* leukemia that highly expresses miR-125b. We knocked down the endogenous miR-125b by using LNA oligos labeled with FAM (Figure 5A) and measured the level of living cells of the FAM positive population (transfected with the LNA oligo) after treatment with either Doxorubicin or Staurosporine (see M&M). Figure 5B and C show that upon knocking down miR-125b, the REH cells become more sensitive to Staurosporine and Doxorubicin treatments respectively. These results indicate that the endogenous *mir-125b-2* provides partial protection from apoptosis induced by those agents similarly to our observation in Ba/F3 cells.

A recent publication by Le et al.¹⁵ suggested that miR-125b is a negative regulator of zebrafish and human (but not mouse) p53. We therefore asked if the effects of miR-125b on apoptosis of Ba/F3 and REH cells could be explained by its regulation of p53 protein levels. Thus, we checked the levels of p53 and its main cellular protein target, p21, both under steady state conditions and during apoptotic stress in both human and mouse models. The binding site of miR-125b on the 3'UTR of p53 is different between mouse and human particularly in the "seed" area, an area considered to be the most significant for the recognition of miRNAs and their targets (Figure 6A). Therefore, it was not surprising that in the mouse Ba/F3 model we have not observed any changes in the level of p53 neither under steady state conditions nor under apoptotic conditions of IL-3 withdrawal (data not shown) or Doxorubicin treatment (Figure 6B and C). Surprisingly, knocking down miR-125b in the human REH cells did not alter p53 protein levels or p21 levels. Furthermore, treating the REH cells with Doxorubicin resulted in similar elevation of p53 levels in the cells transfected with a control LNA oligo or a miR-125b antisense LNA oligo, suggesting that p53 is not a target of miR-125b in these cells. This is further substantiated in the lack of alteration in the levels of p21 (Figure 6D and F). Similarly there was no effect of miR-125b knockdown on p53 or p21 levels upon treatment with Staurosporine (Figure 6E and F). Thus p53 levels are not regulated by miR-125b in REH cells.

Discussion

Here, we describe that expression of the *hsa-mir-125b-2* cluster residing on chr 21 characterizes *ETV6/RUNX1* leukemias compared to other “chromosome 21” leukemias. We demonstrate that this miRNA cluster is not regulated by the *ETV6/RUNX1* fusion protein and therefore hypothesize that its expression is an independent event occurring during the evolution of these leukemias. We further demonstrate by overexpression and knockdown studies that *mir-125b-2* provides a survival advantage by suppressing apoptosis and caspase 3 activation in response to growth inhibitory conditions.

This study was prompted by the hypothesis, that increased expression of chr 21 miRNAs may explain the leukemogenic role of trisomy 21 in ALL of Down Syndrome (DS) and in sporadic HHD leukemias (which uniformly contain either 3 or 4 copies of chromosome 21). This hypothesis was based on our previous observations that trisomies are usually associated with increased expression of multiple genes from the trisomic chromosomes²² and by increased expression in DS AML of the miRNAs belonging to the *hsa-mir-125b-2* cluster²⁸. Furthermore, increased expression of all chr 21 miRNAs has been recently reported in fetal heart and fetal hippocampus of DS patients²⁹. Our findings that the expression of those miRNAs in B cell precursor ALLs was *not* correlated to gene dosage but rather to the leukemia subtype were therefore unexpected. Increased expression levels of the *hsa-mir-125b-2* cluster were observed in *ETV6/RUNX1* ALL, while *hsa-miR-155* was similarly expressed in all subtypes. Furthermore, the expression of the *hsa-mir-125b-2* cluster was specific to the leukemic cells, as it was not observed in remission samples.

ETV6/RUNX1 is the most common translocation in childhood ALL. Similarly to HHD ALL, these are B cell precursor leukemias with excellent prognosis on contemporary treatment protocols³⁰. The translocation is necessary for the initiation of a preleukemic clone but insufficient for the evolution of leukemia^{7, 8, 31}. Little is known about the acquired somatic oncogenic events that promote the progression of an *ETV6/RUNX1* preleukemic clone into a frank leukemia. The observation that the *hsa-mir-125b-2* cluster is not regulated by the *ETV6/RUNX1* fusion protein, suggests that its overexpression might be such an independent progression event.

MiRNAs have been shown to be dysregulated in cancer in tissue and cancer type-specific patterns. While one miRNA acts as an oncogene in one type of cancer, the same miRNA serves as a tumor suppressor in another. In breast cancer miR-125b has been found to suppress *ERBB2 (HER2)* and *ERBB3 (HER3)* expression²⁶ and thus functions as tumor suppressor. Conversely, a recent study suggested that miR-125b enhances growth and survival of prostate cancer and glioma cells^{12, 27}. To test the hypothesis that the increased expression of *mir-125b-2* in *ETV6/RUNX1* leukemia has a similar pro-survival role in hematopoietic cells we utilized the IL-3 dependent pro-B Ba/F3 cells. These cells are considered as a standard screening tool to identify leukemia kinase oncogenes^{25, 32-34}. Functional analysis of *hsa-mir-125b-2* in Ba/F3 cells demonstrated that this miRNA conferred growth factor independence by blocking apoptosis induced by IL3 withdrawal through delayed activation of caspase 3.

Cytokines such as IL3 promote cell survival through activation of JAK/STAT and related signal transduction pathways^{35–38}. The role of mir125b in protecting from death caused by silencing of pro-survival kinase regulated pathways is demonstrated by the relative resistance it endowed Ba/F3 cells to three kinase inhibitors in decreasing specificity from JAK inhibitor 1 to Staurosporine. However the anti-apoptotic effect of miR-125b is not limited to kinases as modest but consistent and statistically significant resistance to apoptosis was also conferred to cells treated by the DNA damaging drug Doxorubicin. The anti-apoptotic phenotype was not an artifact of miRNA overexpression in Ba/F3 cells. Its relevance to *ETV6/RUNX1* leukemias is suggested by the sensitization to apoptosis induced by Staurosporine and Doxorubicin after knockdown of the endogenous miR-125b in REH cells.

Like most of the miRNAs, the precise targets of miR-125b are unknown. MiRNAs are believed to regulate many targets and currently bioinformatic target prediction algorithms are rather limited³⁹. A recent study suggested that miR-125b reduces the level of the pro-apoptotic protein BAK1²⁷ thus blocking apoptosis. Though, we have not seen any reduction in BAK1 in either Ba/F3 or HEK293T cells over-expressing *hsa-mir-125b-2* (data not shown). We also have not detected any observable changes in several related pro-apoptotic proteins including BIM, BID, BAX and MCL-1 (data not shown). The recent report demonstrating that p53 is negatively regulated by miR-125b¹⁵ is intriguing since p53 is a major regulator of apoptosis in response to a variety of stresses including Doxorubicin. However our experiments do not support that report. Similarly to the observations by Le et al, and consistent with the lack of conservation of the miR-125b seed region in the 3'UTR region of mouse p53, we have not seen any alteration of the mouse p53 protein levels in Ba/F3 cells over-expressing miR-125b. Moreover the very efficient knockdown conferred by specific LNA oligos in REH cells did not lead to any alterations in the human p53 levels or its target p21 before or after treatment with Doxorubicin. Thus, at least in these hematopoietic cells p53 is not regulated by miR-125b.

Two provoking recent proteomic studies demonstrate that single miRNAs induce very small alterations in a large number of proteins, and affect functional pathways by acting as sensitive “Rheostats”^{40, 41}. While such small alterations putatively induced by miR-125b in proteins that regulate cell survival may not be significant under steady state conditions, they could provide growth advantage during periods of growth factor deprivation.

The pathogenesis of *ETV6/RUNX1* leukemias has been a mystery. *In vivo* modeling of *ETV6/RUNX1* leukemias by many laboratories has been proven experimentally challenging and resulting at most in a preleukemic condition³¹. A recent analysis of a “knock-in” mouse model in which *ETV6/RUNX1* was expressed from the *ETV6* promoter has demonstrated an increase in hematopoietic stem cells (HSC). Intriguingly the HSC expressing the fusion protein were markedly more sensitive to apoptosis induced by cytokine withdrawal⁴². We propose that *hsa-mir-125b-2* and possibly the two other miRNAs in this cluster may collaborate with *ETV6/RUNX1* in the leukemogenic process by providing survival advantage under growth inhibitory conditions. It would be interesting to test this hypothesis in that mouse model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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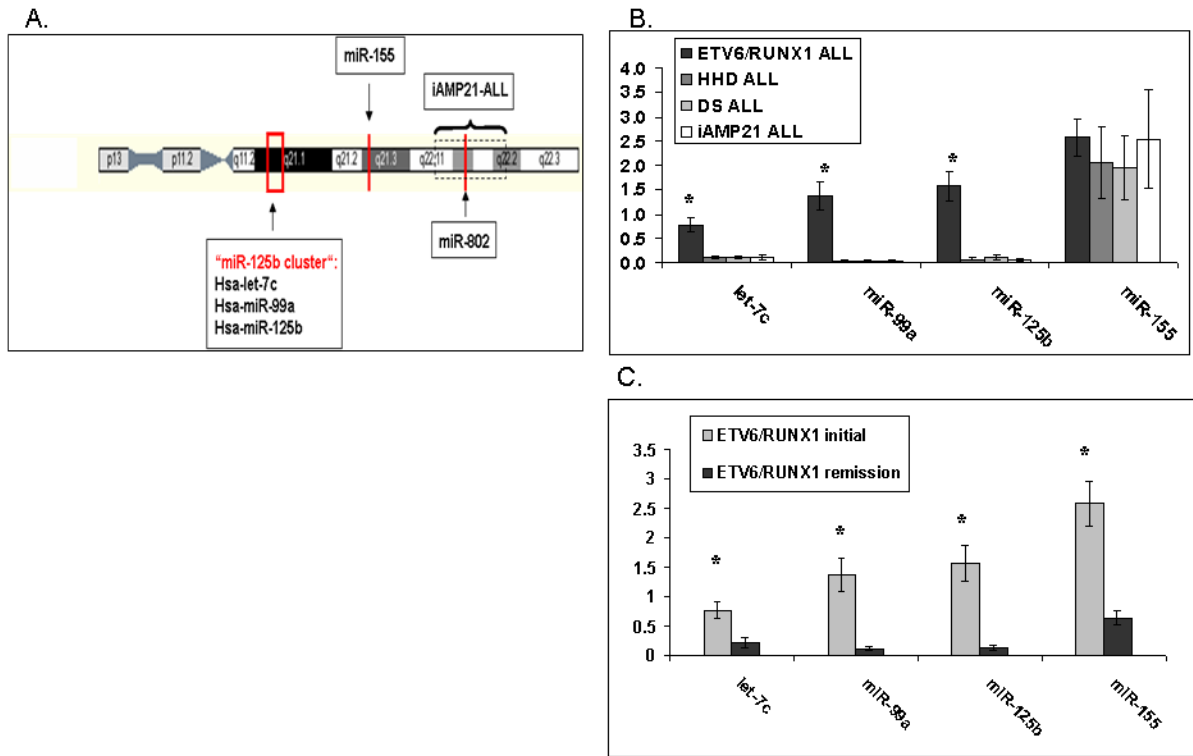


Figure 1. Expression of miRNAs encoded on Chromosome 21 in different leukemia samples
(A) Schematic representation of the location of miRNAs encoded on chr 21. The level of the mature hsa-miR-802 could not be measured because of the lack of an appropriate qRT-PCR based assay. **(B–C)** MiRNA expression was determined by qRT-PCR using the TaqMan® *MicroRNA Assays* from *Applied Biosystems*. The numbers on the Y axis represent relative expression levels of mature miRNA normalized to an internal control (RNU43). Standard errors are indicated. Asterisk marks significance (T-TEST). **(B)** Expression of the chr 21 miRNAs in diagnostic bone marrow samples of different ALL subtypes: *ETV6/RUNX1* (24 patients), Down Syndrome (DS) (10 patients), High Hyperdiploid (10 patients), iAMP21 (7 patients) P<0.05 (ANOVA). **(C)** Comparison of miRNA expression between diagnosis and remission samples of *ETV6/RUNX1* leukemias P<0.005 (T-TEST).

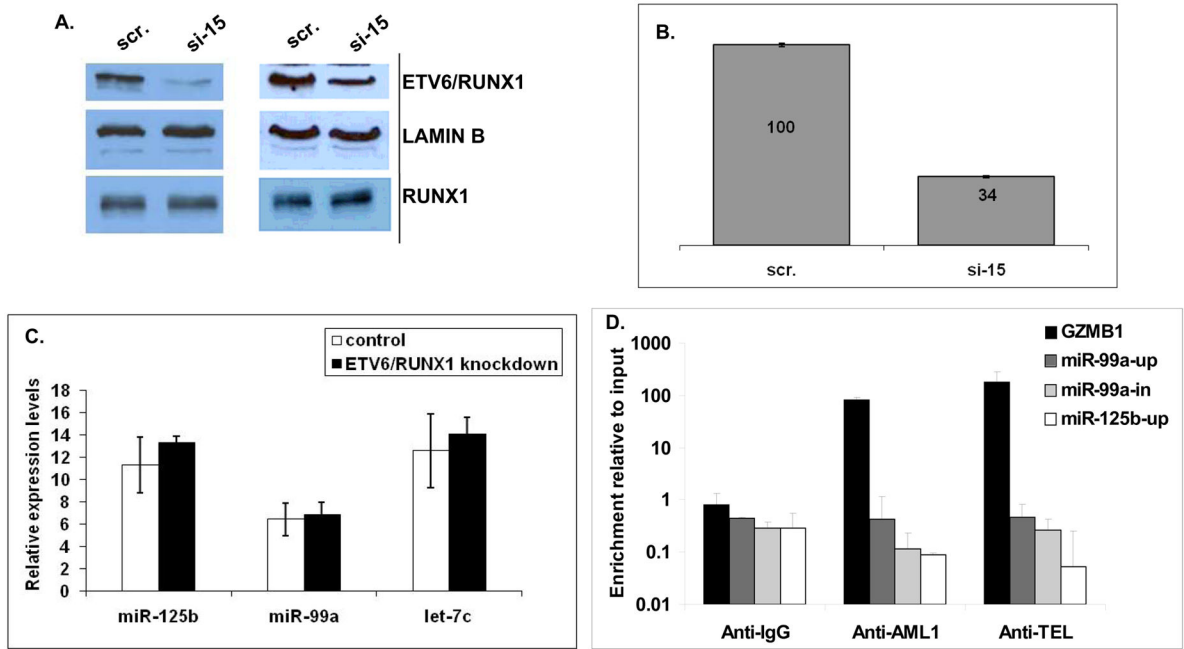


Figure 2. The *hsa-mir-125b-2* cluster is not a target of the *ETV6/RUNX1* fusion protein shown by knockdown of *ETV6/RUNX1* in REH cells

REH cells were transfected with siRNA directed against the fusion part of the *ETV6* and *RUNX1* genes (see methods). (A) Western blot analysis of *ETV6/RUNX1* knockdown in REH cells: Scr- scramble siRNA, 15- mixture of siRNAs 1 and 5. Two independent experiments are shown (B) A histogram showing quantification of the average *ETV6/RUNX1* protein level as detected by two independent western blots, determined by the ImageJ software (C) No significant change in expression levels of the *hsa-mir-125b-2* cluster could be observed in REH cells after knockdown of *ETV6/RUNX1* as determined by qRT-PCR. An average of three independent experiments is shown. Expression levels are normalized to an internal control (RNU43). (D) QRT-PCR of ChIP analysis with anti-*ETV6*, anti-*RUNX1*, anti-IgG (served as negative control) or anti-TBP (served as positive control – see M&M). Granzyme B (GZMB) was used as positive control for the binding of *RUNX1*. The Y-axis indicates the enrichment normalized to GAPDH from sample and input. Three independent experiments were performed. Standard errors are indicated.

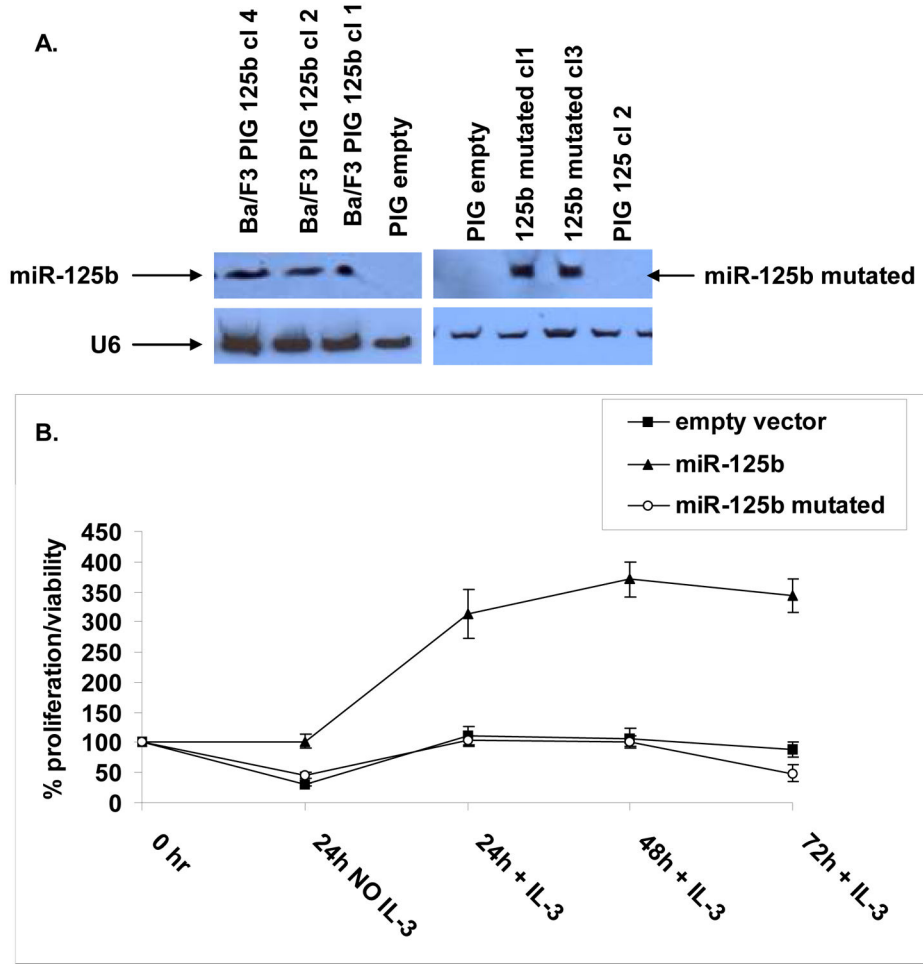


Figure 3. *Hsa-mir-125b-2* confers growth advantage in Ba/F3 cells after transient IL-3 deprivation
 Ba/F3 cells were transduced with an empty vector, a vector expressing *mir-125b-2* or mutated *mir-125b-2*. (A) Northern blot analysis showing representative clones over-expressing *hsa-mir-125b-2* or mutated *hsa-mir-125b-2*. (B) Ba/F3 cells transduced with either empty vector, a vector expressing *hsa-mir-125b-2* or mutated *hsa-mir-125b-2* were cultured in the absence of IL-3 for 24 hours. After 24 hours IL-3 was added to the medium and cell proliferation was measured by MTT every 24 hours. The graph shows an average of at least three different clones and three experiments. Standard errors are indicated. Experiments were repeated 5 times.

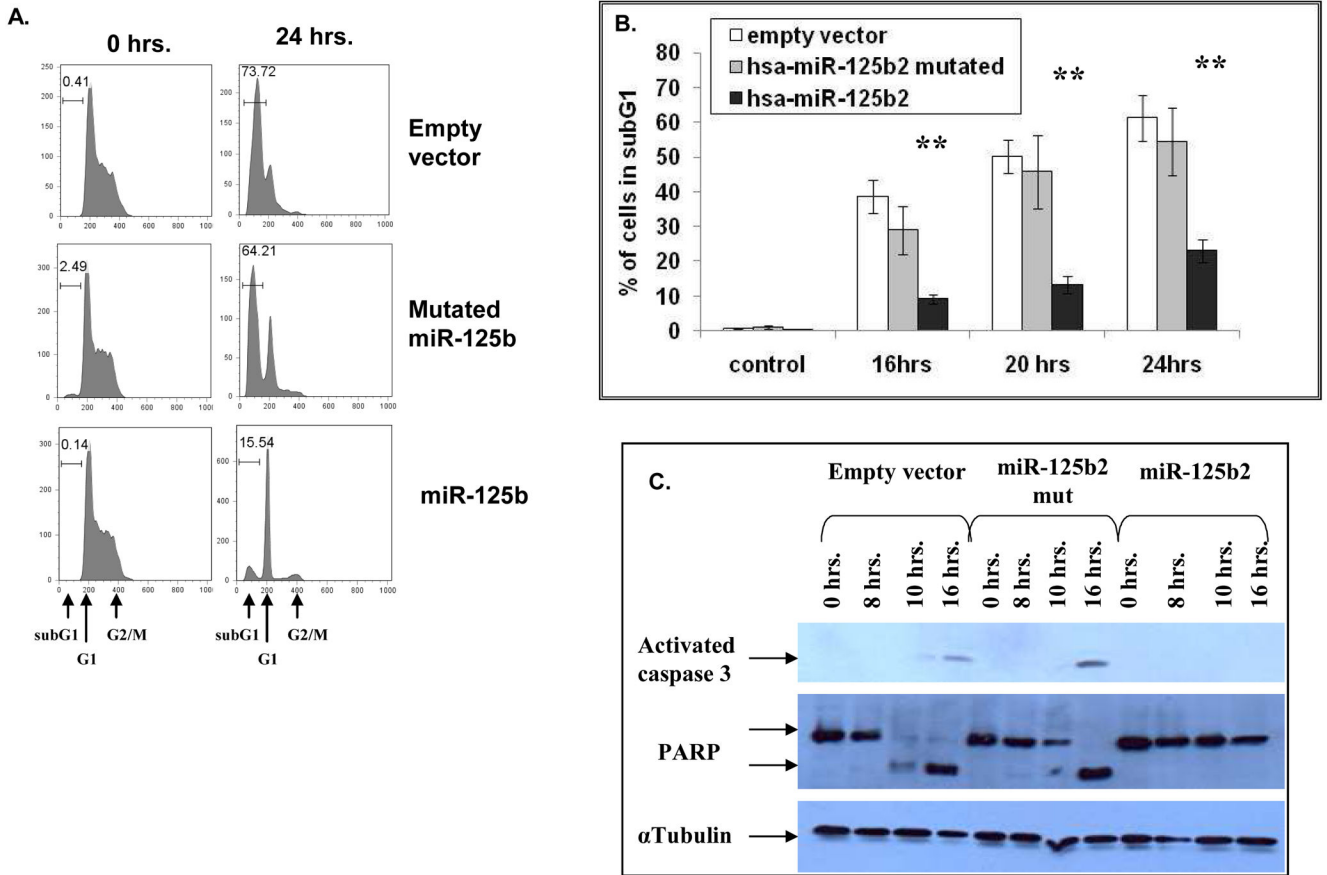


Figure 4. *Hsa-mir-125b-2* has an anti-apoptotic effect in Ba/F3 cells

(A and B) Quantification of apoptosis by measuring the subG1 fraction of Ba/F3 cells stained with Propidium Iodide, comparing wildtype cells, cells over-expressing *hsa-mir-125b-2* and cells over-expressing mutated *mir-125b-2*. (A) A representative example before (time 0) and 24 hours after IL-3 withdrawal. (B) Kinetics of apoptosis with time after IL-3 withdrawal. Asterisks marks significance of $p < 0.005$ as calculated by ANOVA. Standard errors are indicated. (C) Western blot analysis showing lack of activation of caspase 3 and cleavage of PARP following removal of IL-3 in Ba/F3 cells over-expressing *hsa-mir-125b-2* in comparison to wildtype Ba/F3 and those over-expressing mutated *hsa-mir-125b-2*. Experiments were repeated 5 times.

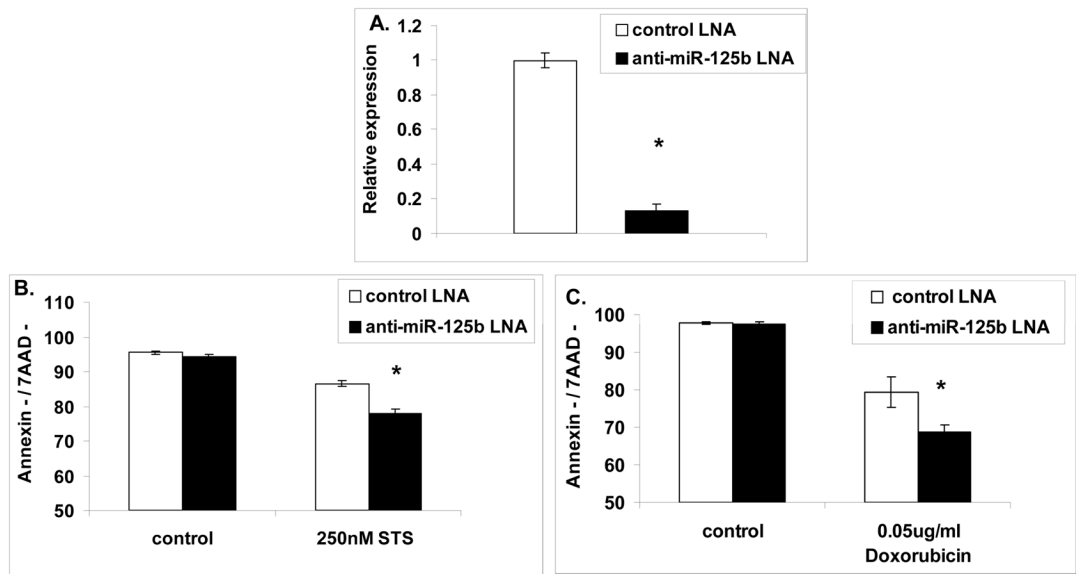


Figure 5. The endogenous hsa-miR-125b protects *ETV6/RUNX1* human leukemia cells from apoptosis

(A) QRT-PCR analysis of the knockdown efficiency of hsa-miR-125b in REH cells using a specific miR-125b antisense LNA oligo or a control oligo. Y axis represents relative expression to the internal control RNU43. Eight independent experiments are shown. (B and C) Knockdown of miR-125b in REH cells increases the sensitivity to Staurosporine or Doxorubicin treatments respectively as indicated by decreased percentage of living cells (Annexin/7AAD negative). Data are means \pm SD of four independent experiments for each treatment. * $P < 0.05$ as calculated by T-TEST.

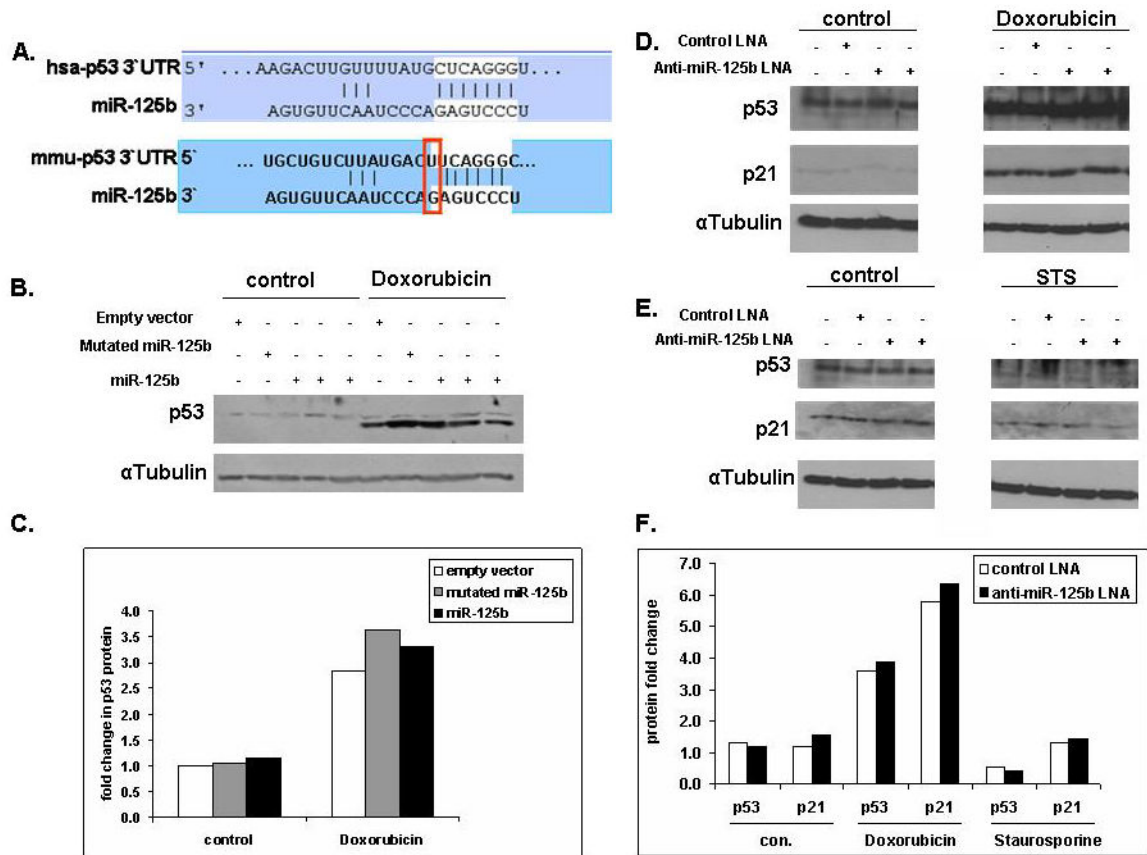


Figure 6. P53 is not a target of miR-125b in Ba/F3 and REH cells

(A) MiR-125b binding site in the 3'UTR of the human and mouse p53 as predicted by TargetScan. Marked in white is the "seed" area of miR-125b, also marked is the single different nucleotide in the potential miR-125b binding site in the 3'UTR of the human and mouse p53. (B) Overexpression of miR-125b in mouse Ba/F3 cells does not down-regulate *endogenous* p53 levels. A representative western blot of *endogenous* p53 in Ba/F3 cells transduced with either the empty vector or the vector expressing mutated miR-125b or miR-125b. The cells were treated for 4 hours with 0.2 μ g/ml Doxorubicin. (C) Quantification of p53 protein level determined by western blot using the ImageJ software, normalized to α Tubulin and presented as fold change relative to the level of p53 in Ba/F3 cells transduced with an empty vector. (D and E) *Endogenous* human p53 and p21 level following *endogenous* miR-125b knockdown in the human REH cells. Following the knockdown the cells were treated with either 0.05 μ g/ml Doxorubicin (D) or Staurosporine (E). (F) Quantification of p53 and p21 protein levels determined by western blot using the ImageJ software, normalized to α Tubulin level and presented as fold change relative to p53 or p21 levels respectively in REH cells transfected with a control LNA oligo.