Published in final edited form as:

Cancer Lett. 2016 March 28; 372(2): 157–165. doi:10.1016/j.canlet.2015.12.032.

Identification of MLL-fusion/MYC–miR-26–TET1 signaling circuit in *MLL*-rearranged leukemia

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Abstract

Expression of functionally important genes is often tightly regulated at both transcriptional and post-transcriptional levels. We reported previously that TET1, the founding member of the TET methylcytosine dioxygenase family, plays an essential oncogenic role in *MLL*-rearranged acute myeloid leukemia (AML) where it is overexpressed owing to *MLL*-fusion-mediated direct up-regulation at the transcriptional level. Here we show that the overexpression of *TET1* in *MLL*-rearranged AML also relies on the down-regulation of miR-26a, which directly negatively regulates *TET1* expression at the post-transcriptional level. Through inhibiting expression of *TET1* and its downstream targets, forced expression of miR-26a significantly suppresses the growth/viability of human *MLL*-rearranged AML cells, and substantially inhibits *MLL*-fusion-mediated mouse hematopoietic cell transformation and leukemogenesis. Moreover, c-Myc, an oncogenic transcription factor up-regulated in *MLL*-rearranged AML, mediates the suppression of miR-26a expression at the transcriptional level. Collectively, our data reveal a previously unappreciated signaling pathway involving the *MLL*-fusion/MYC–miR-26a–TET1 signaling circuit, in which miR-26a functions as an essential tumor-suppressor mediator and its transcriptional repression is required for the overexpression and oncogenic function of *TET1* in *MLL*-rearranged AML. Thus, restoration of miR-26a expression/function holds therapeutic potential to treat *MLL*-rearranged AML.

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CONFLICT OF INTEREST STATEMENT

NONE

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Keywords

MLL-rearranged leukemia; miR-26a; *TET1*; *MYC*; post-transcription regulation

1. INTRODUCTION

Ten-eleven translocation (Tet) proteins (including Tet1/2/3) are a family of methylcytosine dioxygenases that can convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [1; 2], resulting in active or passive DNA demethylation [3–5]. *TET1*, the founding member of the *TET* gene family, was first identified as a fusion partner of the mixed lineage leukemia (*MLL*) gene associated with t(10;11)(q22;q23) in acute myeloid leukemia (AML) [6; 7]. The critical feature of *MLL*-rearrangements is the generation of a chimeric transcript consisting of 5' *MLL* and 3' sequences of a partner gene [8–10]. While approximately 80% of *MLL*-fusion genes in AML involving *AF9*, *AF6*, *AF10*, *ELL* or *ENL*, the *MLL-TET1* rearrangement is very rare in AML [6–11]. In recent years, substantial down-regulation of all three *TET* genes has been reported in various solid tumors [12–16]. Thus, one may expect that all three *TET* genes are tumor suppressors in various cancers. Indeed, *TET1* was shown to be an essential tumor suppressor in prostate and breast cancers [17; 18], and *TET2* was recognized as a tumor suppressor with frequent loss-of-function mutations in myeloid malignancies (but rarely in *MLL*-rearranged leukemia) [19–24].

Surprisingly, we recently found that *TET1* (but not *TET2/TET3*) was significantly up-regulated in *MLL*-rearranged AML samples compared to normal hematopoietic cell controls [25]. We subsequently showed that wild-type *MLL* and especially *MLL*-fusion proteins bind to the promoter region of *TET1* and promote its expression at the transcriptional level directly in both human and mouse hematopoietic stem/progenitor cells, which is associated with an increased level of 5hmC [25]. Furthermore, depletion of *Tet1* expression by small hairpin RNAs (shRNAs) or genetic knockout significantly inhibits *MLL-AF9*-mediated mouse hematopoietic cell transformation *in vitro* and leukemogenesis *in vivo* [25]. In addition, knockdown of *TET1* expression by small interfering RNA (siRNA) oligos significantly decreases viability/growth and increases apoptosis of human *MLL-AF9* leukemic cells [25]. We further revealed that several essential downstream direct target genes of *MLL* fusions, such as *HOXA9*, *MEIS1*, and *PBX3* that have been shown to be critical for the development, maintenance and leukemia stem cells (LSCs) self-renewal of *MLL*-rearranged leukemia [8; 26–38], are also direct target genes of *TET1* in *MLL*-rearranged leukemia [25]. Consistent with our observation that *TET1* plays an oncogenic role in *MLL*-rearranged AML, Xu and colleagues reported very recently that while *Tet2* knockout caused relatively rapid myeloid malignancies, knockout of *Tet1* in *Tet2* knockout mice substantially decreased the incidence and delayed the onset of myeloid malignancies [39], further demonstrating the essential oncogenic function of *TET1* in the pathogenesis of myeloid malignancies. Therefore, giving the functional importance of *TET1*, it is possible that the expression of *TET1* might be carefully controlled at both the transcriptional and post-transcriptional levels in normal cells, and disruption of such control could be important for *TET1* to maintain a high expression level and thereby exert its critical oncogenic role in myeloid malignancies such as *MLL*-rearranged AML.

MicroRNAs (miRNAs), a family of small, non-coding RNAs that post-transcriptionally negatively regulate expression of their targets, play critical regulatory roles in virtually all bioprocesses in normal development [40] and have been implicated in the pathogenesis of various types of cancers, including leukemia [33; 41–47]. Given the fact that most mammalian mRNAs are conserved targets of miRNAs [48], *TET1* is highly likely also under negative regulation of miRNAs at the post-transcriptional level. However, whether and (if so) which miRNAs regulate expression of *TET1* in leukemia is unclear.

In order to effectively maintain the high expression level of *TET1* in *MLL*-rearranged AML [25], it would be important for the cancer cells to diminish miRNA-mediated negative regulation on *TET1* expression. Here we show that *TET1* is a direct target gene of miR-26a, and the repression of miR-26a expression, which is likely owing to the negative regulation mediated by *MLL*-fusion proteins and MYC, is essential for *TET1* to maintain a high expression level in *MLL*-rearranged AML cells. Forced expression of miR-26a significantly decreased expression of *Tet1* and exhibited *MLL*-fusion-mediated cell transformation and leukemogenesis. The inhibitory effect of miR-26a on the growth and viability of human *MLL*-rearranged AML cells can be reversed by co-expressed *Tet1* or *Hoxa9*, a downstream target of Tet1. Collectively, our data suggest that miR-26a, a miRNA significantly down-regulated in *MLL*-rearranged AML cells due to the negative regulation mediated by *MLL*-fusion proteins and MYC, plays a critical tumor-suppressor role in the pathogenesis of *MLL*-rearranged leukemia by directly targeting *Tet1* and its downstream signaling.

2. MATERIALS AND METHODS

2.1 Exiqon microRNA expression profiling assays

As described previously [47; 49; 50], miRNA expression profiling assays of a cohort of AML samples along with normal control samples were performed by Exiqon (Woburn, MA) using the miRCURY LNA arrays (v10.0; covering 757 human miRNAs). The quantified signals were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm [51].

2.2 Cell culture and transfection

MONOMAC-6 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% HEPES, 2 mM L-Glutamine, 100×Non-Essential Amino Acid (Invitrogen), 1 mM sodium pyruvate, 9 µg/ml insulin (Invitrogen) and 1% penicillin-streptomycin. Plasmids were transfected into MONOMAC-6 cells with Cell Line Nucleofector Kit V following program T-027 using the Amaxa® Nucleofector® Technology (Amaxa Biosystems, Berlin, Germany). Experiments were performed 48 hours after transfection.

The *MLL*-ENL-ERTm cell line was kept in RPMI 1640 supplemented with interleukin 3 (IL-3), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/ml; SCF 100 ng/ml; 10% FBS and 1% penicillin-streptomycin. 4-Hydroxy-tamoxifen (4-OHT) (Sigma-Aldrich, St. Louis, MO) was added at a 100 nM final concentration as a 1 mM stock solution in ethanol.

Retrovirus was used to transduce MLL-ENL-ERTm cells. Retrovirus for each construct was produced in 293T cells by co-transfecting the retroviral construct and pCL-Eco packaging vector (IMGENEX, San Diego, CA) as previously described[52; 53]. Rat1a cells were used to determine the viral titer. BM progenitor Cells were co-transduced with MSCV-neo vector or MSCV-neo-MLL-AF9 together with MSCV-PIG vector or MSCV-PIG-miR-26a, or MSCV-PIG-miR-29a constructs, respectively, through “spinoculation”.

2.3 Cloning

As described previously [25], the DNA sequence of *Tet1*, encoding the mouse *Tet1* gene C-terminal 673 amino acids including both the CXXC domain and the catalytic domain (based on NM_027384 and GU079948) was synthesized by GenScript Corp. (Piscataway,NJ), and then ligated into a retroviral vector, namely MSCVpuro at XhoI/ EcoRI site. The miR-26a and miR-29a precursors were amplified by PCR using primers: miR-26a forward: 5'-AATGAATTCTGGCATAGCAAGAAT-3', reverse: 5'-ACACTCGAGACAAGACTCCTCGTT-3', and miR-29a forward: 5'-AATATCTCGAGGCCTGGGT TAAAGA-3', reverse: 5'-GATCTGAATTCTATTGACTCCCT CGCT-3', and were subsequently cloned into the XhoI and EcoRI sites of the retrovirus vector MSCV-PIG. The 3'UTR of *TET1* containing putative binding sites for miR-26a was amplified by PCR using the primers: 3'UTR-1 forward: 5'-ATAACTAGTCCCTCTTAATGCCTTT GCTAGT-3', reverse: 5'-ATAAAGCTTACTGCAGTTAACAAGATGGAACT-3', and 3'UTR-2 forward: 5'-ATAACTAGTTTCCCTACTATCATCACATGCCT-3', reverse: 5'-ATAAAGCTTTTGAAGCAG CTGAAGCAAT-3'. Their relative positions (refer to NCBI reference sequence NM_030625.2) are 3'UTR-1: 6928 ~ 7668, and 3'UTR-2: 8081 ~ 8852. These fragments were cloned into a luciferase reporter plasmid (pMIR-REPORTER, Ambion) at SpeI/HindIII sites. The mutant of TET1-3'UTR-1 was synthesized by GenScript Corp based on the sequence shown in Figure 4a, and then was ligated into a luciferase reporter plasmid.

2.4 Apoptosis and cell viability assays

MONOMAC6 cells were transfected with miR-26a, miR-29a, miR-26a+Tet1, miR-26a+Hoxa9, or Control Vector with 2 million cells in 2 ml of medium. 48 hours after transfection, cells were collected and seeded with requested concentration. Cell apoptosis and viability were assessed using ApoLive-Glo Multiplex Assay Kit (Promega, Madison, WI) following the corresponding manufacturer's manuals.

2.5 Cell proliferation/growth assays

Two million of MONOMAC6 cells were electroporated with miR-26a, miR-29a, miR-26a+Tet1, miR-26a+Hoxa9, miR-29a+TET1, miR-29a+Hoxa9, or Control Vector. 24 hours after transfection, cells were seeded into 96-well plates at the concentration of 10000 cells/well in triplicates. Cell numbers were counted at the indicated days.

2.6 RNA Extraction and Quantitative RT-PCR analysis

Total RNAs were isolated using the miRNeasy kit (Qiagen, Valencia, CA). For mRNA expression, 200 ng RNA was reverse transcribed into cDNA in a total reaction volume 10 μ l with the Qiagen's RT kit according to the manufacturer's instructions. And, quantitative real-time PCR analysis was performed with 0.5 μ l cDNA using SYBR green PCR master mix (Qiagen, Valencia, CA) in an AB 7900HT instrument (Applied Biosystems, Foster City, CA). GAPDH was used as endogenous control. All of samples were run with triplication.

2.7 Immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and ruptured with RIPA buffer (Pierce, Rockford, IL) containing 5 mM EDTA, PMSF, cocktail inhibitor, and phosphatase inhibitor cocktail. Cell extracts were microcentrifuged for 20 min at 10000 x g and supernatants were collected. Cell lysates (20 μ l) were resolved by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at 4 °C with anti-HOXA9 antibody (Millipore, Billerica, MA) or anti-Tet1 (Y-14) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or 1 hour at room temperature with anti-Pgk1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Membranes were washed 30 min with Tris-buffered saline containing 0.1% Tween 20, incubated for 1 hour with appropriate secondary antibodies conjugated to horseradish peroxidase, and developed using chemiluminescence substrates.

2.8 *In vitro* colony forming and replating assays

Hematopoietic progenitor cells (i.e., Lin⁻) were obtained from a cohort of 6-weeks-old B6.SJL (CD45.1) mice five days after 5-FU treatment (150 mg/kg) using the Mouse Lineage Cell Depletion Kit (Miltenyi Biotec Inc., Auburn, CA), and were then co-transduced with MSCV-PIG+MSCVneo (negative control), MSCV-PIG+MSCVneo-MLL-AF9 (positive control), MSCV-PIG-miR-26a+MSCVneo-MLL-AF9, and MSCV-PIG-miR-29a+MSCVneo-MLL-AF9 through "spinoculation". Then, two aliquots of 2×10^4 of the infected cells were plated into two 35 mm Nunc petri dishes in 1.5 ml of Methocult M3231 methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) containing 10 ng/ml each of murine recombinant IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis, MN) and 30 ng/ml of murine recombinant stem cell factor (Sandoz, Holzkirchen, Germany), along with 1.0 mg/ml of G418 (Gibco BRL, Gaithersburg, MD) and 2.5 μ g/ml of puromycin (Sigma, St. Louis, MO). Cultures were incubated at 37° C in a humidified atmosphere of 5% CO₂ in air for 6–7 days. Cells were collected and replated with two aliquots of 2×10^4 of colony cells into two dishes every 7 days up to three or four passages.

2.9 Primary Bone Marrow Transplantation (i.e., *in vivo* reconstitution) assay

For primary bone marrow transplantation (BMT) assay, donor cells were prepared from B6.SJL (CD45.1) mice. To select double-transduction-positive cells, we conducted colony-forming assay first. MSCV-PIG+MSCVneo (negative control), MSCV-PIG+MSCVneo-MLL-AF9 (positive control), MSCV-PIG-miR-26a+MSCVneo-MLL-AF9, and MSCV-

PIG-miR-29a+MSCVneo-MLL-AF9 transduced cells were collected from first passage of colony-forming assay, and after washing with PBS twice, the cells were transplanted *via* tail vein injection into lethally irradiated (960 rads, 96 rads/min, γ -rays) 8–10-week-old C57BL/6(CD45.2) recipient mice. For each recipient mouse, a total of 0.25×10^6 donor cells and a radioprotective dose of whole bone marrow cells (1×10^6) freshly harvested from a C57BL/6 mouse were transplanted.

2.10 Histopathology

Leukemic mice were euthanized by CO₂ inhalation if they showed signs of systemic illness, and some negative control recipient mice were also sacrificed at some time points (though they did not develop leukemia) to collect specimens as controls for further analyses. Portions of the spleen and liver were collected and fixed in formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). BM Cells were isolated from the tibia and femur. 50,000 cells were washed twice and were diluted in 200 μ l of cold MACS Buffer. Each sample was loaded into the appropriate well of the cytospin, and then spun at 2000 rpm for 2 min. Blood smear and BM cytospin slides were stained with Wright-Giemsa.

2.11 Genomic DNA extraction

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.12 5-hmC labelling reaction and dot-blot assay

The 5-hmC labelling reactions were performed in a 20- μ l solution containing 50 mM HEPES buffer (pH 7.9), 25 mM MgCl₂, 3 μ g sonicated genomic DNA (100–500 bp), 100 μ M UDP-6-N₃-Glc, and 1 μ M β -GT. The reactions were incubated for 1 hour at 37 °C. After the reaction, the DNA substrates were purified with DNA purification kit (Qiagen) and eluted in H₂O. The click chemistry was performed with addition of 150 μ M dibenzocyclooctyne modified biotin into the DNA solution, and the reaction mixture was incubated for 2 hours at 37 °C. The DNA samples were then purified by DNA purification kit (Qiagen). 600 ng labeled genomic DNA sample was spotted on an Amersham Hybond-N⁺ membrane (GE Healthcare). DNA was fixed to the membrane by Stratagene UV Stratalinker 2400 (auto-crosslink). The membrane was then blocked with 5% BSA and incubated with Avidin-HRP (1:40,000) (Bio-Rad, Hercules, CA), which was visualized by enhanced chemiluminescence. Quantification was calculated using a working curve generated by 1–8 ng of 32 bp synthetic biotin-5-N₃-gmC-containing DNA.

2.13 Statistical software

The miRNA array data analysis, as well as qPCR data analyses were conducted by use of Partek Genomics Suite (Partek Inc, St. Louis, MI), The t-test, Kaplan-Meier method, and log-rank test, etc. were performed with WinSTAT (R. Fitch Software) and/or Partek Genomics Suite (Partek Inc, St. Louis, MI).

3. RESULTS

3.1 miR-26 and miR-29 are significantly down-regulated in human MLL-rearranged AML and are potential miRNA regulators of TET1

Because expression of *TET1* is significantly up-regulated in *MLL*-rearranged AML [25], we presumed that its critical miRNA regulators might be down-regulated in *MLL*-rearranged AML. Thus, we sought to identify miRNAs that are potential regulators of *TET1* and significantly down-regulated in *MLL*-rearranged AML. As described previously [47; 49; 50], we have conducted miRNA expression profiling assays of a large cohort of AML samples along with normal control samples by use of Exiqon miRNA microarrays. With the miRNA expression profiling data, here we compared expression of 527 individual miRNAs between 10 primary *MLL*-rearranged AML samples and 6 normal bone marrow (BM) CD34⁺ hematopoietic stem/progenitor cell (HSPC) samples using a statistic method, namely SAM (Significant Analysis of Microarrays) [54]. As shown in Figure 1A, a total of 11 individual miRNAs (including miR-26a, miR-26b, miR-29a, miR-29b, miR-150, miR-376a, miR-495, miR-585, miR-612, miR-638 and miR-768-3p) are significantly down-regulated in the *MLL*-rearranged AML samples related to the normal CD34⁺ HSPC samples. Amongst these 11 down-regulated miRNAs, only miR-26a, miR-26b, miR-29a and miR-29b are predicted by all five major miRNA-target prediction algorithms, including TargetScan [55], miRanda [56], Pictar [57], miRDB [58] and miRWalk [59], as potential miRNA regulators of *TET1*. Therefore, we focused on these four miRNAs for further studies.

3.2 Expression of miR-26 and miR-29 is repressed by MLL-fusion proteins

As miR-26a/b and miR-29a/b are significantly down-regulated in human *MLL*-rearranged primary AML cells, we sought to investigate whether their expression is affected by *MLL*-fusion proteins. To this end, we retrovirally transduced MSCVneo-*MLL-AF9*, MSCVneo-*MLL-AF10*, or MSCVneo-*MLL-ENL* into mouse BM progenitor cells and transduction-positive cells were selected by G418 in methycellulose medium as described previously [25; 46; 47]. After 7 days of selection, colony cells were collected and expression of miR-26a/b and miR-29a/b were detected by quantitative PCR (qPCR). As shown in Figure 1B, forced expression of *MLL-AF9*, *MLL-AF10*, and *MLL-ENL* all caused a significant down-regulation of miR-26a/b and miR-29a/b in expression. Furthermore, we also conducted a loss-of-function assay by use of a *MLL-ENL*-estrogen receptor inducible (ERTm) mouse myeloid cell line carrying tamoxifen-inducible *MLL-ENL* [28; 60]. As shown in Figure 1C, after withdrawal of 4-hydroxytamoxifen (4-OHT), while expression of *MLL-ENL* was significantly decreased during a period of 10 days, expression of miR-26a/b and miR-29a/b was steadily and significantly increased. Taken together, these data suggest that *MLL*-fusion proteins negatively regulate expression of both miR26 and miR-29 family members in hematopoietic cells.

3.3 miR-26a and miR-29a significantly inhibit the growth and viability of human MLL-rearranged AML cells

To investigate the pathological function of miR-26 and miR-29 in *MLL*-rearranged AML, we cloned miR-26a and miR-29a, as the representatives of the miR-26 family and miR-29 family, respectively, into MSCV-PIG, a retroviral vector containing GFP and puromycin-

resistant elements [42]. We then transfected MSCV-PIG-miR-26a, MSCV-PIG-miR-29a or empty MSCV-PIG plasmid into MONOMAC-6 cells, a human AML cell line carrying t(9;11)/*MLL-AF9*. As shown in Figure 2A, forced expression of miR-29a and especially miR-26a significantly and consistently inhibited the growth of MONOMAC-6 cells from day 4 post-transfection. Accordingly, overexpression of miR-26a and miR-29a also significantly inhibited the viability of MONOMAC-6 cells (Fig. 2B). Conversely, overexpression of miR-29a and especially miR-26a significantly promoted apoptosis in MONOMAC-6 cells (Fig. 2C).

3.4 miR-26a significantly inhibits MLL-AF9-induced cell transformation in vitro and leukemogenesis in vivo

We next performed *in vitro* colony-forming/replating assays (CFA) and *in vivo* mouse BM transplantation (BMT) assays to investigate the roles of miR-26a and miR-29a in cell transformation and leukemogenesis induced by MLL-fusion proteins. In the *in vitro* CFA assays, we found that forced expression of miR-29a and especially miR-26a significantly inhibited the colony-forming capacity of normal mouse BM progenitor (lineage negative; Lin-) cells induced by MLL-AF9 after replating (Fig. 3A). Through the *in vivo* mouse BMT assays, we showed that forced expression of miR-26a significantly inhibited leukemogenesis mediated by MLL-AF9, whereas miR-29a exhibited no significant inhibitory effect on MLL-AF9-induced leukemogenesis (Fig. 3B). Forced expression of miR-26a, but not miR-29a, substantially diminished the leukemic phenotype induced by MLL-AF9 in various tissues, showing a significant decrease in the proportion of immature AML blast cells in peripheral blood (PB) and BM, as well as a better maintenance of structure and less leukemic invasion in spleen and liver tissues (Fig. 3C). Taken together, these data suggest that miR-26a, but not miR-29a, exhibits a significant inhibitory effect on MLL-fusion-induced leukemogenesis. Thus, repression of miR-26a is likely a required event in the pathogenesis of *MLL*-rearranged AML.

3.5 miR-26a directly targets Tet1 and its downstream signaling in MLL-rearranged AML cells

Given the functional importance of miR-26a in *MLL*-rearranged AML as demonstrated in the above functional studies, we next investigated whether *TET1* is a direct target gene of miR-26a in *MLL*-rearranged AML. First, we cloned two 3'UTR fragments of *TET1* that contain different putative target sites of miR-26a, into luciferase reporter vectors, and named them as TET1-3'UTR-1 and TET1-3'UTR-2. Then, luciferase reporter assays were conducted. As shown in Figure 4A, forced expression of miR-26a significantly repressed luciferase activity of TET1-3'UTR-1, but not that of TET1-3'UTR-2, suggesting that TET1-3'UTR-1, but not TET1-3'UTR-2, contains a genuine target site of miR-26a. To confirm this, we also cloned a mutant of TET1-3'UTR-1, namely TET1-3'UTR-Mut, in which the predicted target site of miR-26a was mutated. As expected, miR-26a exhibited no inhibitory effect on luciferase activity of TET1-3'UTR-Mut (Fig. 4A), demonstrating that this target site is a genuine one for miR-26a. Consistent with the data that *TET1* is a direct target gene of miR-26a, we showed that when the MLL-ENL-ERTm cells were cultured without 4-OHT, expression of miR-26a and *Tet1* was steadily up-regulated and down-regulated, respectively (Fig 4B).

As one important function of TET1 is converting 5mC to 5hmC, we next investigated whether forced expression of miR-26a could inhibit 5hmC level in *MLL*-rearranged AML cells. Our Dot blot analysis showed that while the 5hmC level was significantly up-regulated by forced expression of *MLL*-AF9, largely due to the up-regulation of Tet1 [25], co-expressed miR-26a thoroughly reversed the up-regulation of 5hmC level (Fig. 4C). Thus, our data suggests that miR-26a directly targets Tet1, and thereby affects the global 5hmC level in leukemic cells.

We reported previously that Tet1 directly mediates the transcriptional up-regulation of a set of target genes including *Hoxa* genes, *Meis1*, *Pbx3*, and *Flt3* in *MLL*-rearranged leukemia cells [25]. As expected, we found that forced expression of miR-26a could significantly inhibit expression of Tet1 and its downstream target genes in *MLL*-AF9-transduced colony-forming cells (Fig. 4D). Similarly, forced expression of miR-26a significantly inhibited expression of *Tet1* (but not *Tet2*) and *Hoxa9* in *MLL*-AF9-induced mouse AML cells as detected by qPCR and Western blotting (Figs. 4E and F).

To investigate whether *Tet1* is a functionally important target gene of miR-26 in *MLL*-rearranged leukemic cells, we co-transfected *Tet1* or *Hoxa9*, a downstream target gene of Tet1, with miR-26a into human *MLL*-rearranged AML cells, and then performed cell proliferation, apoptosis, and viability assay. As expected, co-transfection of *Tet1* or *Hoxa9* with miR-26a could largely reverse the effects of miR-26a on cell growth/proliferation (Fig. 5A), viability (Fig. 5B) and apoptosis (Fig. 5C) of MONOMAC-6 AML cells. Collectively, our data suggests that TET1 and its downstream targets are essential (direct or indirect) targets of miR-26a, and the repression of miR-26a is required for their up-regulation in *MLL*-rearranged AML cells.

3.6 Myc mediates the transcriptional repression of miR-26a

We have shown previously that Myc, a critical downstream target of *MLL*-fusion proteins that is up-regulated in *MLL*-rearranged AML cells [47; 61], plays an essential role in inhibiting the expression of miR-150 [47]. Interestingly, while Myc can promote the primary transcription of miR-150, it recruits Lin28 to inhibit the maturation of miR-150, leading to the significant down-regulation of mature miR-150 in AML cells [47]. To analyze the effect of Myc on miR-26a expression, we collected BM mononuclear cells from wild-type and *Myc*^{-/-} mice, and then analyzed expression levels of the primary, precursor, and mature miR-26a, as well as that of its direct target gene, i.e., *Tet1*, by q-PCR assay. As shown in Figure 6A, knockout of *Myc* resulted in a significant increase in expression of immature (pri-miR-26a and pre-miR-26a) and mature miR-26a, associated with a significant decrease in *Tet1* expression. To assess the effect of MYC on miR-26a expression in *MLL*-rearranged AML cells, we transfected *MYC* siRNA oligos (siMYC) into human MONOMAC-6 AML cells. As expected, we found that knockdown of endogenous expression of *MYC* in MONOMAC-6 cells resulted in a significant increase and decrease in expression of miR-26a and *TET1*, respectively (Fig. 6B). Thus, our data suggests that Myc is a critical mediator that represses expression of miR-26a at the transcriptional level and thereby highly likely contributes to the down-regulation of miR-26a in *MLL*-rearranged AML cells.

4. DISCUSSION

In the present study, we show that miR-26a/b and miR-29a/b, putative miRNA regulators of *TET1*, are significantly down-regulated in *MLL*-rearranged AML, in which *TET1* is significantly up-regulated. While both miR-26a and miR-29a exhibit significant inhibitory effects on the growth and viability of human *MLL*-rearranged AML cells *in vitro*, only miR-26a shows a significant inhibitory effect on the development of *MLL*-fusion-mediated AML *in vivo*. Our luciferase reporter and mutagenesis assays confirmed that *TET1* is a direct target gene of miR-26a, and our further regulatory and functional studies demonstrated that *TET1* and its downstream targets are functionally important targets of miR-26a in *MLL*-rearranged AML. We also show that MYC likely participates in the repression of miR-26a through inhibiting its primary transcription in *MLL*-rearranged AML. Collectively, our data uncovers an important signaling pathway involving the *MLL*-fusion/MYC-miR-26a-*TET1* signaling circuit in *MLL*-rearranged leukemia (see Fig. 6C).

In different types of cancers, miR-26a has been reported to be either up-regulated or down-regulated, and exhibits either an oncogenic or a tumor-suppressor function [62–68], suggesting the expressional regulation and pathological function of miR-26a are cell context dependent. A set of target genes of miR-26a, such as *HGMA1* [62], *EZH2* [63], *MCL-1* [64], *FGF9* [65] and *GSK-GSK-3 β* [67], have been reported previously. In our studies, we show that *TET1* is a *bona fide* target gene of miR-26a in *MLL*-rearranged AML. Interestingly, a recent study also reported that miR-26a targets *TET1* in normal pancreatic cells, and that miR-26a and *Tet1* are up-regulated and down-regulated, respectively, during murine pancreatic cell differentiation [69]. Thus, our data together with Fu et al.'s study [69] suggest that the direct regulation of *TET1* expression by miR-26a is a conserved regulatory mode that exists in different tissues and in both cancer and normal cell development, further highlighting the functional importance of this post-transcriptional regulation.

We reported previously that MYC mediated the down-regulation of miR-150 in various subtypes of AML, especially in *MLL*-rearranged AML, through inhibiting the maturation process of miR-150 while promoting its primary transcription [47]. Here we show that Myc likely directly inhibits the primary transcription of miR-26a. Thus, the present study together with our previous study [47] suggest that Myc may exert distinct mechanisms to suppress expression of different tumor-suppressor miRNAs in AML. In addition, our data also suggests that Myc can positively regulate expression of *TET1* and enhance its downstream signaling through repressing expression of miR-26a, a critical negative regulator of *TET1* in *MLL*-rearranged AML.

In summary, our data suggest that the down-regulation of miR-26a is likely a required event for the overexpression and oncogenic function of *TET1* in *MLL*-rearranged AML, in which miR-26a exhibits a significant anti-tumor effect. *MLL*-rearranged AML is usually associated with poor prognosis and the majority of the patients cannot be cured by standard chemotherapy [8; 10; 60; 70–75]. Thus, it is urgent to develop effective novel therapeutic strategies to treat this presently therapy-resistant disease. Given the potent oncogenic role of *TET1* and its downstream signaling in *MLL*-rearranged AML [25] and the minor side effect of *Tet1* knockout on normal hematopoiesis [76], restoration of the expression/function of

miR-26a holds great therapeutic potential to treat *MLL*-rearranged AML. Various types of nanoparticles have been developed to deliver miRNA oligos to target leukemic cells (e.g. [77–79]). Thus, employing miR-26a-based nanoparticles, alone or in combination with other therapeutic agents, may represent an effective novel therapeutic strategy to treat *MLL*-rearranged AML, a type of dismal cancer that is resistant to contemporary therapies. Overall, our data not only provides novel insight into our understanding of the complex molecular mechanisms underlying the pathogenesis of *MLL*-rearranged leukemia, but may also lead to the development of novel, more effective therapeutic strategies to treat this type of dismal disease.

Acknowledgments

The authors thank Drs. Gregory Hannon, Scott Hammond, Lin He, and Scott Armstrong for providing retroviral constructs. This work was supported in part by the National Institutes of Health (NIH) R01 Grants CA178454 and CA182528 (J.C.), Leukemia & Lymphoma Society (LLS) Translational Research Grant (J.C.), American Cancer Society (ACS) Research Scholar grant (J.C.), Gabrielle's Angel Foundation for Cancer Research (J.C., H.H., X.J. and Z.L.), The University of Chicago Committee on Cancer Biology (CCB) Fellowship Program (X.J.), and LLS Special Fellowship (Z.L.).

Abbreviations

<i>MLL</i>	mixed lineage leukemia
AML	acute myeloid leukemia
qRT-PCR	quantitative real-time polymerase chain reaction
ChIP	chromatin immunoprecipitation
BM	bone marrow
BMT	bone marrow transplantation
PB	peripheral blood
SP	spleen
SAM	Significance Analysis of Microarrays
Lin⁻	lineage-negative

References

1. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by *MLL* partner TET1. *Science*. 2009; 324:930–935. [PubMed: 19372391]
2. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*. 2010; 466:1129–1133. [PubMed: 20639862]
3. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science*. 2011; 333:1303–1307. [PubMed: 21817016]
4. Guo JU, Su Y, Zhong C, Ming GL, Song H. Hydroxylation of 5-Methylcytosine by TET1 Promotes Active DNA Demethylation in the Adult Brain. *Cell*. 2011

5. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011; 333:1300–1303. [PubMed: 21778364]
6. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y. LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). *Cancer Res*. 2002; 62:4075–4080. [PubMed: 12124344]
7. Lorsch RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). *Leukemia*. 2003; 17:637–641. [PubMed: 12646957]
8. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat Rev Cancer*. 2007; 7:823–833. [PubMed: 17957188]
9. Popovic R, Zeleznik-Le NJ. MLL: how complex does it get? *J Cell Biochem*. 2005; 95:234–242. [PubMed: 15779005]
10. Slany RK. The molecular biology of mixed lineage leukemia. *Haematologica*. 2009; 94:984–993. [PubMed: 19535349]
11. Meyer C, Hofmann J, Burmeister T, Groger D, Park TS, Emerenciano M, Pombo de Oliveira M, Renneville A, Villarese P, Macintyre E, Cave H, Clappier E, Mass-Malo K, Zuna J, Trka J, De Braekeleer E, De Braekeleer M, Oh SH, Tsaur G, Fechina L, van der Velden VH, van Dongen JJ, Delabesse E, Binato R, Silva ML, Kustanovich A, Aleinikova O, Harris MH, Lund-Aho T, Juvonen V, Heidenreich O, Vormoor J, Choi WW, Jarosova M, Kolenova A, Bueno C, Menendez P, Wehner S, Eckert C, Talmant P, Tondeur S, Lippert E, Launay E, Henry C, Ballerini P, Lapillone H, Callanan MB, Cayuela JM, Herbaux C, Cazzaniga G, Kakadiya PM, Bohlander S, Ahlmann M, Choi JR, Gameiro P, Lee DS, Krauter J, Cornillet-Lefebvre P, Te Kronnie G, Schafer BW, Kubetzko S, Alonso CN, zur Stadt U, Sutton R, Venn NC, Izraeli S, Trakhtenbrot L, Madsen HO, Archer P, Hancock J, Cerveira N, Teixeira MR, Lo Nigro L, Moricke A, Stanulla M, Schrappe M, Sedek L, Szczepanski T, Zwaan CM, Coenen EA, van den Heuvel-Eibrink MM, Strehl S, Dworzak M, Panzer-Grumayer R, Dingermann T, Klingebiel T, Marschalek R. The MLL recombinome of acute leukemias in 2013. *Leukemia*. 2013; 27:2165–2176. [PubMed: 23628958]
12. Guo JU, Su Y, Zhong C, Ming GL, Song H. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell*. 2011; 145:423–434. [PubMed: 21496894]
13. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget*. 2011; 2:627–637. [PubMed: 21896958]
14. Kudo Y, Tateishi K, Yamamoto K, Yamamoto S, Asaoka Y, Ijichi H, Nagae G, Yoshida H, Aburatani H, Koike K. Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. *Cancer Sci*. 2012; 103:670–676. [PubMed: 22320381]
15. Yang H, Liu Y, Bai F, Zhang JY, Ma SH, Liu J, Xu ZD, Zhu HG, Ling ZQ, Ye D, Guan KL, Xiong Y. Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene*. 2013; 32:663–669. [PubMed: 22391558]
16. Lian CG, Xu Y, Ceol C, Wu F, Larson A, Dresser K, Xu W, Tan L, Hu Y, Zhan Q, Lee CW, Hu D, Lian BQ, Kleffel S, Yang Y, Neiswender J, Khorasani AJ, Fang R, Lezcano C, Duncan LM, Scolyer RA, Thompson JF, Kakavand H, Houvras Y, Zon LI, Mihm MC Jr, Kaiser UB, Schatton T, Woda BA, Murphy GF, Shi YG. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell*. 2012; 150:1135–1146. [PubMed: 22980977]
17. Hsu CH, Peng KL, Kang ML, Chen YR, Yang YC, Tsai CH, Chu CS, Jeng YM, Chen YT, Lin FM, Huang HD, Lu YY, Teng YC, Lin ST, Lin RK, Tang FM, Lee SB, Hsu HM, Yu JC, Hsiao PW, Juan LJ. TET1 Suppresses Cancer Invasion by Activating the Tissue Inhibitors of Metalloproteinases. *Cell Rep*. 2012; 2:568–579. [PubMed: 22999938]
18. Sun M, Song CX, Huang H, Frankenberger CA, Sankarasharma D, Gomes S, Chen P, Chen J, Chada KK, He C, Rosner MR. HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis. *Proc Natl Acad Sci USA*. 2013; 110:9920–9925. [PubMed: 23716660]
19. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lecluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C,

- Romana SP, Dessen P, Soulier J, Viguie F, Fontenay M, Vainchenker W, Bernard OA. Mutation in TET2 in myeloid cancers. *N Engl J Med*. 2009; 360:2289–2301. [PubMed: 19474426]
20. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, Malinge S, Yao J, Kilpivaara O, Bhat R, Huberman K, Thomas S, Dolgalev I, Heguy A, Paietta E, Le Beau MM, Beran M, Tallman MS, Ebert BL, Kantarjian HM, Stone RM, Gilliland DG, Crispino JD, Levine RL. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood*. 2009; 114:144–147. [PubMed: 19420352]
 21. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*. 2010; 468:839–843. [PubMed: 21057493]
 22. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, Do Cruzeiro M, Delhommeau F, Arnulf B, Stern MH, Godley L, Opolon P, Tilly H, Solary E, Duffourd Y, Dessen P, Merle-Beral H, Nguyen-Khac F, Fontenay M, Vainchenker W, Bastard C, Mercher T, Bernard OA. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell*. 2011; 20:25–38. [PubMed: 21723201]
 23. Li Z, Cai X, Cai CL, Wang J, Zhang W, Petersen BE, Yang FC, Xu M. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood*. 2011; 118:4509–4518. [PubMed: 21803851]
 24. Moran-Crusio K, Reavie L, Shih A, Abdel-Wahab O, Ndiaye-Lobry D, Lobry C, Figueroa ME, Vasanthakumar A, Patel J, Zhao X, Perna F, Pandey S, Madzo J, Song C, Dai Q, He C, Ibrahim S, Beran M, Zavadil J, Nimer SD, Melnick A, Godley LA, Aifantis I, Levine RL. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell*. 2011; 20:11–24. [PubMed: 21723200]
 25. Huang H, Jiang X, Li Z, Li Y, Song CX, He C, Sun M, Chen P, Gurbuxani S, Wang J, Hong GM, Elkahoul AG, Arnovitz S, Wang J, Szulwach K, Lin L, Street C, Wunderlich M, Dawlaty M, Neilly MB, Jaenisch R, Yang FC, Mulloy JC, Jin P, Liu PP, Rowley JD, Xu M, He C, Chen J. TET1 plays an essential oncogenic role in MLL-rearranged leukemia. *Proc Natl Acad Sci USA*. 2013; 110:11994–11999. [PubMed: 23818607]
 26. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, Xia X, Jesneck J, Bracken AP, Silverman LB, Kutok JL, Kung AL, Armstrong SA. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. *Cancer Cell*. 2008; 14:355–368. [PubMed: 18977325]
 27. Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR, Armstrong SA. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*. 2006; 442:818–822. [PubMed: 16862118]
 28. Zeisig BB, Milne T, Garcia-Cuellar MP, Schreiner S, Martin ME, Fuchs U, Borkhardt A, Chanda SK, Walker J, Soden R, Hess JL, Slany RK. Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization. *Mol Cell Biol*. 2004; 24:617–628. [PubMed: 14701735]
 29. Kumar AR, Li Q, Hudson WA, Chen W, Sam T, Yao Q, Lund EA, Wu B, Kowal BJ, Kersey JH. A role for MEIS1 in MLL-fusion gene leukemia. *Blood*. 2009; 113:1756–1758. [PubMed: 19109563]
 30. Wong P, Iwasaki M, Somervaille TC, So CW, Cleary ML. Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. *Genes Dev*. 2007; 21:2762–2774. [PubMed: 17942707]
 31. Li Z, Zhang Z, Li Y, Arnovitz S, Chen P, Huang H, Jiang X, Hong GM, Kunjamma RB, Ren H, He C, Wang CZ, Elkahoul AG, Valk PJ, Dohner K, Neilly MB, Bullinger L, Delwel R, Lowenberg B, Liu PP, Morgan R, Rowley JD, Yuan CS, Chen J. PBX3 is an important cofactor of HOXA9 in leukemogenesis. *Blood*. 2013; 121:1422–1431. [PubMed: 23264595]
 32. Ayton PM, Cleary ML. Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. *Genes Dev*. 2003; 17:2298–2307. [PubMed: 12952893]
 33. Chen J, Odenike O, Rowley JD. Leukaemogenesis: more than mutant genes. *Nat Rev Cancer*. 2010; 10:23–36. [PubMed: 20029422]

34. Chen W, Kumar AR, Hudson WA, Li Q, Wu B, Staggs RA, Lund EA, Sam TN, Kersey JH. Malignant transformation initiated by MLL-AF9: gene dosage and critical target cells. *Cancer Cell*. 2008; 13:432–440. [PubMed: 18455126]
35. Faber J, Krivtsov AV, Stubbs MC, Wright R, Davis TN, van den Heuvel-Eibrink M, Zwaan CM, Kung AL, Armstrong SA. HOXA9 is required for survival in human MLL-rearranged acute leukemias. *Blood*. 2009; 113:2375–2385. [PubMed: 19056693]
36. Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell*. 2006; 10:257–268. [PubMed: 17045204]
37. Somervaille TC, Matheny CJ, Spencer GJ, Iwasaki M, Rinn JL, Witten DM, Chang HY, Shurtleff SA, Downing JR, Cleary ML. Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. *Cell Stem Cell*. 2009; 4:129–140. [PubMed: 19200802]
38. Wei J, Wunderlich M, Fox C, Alvarez S, Cigudosa JC, Wilhelm JS, Zheng Y, Cancelas JA, Gu Y, Jansen M, Dimartino JF, Mulloy JC. Microenvironment determines lineage fate in a human model of MLL-AF9 leukemia. *Cancer Cell*. 2008; 13:483–495. [PubMed: 18538732]
39. Zhao Z, Chen L, Dawlaty MM, Pan F, Weeks O, Zhou Y, Cao Z, Shi H, Wang J, Lin L, Chen S, Yuan W, Qin Z, Ni H, Nimer SD, Yang FC, Jaenisch R, Jin P, Xu M. Combined Loss of Tet1 and Tet2 Promotes B Cell, but Not Myeloid Malignancies, in Mice. *Cell Rep*. 2015; 13:1692–1704. [PubMed: 26586431]
40. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004; 5:522–531. [PubMed: 15211354]
41. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature*. 2005; 435:834–838. [PubMed: 15944708]
42. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature*. 2005; 435:828–833. [PubMed: 15944707]
43. Marcucci G, Radmacher MD, Maharry K, Mrozek K, Ruppert AS, Paschka P, Vukosavljevic T, Whitman SP, Baldus CD, Langer C, Liu CG, Carroll AJ, Powell BL, Garzon R, Croce CM, Koltitz JE, Caligiuri MA, Larson RA, Bloomfield CD. MicroRNA expression in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008; 358:1919–1928. [PubMed: 18450603]
44. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov*. 2010; 9:775–789. [PubMed: 20885409]
45. Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. *Blood*. 2011; 117:1121–1129. [PubMed: 21045193]
46. Li Z, Huang H, Chen P, He M, Li Y, Arnovitz S, Jiang X, He C, Hyjek E, Zhang J, Zhang Z, Elkahoul A, Cao D, Shen C, Wunderlich M, Wang Y, Neilly MB, Jin J, Wei M, Lu J, Valk PJ, Delwel R, Lowenberg B, Le Beau MM, Vardiman J, Mulloy JC, Zeleznik-Le NJ, Liu PP, Chen J. miR-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumour suppressor in MLL-rearranged leukaemia. *Nat Commun*. 2012; 2:688. [PubMed: 22353710]
47. Jiang X, Huang H, Li Z, Li Y, Wang X, Gurbuxani S, Chen P, He C, You D, Zhang S, Wang J, Arnovitz S, Elkahoul A, Price C, Hong GM, Ren H, Kunjamma RB, Neilly MB, Matthews JM, Xu M, Larson RA, Le Beau MM, Slany RK, Liu PP, Lu J, Zhang J, Chen J. Blockade of miR-150 maturation by MLL-fusion/MYC/LIN-28 is required for MLL-associated leukemia. *Cancer Cell*. 2012; 22:524–535. [PubMed: 23079661]
48. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009; 19:92–105. [PubMed: 18955434]
49. Jiang X, Huang H, Li Z, He C, Li Y, Chen P, Gurbuxani S, Arnovitz S, Hong GM, Price C, Ren H, Kunjamma RB, Neilly MB, Salat J, Wunderlich M, Slany RK, Zhang Y, Larson RA, Le Beau MM, Mulloy JC, Rowley JD, Chen J. miR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia. *Proc Natl Acad Sci USA*. 2012; 109:19397–19402. [PubMed: 23132946]

50. Chen P, Price C, Li Z, Li Y, Cao D, Wiley A, He C, Gurbuxani S, Kunjamma RB, Huang H, Jiang X, Arnovitz S, Xu M, Hong GM, Elkahoul AG, Neilly MB, Wunderlich M, Larson RA, Le Beau MM, Mulloy JC, Liu PP, Rowley JD, Chen J. miR-9 is an essential oncogenic microRNA specifically overexpressed in mixed lineage leukemia-rearranged leukemia. *Proc Natl Acad Sci USA*. 2013; 110:11511–11516. [PubMed: 23798388]
51. Berger JA, Hautaniemi S, Jarvinen AK, Edgren H, Mitra SK, Astola J. Optimized LOWESS normalization parameter selection for DNA microarray data. *BMC Bioinformatics*. 2004; 5:194. [PubMed: 15588297]
52. Li Z, Lu J, Sun M, Mi S, Zhang H, Luo RT, Chen P, Wang Y, Yan M, Qian Z, Neilly MB, Jin J, Zhang Y, Bohlander SK, Zhang DE, Larson RA, Le Beau MM, Thirman MJ, Golub TR, Rowley JD, Chen J. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc Natl Acad Sci U S A*. 2008; 105:15535–15540. [PubMed: 18832181]
53. Mi S, Li Z, Chen P, He C, Cao D, Elkahoul A, Lu J, Pelloso LA, Wunderlich M, Huang H, Luo RT, Sun M, He M, Neilly MB, Zeleznik-Le NJ, Thirman MJ, Mulloy JC, Liu PP, Rowley JD, Chen J. Aberrant overexpression and function of the miR-17-92 cluster in MLL-rearranged acute leukemia. *Proc Natl Acad Sci U S A*. 2010; 107:3710–3715. [PubMed: 20133587]
54. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA*. 2001; 98:5116–5121. [PubMed: 11309499]
55. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
56. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in *Drosophila*. *Genome Biol*. 2003; 5:R1. [PubMed: 14709173]
57. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet*. 2005; 37:495–500. [PubMed: 15806104]
58. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Research*. 2015; 43:D146–152. [PubMed: 25378301]
59. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nature Methods*. 2015; 12:697. [PubMed: 26226356]
60. Mueller D, Garcia-Cuellar MP, Bach C, Buhl S, Maethner E, Slany RK. Misguided transcriptional elongation causes mixed lineage leukemia. *PLoS Biol*. 2009; 7:e1000249. [PubMed: 19956800]
61. Zeisig BB, Schreiner S, Garcia-Cuellar MP, Slany RK. Transcriptional activation is a key function encoded by MLL fusion partners. *Leukemia*. 2003; 17:359–365. [PubMed: 12592336]
62. Lin Y, Chen H, Hu Z, Mao Y, Xu X, Zhu Y, Wu J, Li S, Mao Q, Zheng X, Xie L. miR-26a inhibits proliferation and motility in bladder cancer by targeting HMGA1. *FEBS Lett*. 2013; 587:2467–2473. [PubMed: 23796420]
63. Yu L, Lu J, Zhang B, Liu X, Wang L, Li SY, Peng XH, Xu X, Tian WD, Li XP. miR-26a inhibits invasion and metastasis of nasopharyngeal cancer by targeting EZH2. *Oncol Lett*. 2013; 5:1223–1228. [PubMed: 23599767]
64. Gao J, Li L, Wu M, Liu M, Xie X, Guo J, Tang H. MiR-26a inhibits proliferation and migration of breast cancer through repression of MCL-1. *PLoS One*. 2013; 8:e65138. [PubMed: 23750239]
65. Deng M, Tang HL, Lu XH, Liu MY, Lu XM, Gu YX, Liu JF, He ZM. miR-26a suppresses tumor growth and metastasis by targeting FGF9 in gastric cancer. *PLoS One*. 2013; 8:e72662. [PubMed: 24015269]
66. Huse JT, Brennan C, Hambardzumyan D, Wee B, Pena J, Rouhanifard SH, Sohn-Lee C, le Sage C, Agami R, Tuschl T, Holland EC. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev*. 2009; 23:1327–1337. [PubMed: 19487573]
67. Zhang J, Han C, Wu T. MicroRNA-26a promotes cholangiocarcinoma growth by activating beta-catenin. *Gastroenterology*. 2012; 143:246–256 e248. [PubMed: 22484120]
68. Suh JH, Choi E, Cha MJ, Song BW, Ham O, Lee SY, Yoon C, Lee CY, Park JH, Lee SH, Hwang KC. Up-regulation of miR-26a promotes apoptosis of hypoxic rat neonatal cardiomyocytes by

- repressing GSK-3 β protein expression. *Biochem Biophys Res Commun.* 2012; 423:404–410. [PubMed: 22664106]
69. Fu X, Jin L, Wang X, Luo A, Hu J, Zheng X, Tsark WM, Riggs AD, Ku HT, Huang W. MicroRNA-26a targets ten eleven translocation enzymes and is regulated during pancreatic cell differentiation. *Proc Natl Acad Sci USA.* 2013; 110:17892–17897. [PubMed: 24114270]
 70. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med.* 1999; 341:1051–1062. [PubMed: 10502596]
 71. Rowley JD. Chromosomal translocations: revisited yet again. *Blood.* 2008; 112:2183–2189. [PubMed: 18779403]
 72. Chen CS, Sorensen PH, Domer PH, Reaman GH, Korsmeyer SJ, Heerema NA, Hammond GD, Kersey JH. Molecular rearrangements on chromosome 11q23 predominate in infant acute lymphoblastic leukemia and are associated with specific biologic variables and poor outcome. *Blood.* 1993; 81:2386–2393. [PubMed: 8481519]
 73. Rowley JD. Seminars from the University of Minnesota. Chromosome translocations: dangerous liaisons. *J Lab Clin Med.* 1998; 132:244–250. [PubMed: 9794694]
 74. Slany RK. When epigenetics kills: MLL fusion proteins in leukemia. *Hematol Oncol.* 2005; 23:1–9. [PubMed: 16118769]
 75. Muntean AG, Hess JL. The pathogenesis of mixed-lineage leukemia. *Annu Rev Pathol.* 2012; 7:283–301. [PubMed: 22017583]
 76. Dawlaty MM, Ganz K, Powell BE, Hu YC, Markoulaki S, Cheng AW, Gao Q, Kim J, Choi SW, Page DC, Jaenisch R. Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. *Cell Stem Cell.* 2011; 9:166–175. [PubMed: 21816367]
 77. Dorrance AM, Neviani P, Ferencak GJ, Huang X, Nicolet D, Maharry KS, Ozer HG, Hoellbauer P, Khalife J, Hill EB, Yadav M, Bolon BN, Lee RJ, Lee LJ, Croce CM, Garzon R, Caligiuri MA, Bloomfield CD, Marcucci G. Targeting leukemia stem cells in vivo with antagomiR-126 nanoparticles in acute myeloid leukemia. *Leukemia.* 2015; 29:2143–2153. [PubMed: 26055302]
 78. Huang X, Schwind S, Yu B, Santhanam R, Wang H, Hoellbauer P, Mims A, Klisovic R, Walker AR, Chan KK, Blum W, Perrotti D, Byrd JC, Bloomfield CD, Caligiuri MA, Lee RJ, Garzon R, Muthusamy N, Lee LJ, Marcucci G. Targeted delivery of microRNA-29b by transferrin-conjugated anionic lipopolyplex nanoparticles: a novel therapeutic strategy in acute myeloid leukemia. *Clin Cancer Res.* 2013; 19:2355–2367. [PubMed: 23493348]
 79. Biray Avci C, Ozcan I, Balci T, Ozer O, Gunduz C. Design of polyethylene glycol-polyethylenimine nanocomplexes as non-viral carriers: mir-150 delivery to chronic myeloid leukemia cells. *Cell Biol Int.* 2013; 37:1205–1214. [PubMed: 23881828]

HIGHLIGHTS

- miR-26a is significantly down-regulated in *MLL*-rearranged AML
- miR-26a plays a critical anti-leukemic function both *in vitro* and *in vivo*
- *TET1* is a direct target gene of miR-26a in *MLL*-rearranged AML
- *TET1*'s overexpression and oncogenic role in *MLL* AML rely on suppression of miR-26a
- MYC mediates the transcriptional suppression of miR-26a in *MLL*-rearranged AML

Figure 1. Expression of miR-26a/b and miR-29a/b is significantly down-regulated *MLL* in-rearranged AML

(A) Expression profiles of miRNAs that are significantly down-regulated in human *MLL*-rearranged AML samples relative to human normal CD34⁺ bone marrow (BM) hematopoietic stem/progenitor cell (HSPC) samples as detected by Exiqon miRNA microarray assays. Expression data is mean centered and the relative value for each sample is represented by a color, high expression is represented with red and low expression is represented with green (scale shown in the upper left). MLL, *MLL*-rearranged AML; NC, normal control. (B) Quantitative PCR (qPCR) analysis of expression levels of miR-26a, miR-26b, miR-29a, and miR-29b in colony-forming cells of mouse BM progenitor cells retrovirally transduced with individual *MLL*-fusion genes (i.e., MSCVneo-*MLL-AF9*, MSCVneo-*MLL-AF10*, or MSCVneo-*MLL-ENL*), or the control construct (i.e., MSCVneo). (C) Relative expression levels of miR-26a, miR-26b, miR-29a, miR-29b and *MLL-ENL* in MLL-ENL-ERTm cells before and after withdrawal of 4-OHT (days 0, 3, 5, 7, and 10 are shown). *, $P<0.05$; **, $P<0.01$, two-tailed t -test.

Figure 2. Biological effects of miR-26a and miR-29a on human *MLL*-rearranged AML cells
(A) Effect of forced expression of miR-26a or miR-29a on the proliferation/growth of MONOMAC-6/t(9;11) AML cells. Cells were transfected with MSCV-PIG (Control), MSCV-PIG-miR-26a (miR-26a), or MSCV-PIG-miR-29a (miR-29a). Cell numbers were counted every day after transfection for 7 days. **(B,C)** Effect of ectopic expression of miR-26a or miR-29a on the viability **(B)** and apoptosis **(C)** of MONOMAC-6 AML cells. Assays were performed at 48 hours after transfection. *, $P < 0.05$; **, $P < 0.01$, two-tailed t -test.

Figure 3. miR-26a plays a critical tumor-suppressor role in the pathogenesis of *MLL*-rearranged AML

(A) Effects of miR-26a and miR-29a on MLL-AF9-induced transformation /immortalization of normal mouse BM progenitor cells *in vitro*. Normal mouse BM cells were retrovirally transduced with MSCVneo+MSCV-PIG (Control), MSCVneo-MLL-AF9+MSCV-PIG (MLL-AF9), MSCVneo-MLL-AF9+MSCV-PIG-miR-26a (MLL-AF9+miR-26a), or MSCVneo-MLL-AF9+ MSCV-PIG-miR-29a (MLL-AF9+miR-29a), and then colony forming/replating assays (CFA) were conducted. 20,000 cells were plated in each dish for colony-forming in each passage. *, $P < 0.05$; **, $P < 0.01$, two-tailed *t*-test (Note: the *MLL-AF9* group was used as the control group for the statistic analysis). (B) Effects of miR-26a and miR-29a on MLL-AF9-induced leukemogenesis *in vivo*. Colony cells (CD45.1) of the above four groups were collected from the first passage of CFA and used as donor cells for the mouse BM transplantation (BMT) assays. 2.5×10^5 donor cells were transplanted into each lethally irradiated recipient mouse (CD45.2) with five recipient mice per group. Kaplan-Meier curves are shown. The *P* values were detected by log-rank test. (C) Wright-Giemsa stained BM cell cytopsin and peripheral blood (PB) smear, and hematoxylin and eosin (H&E) stained spleen and liver paraffin sections of transplantation recipient mice are shown.

Figure 4. miR-26a directly targets *TET1* at the 3'UTR and forced expression of miR-26a significantly inhibits expression of *Tet1* and its downstream target genes in *MLL-AF9* leukemic cells

(A) Luciferase reporter and mutagenesis assays were performed to determine whether miR-26a directly targets *TET1* at the 3'UTR. Two 3'UTR fragments of *TET1* containing two independent putative target sites of miR-26a were cloned into the luciferase reporter vector and used in the assays. A mutant form of *TET1*-3'UTR-1 fragment with mutations in the putative miR-26a target site was also constructed and used in the assays. (B) Expression level changes of miR-26a and *Tet1* in MLL-ENL-ERTm cells before and after withdrawal of 4-OHT (days 0, 3, 5, 7, and 10 are shown). (C) 5hmC level was detected in genomic DNA of BM cells collected from the Control, MLL-AF9 and MLL-AF9+miR-26a group mice (see Fig. 3B for sample information) at the end point by dot-blot assays. Mean±SD values were shown for 3 independent samples per group. (D) Effect of miR-26a on expression of *Tet1* and its six downstream target genes in colony cells collected from the secondary passage of CFA assays (see Fig. 3A for sample information). (E,F) Effect of miR-26a on expression of *Tet1*, *Tet2*, and *Hoxa9* at the RNA (E) and protein (F) levels in BM cells of the transplanted mice (see Fig. 3B for sample information). *, $P < 0.05$; **, $P < 0.01$, two-tailed t -test.

Figure 5. *TET1* is a functionally important target gene of miR-26a in *MLL*-rearranged AML
(A) Effects of forced expression of miR-26a alone or together with murine *Tet1* or *Hoxa9* on the growth/proliferation of MONOMAC-6 AML cells. Cell numbers were counted at day 0, 3, 5, 7, 9 after transfection. **(B,C)** Effects of forced expression of miR-26a alone or together with *Tet1* or *Hoxa9* on the viability **(B)** and apoptosis **(C)** of MONOMAC-6 AML cells. Assays were conducted 48 hours post transfection. *, $P < 0.05$; **, $P < 0.01$, *t*-test (Note: the control group was used as the statistic control).

Figure 6. Myc mediates the repression of miR-26a expression

(A) Relative expression levels of *Myc*, primary miR-26a (pri-miR-26a), precursor miR-26a (pre-miR-26a), mature miR-26a (miR-26a) and *Tet1* in BM cells of c-*Myc* wild type (Myc_WT) and knockout (Myc_KO) mice as detected by qPCR assay. (B) Relative expression levels of *MYC*, miR-26a and *TET1* in MONOMAC-6 AML cells transfected with *MYC* siRNA (siMYC) or scrambled control siRNA (Control) oligos as detected by qPCR assay. (C) A schematic model of the MLL-fusion/MYC-miR-26a-TET1 signal pathway in *MLL*-rearranged leukemia. MLL fusion proteins increase expression of *TET1* and *MYC*, and the upregulation of *MYC* further inhibits expression of miR-26a, which in turn enhances expression of *TET1* and its downstream signaling. **, $P < 0.01$, *t*-test.