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# An Extensive Network of TET2-Targeting MicroRNAs Regulates Malignant Hematopoiesis

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## SUMMARY

**The Ten-Eleven-Translocation 2 (*TET2*) gene, which oxidates 5-methylcytosine in DNA to 5-hydroxylmethylcytosine (5hmC), is a key tumor suppressor frequently mutated in hematopoietic malignancies. However, the molecular regulation of *TET2* expression is poorly understood. We show that *TET2* is under extensive microRNA (miRNA) regulation, and such *TET2* targeting is an important pathogenic mechanism in hematopoietic malignancies. Using a high-throughput 3′ UTR activity screen, we identify >30 miRNAs that inhibit TET2 expression and cellular 5hmC. Forced expression of *TET2*-targeting miRNAs in vivo disrupts normal hematopoiesis, leading to hematopoietic expansion and/or myeloid differentiation bias, whereas coexpression of *TET2* corrects these phenotypes. Importantly, several *TET2*-targeting miRNAs, including miR-125b, miR-29b, miR-29c, miR-101, and miR-7, are preferentially overexpressed in *TET2*-wild-type acute myeloid leukemia. Our results demonstrate the extensive roles of miRNAs in functionally regulating *TET2* and cellular 5hmC and reveal miRNAs with previously unrecognized oncogenic potential. Our work suggests that *TET2*-targeting miRNAs might be exploited in cancer diagnosis.**

## INTRODUCTION

The recently discovered Ten-Eleven-Translocation (*TET*) genes are key players in epigenetic regulation, with important roles in development and cancer. All three TET proteins, including *TET1*, *TET2*, and *TET3*, are enzymes that catalyze the conversion of 5-methylcytosine (5mC) in genomic DNA to 5-hydroxylmethylcytosine (5hmC) and its oxidative derivatives (Ito et al., 2010, 2011; Tahiliani et al., 2009; He et al., 2011). These enzymatic activities are involved in both active and passive DNA demethylation (reviewed in Wu and Zhang, 2011; Cimmino et al., 2011; Shih et al., 2012), the tight regulation of which is essential in defining and safeguarding cellular identities. Accordingly, *TET* gene expression and 5hmC levels are often downregulated in a wide spectrum of cancers (Lian et al., 2012; Hsu et al., 2012; Yang et al., 2013). In particular, haploinsufficient loss-of-function mutations in *TET2* are frequently found in patients with a variety of hematopoietic malignancies, including acute myeloid leukemia (AML), myeloproliferative neoplasms, myelodysplastic syndromes, chronic myelomonocytic leukemia (CMML), and lymphoid malignancies (Cimmino et al., 2011; Shih et al., 2012). In mouse models, homozygous or heterozygous loss of *TET2* results in enhanced hematopoietic stem cell activity and CMML-like malignant progression (Moran-Crusio et al., 2011; Quivoron et al., 2011; Li et al., 2011).

Increasing efforts are underway to incorporate *TET2* mutational status in routine clinical diagnostics to inform molecular pathogenesis and therapeutic outcomes. However, genetic *TET2* mutation analysis is not sufficient to completely capture *TET2* functional deregulation. For example, it was found that a substantial fraction of patients with AML with wild-type *TET2* shows similarly decreased 5hmC levels as *TET2* mutant AMLs (Ko et al., 2010). Hence, it raises the question whether, in addition to genetic mutations and inhibition of enzyme activity (Mardis et al., 2009; Shih et al., 2012), molecular pathways regulating *TET2* expression can serve as an important alternative mechanism in hematopoietic malignancies and should be considered in diagnosis.

Despite the importance of *TET* gene dosage control, much less is known about the mechanisms that regulate *TET* gene expression (Kallin et al., 2012; Wu et al., 2013; Song et al.,

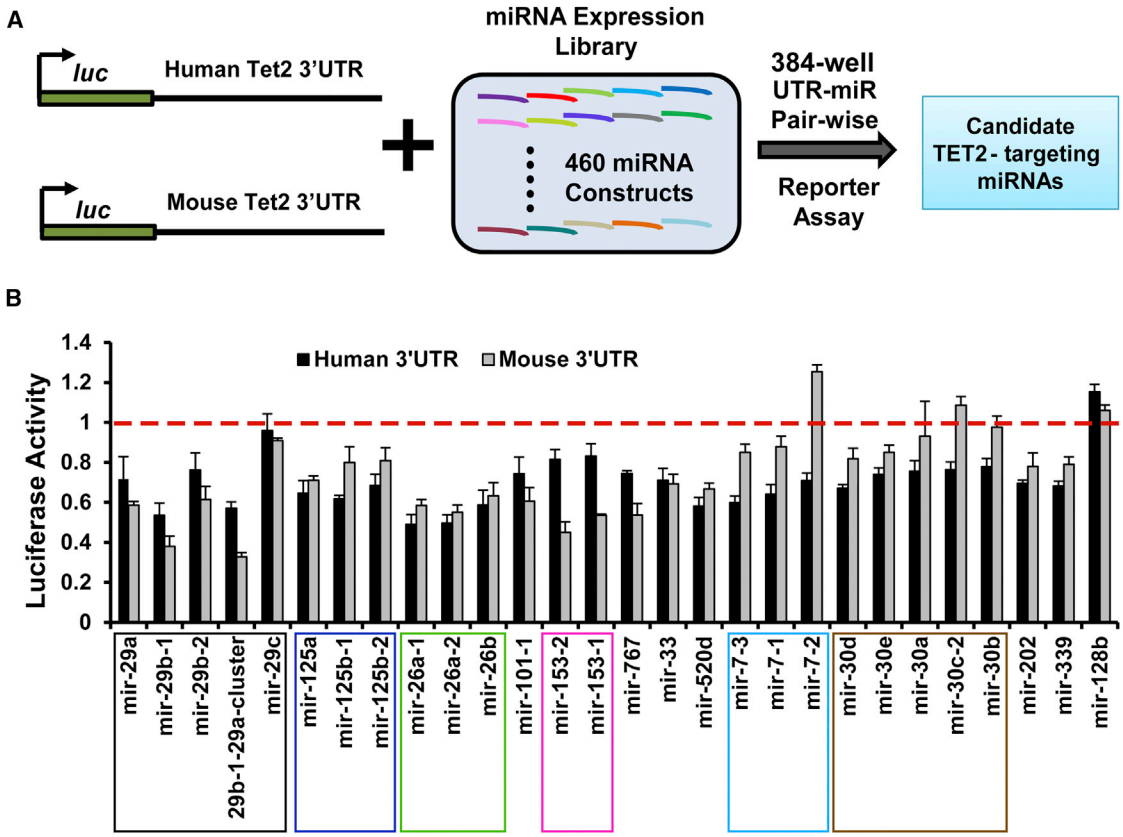


**Figure 1. A 3′ UTR Reporter Screen Identifies Candidate *TET2*-Targeting miRNAs**

(A) A schematic shows the high-throughput screen in which ~460 human miRNA expression constructs were assayed one by one with human or mouse *TET2* 3′ UTR reporters.

(B) Data for a subset of candidate *TET2*-targeting miRNAs identified through the screen, as well as the nontargeting miR-128b, are shown. Normalized luciferase activities are plotted with the red line and with 1 representing averaged luciferase activities of controls. miRNAs that belong to the same family are boxed. Error bars represent SD (n = 4).

See also Figures S1 and S2 and Tables S1 and S6.

2013a, 2013b; Zhang et al., 2013). MicroRNAs (miRNAs) are small noncoding RNAs that downregulate target gene expression by inhibiting target mRNA stability and translatability (Bartel, 2009). Target downregulation by miRNAs is primarily achieved through cognitive sites in the 3′ UTRs, with miRNA binding sites in other regions of target transcript generally contributing much less to functional regulation (Bartel, 2009). However, despite increasing understandings of how miRNAs regulate their targets, faithful identification of miRNA-mediated functional targeting still presents a major challenge.

In this study, we systematically surveyed miRNA-mediated regulation of *TET2* expression, and the roles of *TET2*-targeting miRNAs in abnormal hematopoiesis. Using a high-throughput screen, we identified a large network of miRNAs capable of inhibiting *TET2* expression. Among the *TET2*-targeting miRNAs were those that induce traits associated with malignant hematopoiesis in vivo, and were preferentially expressed in *TET2*-wild-type AMLs. This study reveals a group of miRNAs with previously unrecognized oncogenic potentials in malignant hematopoiesis. Given the limited expression range of *TET2* itself, our data suggest that for cancers with wild-type *TET2* status, in addition to screening IDH1/IDH2 (Shih et al., 2012), *TET2*-targeting miRNAs could be useful diagnostic biomarkers and potential therapeutics.

## RESULTS

### A High-Throughput Reporter Screen Identifies a Large Network of miRNAs that Inhibits *TET2* 3′ UTR

To identify *TET2*-targeting miRNAs, we undertook an unbiased high-throughput screen to identify miRNA-mediated regulation of *TET2* 3′ UTR (Figure 1A). Unlike biochemical identification of miRNA binding regions on target mRNA (Lipchina et al., 2011; Chi et al., 2009; Hafner et al., 2010), this approach produced functional miRNA-target relationships rather than just binding relationships. We first cloned 3′ UTR luciferase reporters of human and mouse *TET2* from the corresponding full-length isoforms. Although several splicing variants of *TET2* have been reported (Langemeijer et al., 2009; Moran-Crusio et al., 2011), only the full-length isoforms encode the catalytic domain in the C terminus, the importance of which was confirmed in a murine knockout study by Quivoron et al. (2011). We next successfully

miniaturized a cell-based reporter assay system, with which we quantified the effects of ~460 individual miRNA constructs (expressing a single miRNA or miRNA cluster) one by one with human or mouse *TET2* 3′ UTR reporters in quadruplicates in 384-well plates. For the vast majority of the assayed miRNA-3′ UTR pairs, the miRNAs had either no or weak effect on the corresponding *TET2* 3′ UTR (Figures S1A and S1B; Table S1). In contrast, 48 miRNA-3′ UTR pairs (see Experimental Procedures) led to a >25% repressive effect (Figures 1B and S1B; Table S1). Compared to two popular computational target prediction algorithms, TargetScan and mirSVR (Grimson et al., 2007; Betel et al., 2010), these inhibitory miRNA-3′ UTR relations include only 13% (32 out of 246) of predicted relations by both algorithms or 9% (44 out of 491) of those predicted by either algorithm, suggesting that the majority of the algorithm-predicted miRNA-3′ UTR pairs had weak or no effect (Figure S1C). In addition, 4 (8.3% of all) inhibitory miRNA-3′ UTR relations were not predicted, and 12 (25%) were only predicted by one of the two algorithms, suggesting a significant level of false negatives by these computational predictions. These data support the importance of defining *TET2*-targeting miRNAs through experimental approaches.

The 48 miRNA-3′ UTR pairs with >25% repression consist of 32 unique miRNAs that repress either human or mouse *TET2* 3′ UTRs. Among such *TET2*-targeting miRNAs, different constructs from the same miRNA family (i.e., sharing the same seed sequence) often led to similar effects, such as miR-29, miR-125, and miR-26 families (Figure 1B). We also noticed that whereas many candidate miRNAs had similar levels of repression on both human and mouse *TET2* 3′ UTRs, some show species specificity in functional targeting. For example, the miR-7 family had a much weaker repression of mouse 3′ UTR compared to human. To validate the screen result, we repeated the luciferase reporter assays on the candidate miRNAs and obtained highly consistent data (Figure S1D). In addition, by mutagenesis, we confirmed that miR-29b, miR-125a, miR-101, and miR-26a regulate *TET2* 3′ UTR through specific binding sites, supporting direct regulation (Figure S2). The data above show that our high-throughput reporter assay approach can reproducibly and systematically reveal miRNA-mediated regulation of *TET2* 3′ UTR by an extensive network of miRNAs.

These candidate *TET2*-targeting miRNAs contain mostly two classes of miRNAs: (1) miRNAs that are not known to be involved in hematopoietic malignancies, and (2) oncogenic miRNAs without fully understood downstream mechanisms. For example, forced expression of miR-29a induces malignant hematopoiesis, yet the relevant miR-29a target for this biology is unknown (Han et al., 2010). On the other hand, miR-29b and miR-29c are not known to be myeloid oncogenes (Han et al., 2010), and miR-29b was instead reported as a tumor suppressor in myeloid leukemia (Garzon et al., 2009a, 2009b; Huang et al., 2013). In contrast to these reports, our data (Figure 1B) suggest that miR-29b and miR-29c could regulate *TET2* and function as hematopoietic oncogenes under certain circumstances. Another example is the miR-125a/125b family, which is known to induce a CMML-like disease (reviewed in Shaham et al., 2012; Guo et al., 2012) via incompletely characterized mechanisms. Our data also suggest that additional *TET2*-targeting miRNAs, such as miR-101, miR-7, and miR-26a/26b, may play oncogenic roles in hematopoietic malignancies, which have not been previously recognized. We thus went on to further characterize the effects of *TET2*-targeting miRNAs in vitro and in vivo.

### *TET2*-Targeting miRNAs Downregulate TET2 Protein and 5hmC Levels in Hematopoietic Cells

To systematically examine the functions of *TET2*-targeting miRNAs, we first determined whether these candidate miRNAs can regulate endogenous *TET2* expression and function in hematopoietic cells. We initially examined hematopoietic cell lines BaF3 (murine) and K562 (human), which express endogenous TET2 protein at detectible levels (Figures S3A and S3B). We individually expressed ~16 candidate miRNAs in BaF3 and K562 cells, as well as a negative control miR-128b. We focused on those miRNAs that target both human and mouse 3′ UTRs, as well as a few that preferentially target the human 3′ UTR. Many of the miRNAs, including those from miR-29, miR-125, and miR-26 families, miR-101, and miR-520d significantly suppressed endogenous TET2 protein expression in both murine and human cells (Figures 2A and S3C). The miRNA-mediated downregulation of *TET2* was also detected on the RNA level (Figure 2B). To confirm the regulation of *TET2* in primary cells, we expressed miR-29b and miR-125a in primary murine bone marrow cells, which similarly decreased TET2 protein levels (Figure 2C). Consistent with the luciferase reporter screen data, miR-7 only reduced TET2 protein level in human but not in murine cells (Figures 2A and S3C). The decreases in endogenous TET2 protein levels were also accompanied by decreases in total cellular 5hmC levels (Figures 2D–2G and S3D), supporting that the function of TET protein(s) was compromised. Importantly, the downregulation of cellular 5hmC by miR-29b or miR-125a could be rescued by expression of a *TET2* cDNA without 3′ UTR (Figures 2F and 2G), further supporting the role of a miRNA-*TET* pathway in the control of cellular 5hmC levels.

To determine whether the regulation by *TET2*-targeting miRNAs is physiologically or pathologically relevant, we first quantified the overexpression levels of miR-125a, miR-29b, miR-101, and miR-26a using quantitative RT-PCR and compared to those seen in human AML samples (see Experimental Procedures for details). Results showed that the overexpression levels of miR-125a and miR-101 were comparable to or lower than those reachable in AML samples, especially when considering the contribution of family members such as miR-125b (Figures S4C and S4D). For miR-29b and miR-26a, the levels of overexpression were ~2- to 10-fold higher than clinical AML samples (Figures S4E and S4F). We thus asked whether *TET2* expression was under the control of endogenous miR-29 and miR-26 family miRNAs. We used miRNA sponges against miR-29 family or miR-26 family, which are decoy targets that inhibit miRNA function (Ebert et al., 2007). In BaF3 cells that express endogenous miR-29b and miR-26a at levels comparable to those in clinical samples (Figures S4E and S4F), both miR-29a/29b/29c sponge and miR-26a/26b sponge led to a significant increase in endogenous TET2 protein level, as well as an increase in cellular 5hmC (Figures 2H–2K). In contrast, miR-125a/125b sponge did not increase TET2 protein because endogenous miR-125a/125b expression is low in BaF3 cells (Figure S4C; Table S2) and thus

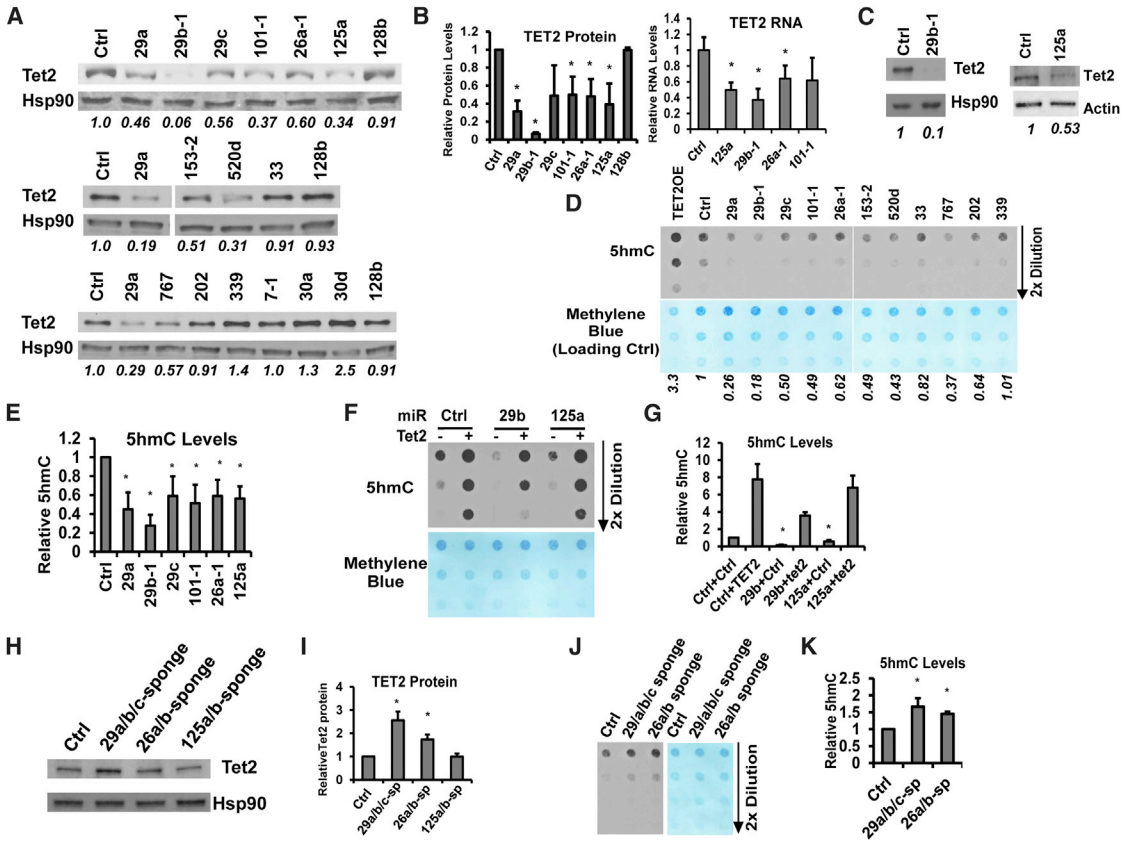


**Figure 2. *TET2*-Targeting miRNAs Regulate *TET2* Expression and Cellular 5hmC Levels**

(A) BaF3 cells were transduced with control vector (Ctrl) or indicated miRNAs, including miR-128b, which does not target *TET2*. TET2 and HSP90 protein levels were determined by western blot. Representative data are shown out of two to three repeats. Quantification of TET2 protein level is indicated, after normalization to HSP90.

(B) Quantification of the effect of indicted miRNAs on TET2 protein or RNA levels in BaF3 cells is shown. Data were quantified from three repeats.

(C) Primary mouse bone marrow cells were transduced with indicated miRNAs. Representative western blots are shown out of two (miR-125a) and three (miR-29b) experiments.

(D) Genomic DNA from BaF3 cells transduced with Ctrl or indicated miRNAs or a *TET2* overexpression vector (TET2OE) was analyzed for 5hmC levels using dot blot assay. Blot was stained with methylene blue to control for loading. Normalized 5hmC levels are indicated. Representative blot is shown out of two to three repeats. Note that the separated 5hmC images were from the same exposure of the same blot and the same for methylene blue.

(E) Quantification of 5hmC data (n = 3) is presented.

(F) *TET2* cDNA or Ctrl was coexpressed with indicated miRNAs. Cellular 5hmC was assayed with dot blot. Representative data are shown out of three repeats.

(G) Quantification of data in (F) (n = 3) is shown. Indicated statistical significance (by an asterisk [*]) was evaluated in comparison to Ctrl+Ctrl.

(H) BaF3 cells were transduced with a Ctrl vector, or sponges that inhibit the miR-29 family, miR-26 family, or miR-125 family. TET2 and HSP90 protein levels were determined by western blot. Representative data are shown from three repeats.

(I) Quantification of western data in (H) (n = 3) is presented.

(J) Cellular 5hmC levels from BaF3 cells transduced with Ctrl or indicated miRNA sponges were determined by dot blot analysis. Representative data are shown from three repeats.

(K) Quantification of 5hmC levels for (J), after normalizing with methylene blue (n = 3), is shown.

Error bars represent SD. *$p < 0.05$.

See also Figures S3 and S4 and Table S2.

serves as a negative control. Taken together, these data demonstrate that the endogenous *TET2* can be regulated by an extensive network of miRNAs, the expression of which contributes to controlling the epigenetic landscape via targeting a central regulator of cellular 5hmC levels.

## Multiple *TET2*-Targeting miRNAs Also Regulate *TET1* and *TET3*

Given that there are three *TET* family proteins and that TET3, like *TET2*, is abundantly expressed in hematopoietic tissues (Ito et al., 2010), we asked whether miR-29b, miR-26a, and several other *TET2*-targeting miRNAs can also regulate *TET1* and *TET3*. Indeed, expression of miR-29 and miR-26 family miRNAs resulted in an inhibition of *TET1* and *TET3* 3′ UTR reporter activities (Figures 3A and 3B). These miRNAs also decreased endogenous *TET3* RNA and protein levels in hematopoietic cells (Figures 3C and 3D). The endogenous TET1 RNA level was too low to be reliably quantified in these cells (data not shown). These data reveal miRNA-mediated regulation of *TET1* and *TET3* and suggest that these specific miRNAs can function as

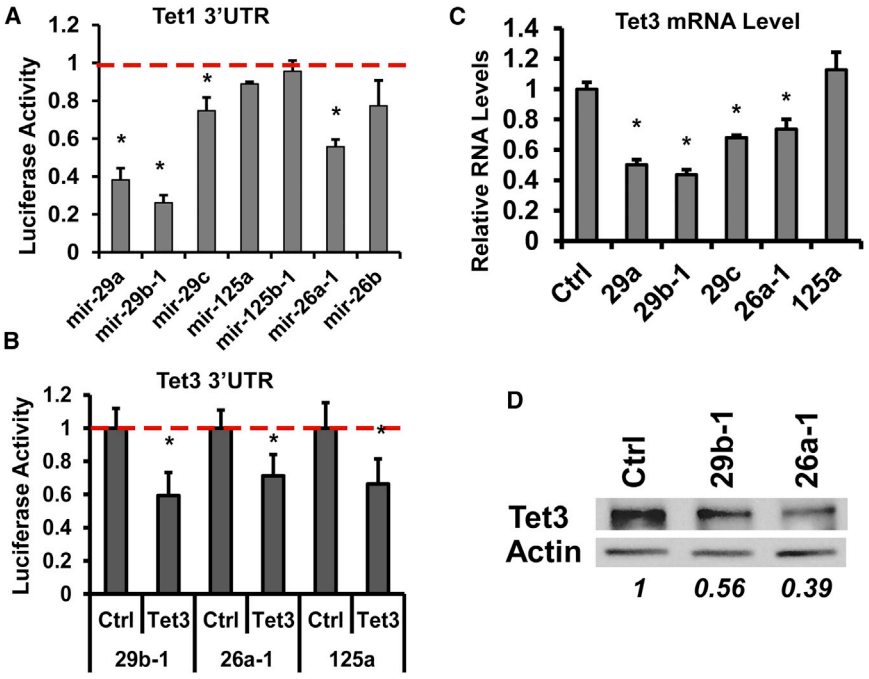


**Figure 3. Some *TET2*-Targeting miRNAs Regulate Additional TET Family Members**

(A) Human *TET1* luciferase reporter was analyzed with indicated miRNAs, with the red line representing Ctrl levels (n = 4).

(B) Mouse *TET3* luciferase reporter was analyzed with indicated miRNAs, with the red line representing Ctrl levels (n = 4).

(C) *TET3* RNA levels in K562 cells transduced with indicated Ctrl or miRNAs were determined by qRT-PCR (n = 3).

(D) TET3 protein levels were analyzed in K562 cells transduced with Ctrl or indicated miRNAs by western blot. Representative blots are shown out of two repeated experiments.

All error bars represent SD. *p < 0.05.

"master regulators" of cellular 5mC to 5hmC conversion by targeting all three *TET* family members.

## Expression of *TET2*-Targeting miRNAs Leads to Malignant Hematopoietic Traits

To determine whether *TET2*-targeting miRNAs can cause malignant hematopoiesis, we next examined the effect of ten miRNAs in vivo. Forced expression of control or candidate miRNAs was delivered through viral transduction of wild-type bone marrow cells, with GFP marking transduced cells, followed by bone marrow transplantation into lethally irradiated host mice. We paid special attention to two hematopoietic traits associated with *TET2* loss, namely biased differentiation into the myeloid lineage, as well as hematopoietic expansion (Moran-Crusio et al., 2011; Quivoron et al., 2011; Li et al., 2011; Ko et al., 2010; Pronier et al., 2011). We used a myeloid bias index to reflect the biased differentiation, which was calculated by ratios of myeloid (Mac1+) versus nonmyeloid cells in peripheral blood transduced (GFP+) versus nontransduced (GFP−) populations (see Experimental Procedures). To quantify hematopoietic expansion, we followed the peripheral blood GFP+ percentage from 3 weeks posttransplantation and on. Among *TET2*-targeting miRNAs, the known oncogenic miR-125a served as a positive control, which led to both increased myeloid bias index, indicating skewed differentiation into myeloid lineage, and an increase in GFP+ percentage over time, indicating hematopoietic expansion (Figures 4A–4C). On the other hand, miR-144, which does not strongly affect *TET2* 3′ UTR, behaved similarly as the control vector (Figures 4A–4C). Consistent with miR-29b functioning as a bona fide oncogene, we observed both increased myeloid bias index and hematopoietic expansion (Figures 4A–4C). In addition, miR-29b mice had splenomegaly, with increased percentage of GFP+Ly6C+Ly6G− monocytes in bone marrow, revealing a CMML-like disease (Figures 4E and 4F). Other candidate miRNAs examined, except for miR-33, displayed these hematopoietic phenotypes to variable severities, and sometimes in unique manners (summarized in Figure S5D). For example, miR-101 expression led to a significant myeloid bias. Interestingly, although the GFP+ cell expansion was not statistically significant in the miR-101 cohorts, two out of ten miR-101 recipients showed persistent hematopoietic expansion, suggesting incomplete penetrance. In contrast, these phenotypes were never observed in cohorts of 12 control recipients (Figures 4A–4C). Another example is miR-26a, which led to a reproducible transient myeloid bias at 3.5 weeks posttransplantation (Figures 4A–4D), with one out of ten mice showing hematopoietic expansion. Multiple other tested miRNAs (Figures S5A–S5D) also led to variable degrees of myeloid bias and hematopoietic expansion, often with incomplete penetrance. Of note, the levels of overexpression in vivo were similar to those observed in vitro (Figures S4C–S4F), and we confirmed that 5hmC levels in vivo could be suppressed by *TET2*-targeting miR-29b and miR-125a (Figure S5E). The differential phenotypes induced by these *TET2*-targeting miRNAs may reflect the different degrees of repression on *TET2*, the level of overexpression, and/or the effects from additional targets. Taken together, we identified previously unrecognized functions for miRNAs in derailing normal hematopoiesis. They converge on inhibiting *TET2* and induce phenotypes associated with hematopoietic malignancy.

## Expression of *TET2* Rescues Malignant Phenotypes of *TET2*-Targeting miRNAs

Given the in vivo phenotypes of *TET2*-targeting miRNAs, we asked whether *TET2* expression can rescue miRNA-induced malignant hematopoiesis. We particularly focused on miR-125a, which potently induces a CMML-like disease in mice (Guo et al., 2012; and reviewed in Shaham et al., 2012), and miR-29b, which we characterized above as a new oncogene. Compared to other *TET2*-targeting miRNAs, another reason to

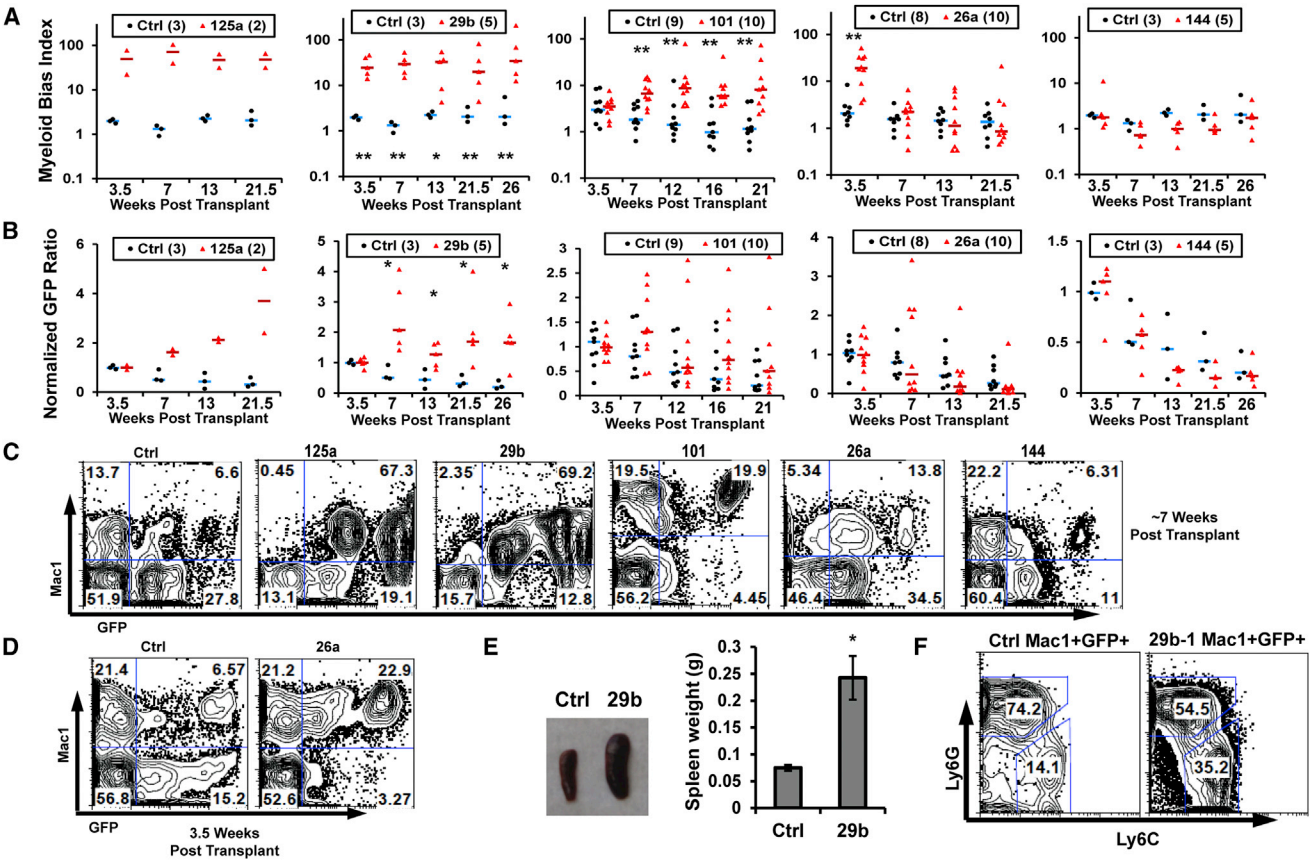


**Figure 4. *TET2*-Targeting miRNAs Induce Abnormal Hematopoiesis In Vivo**

(A and B) Wild-type bone marrow cells were transduced with Ctrl or indicated miRNAs, and transplanted into recipient mice, with GFP-labeling transduced cells. Peripheral blood was analyzed in recipients at the indicated time points, with each dot representing one recipient. (A) Myeloid bias index (frequency ratios of (%GFP+Mac1+/%GFP+Mac1−)/(%GFP−Mac1+/%GFP−Mac1−) was calculated to reflect the biased presence of myeloid cells in transduced population. (B) Normalized GFP ratios were also calculated (by taking the ratio of GFP+/GFP− cells and normalized to the average at 3.5 weeks) to reflect hematopoietic expansion. Numbers of mice per group are indicated in parentheses. The short horizontal bars represent median levels.

(C and D) Representative flow cytometry plots of recipients at ~7 weeks posttransplantation (C) or 3.5 weeks posttransplantation (D) show myeloid marker Mac1 and GFP.

(E) Splenomegaly in miR-29b recipients is shown. A representative image is shown on the left, with pooled spleen weight data shown on the right (n = 4 for Ctrl and n = 5 for 29b).

(F) Bone marrow cells from Ctrl or miR-29b recipients were analyzed for granulocyte (Ly6G+Ly6C−) and monocytes (Ly6G−Ly6C+). Representative flow cytometry plots are shown, after gating on transduced myeloid cell populations (GFP+Mac1+ population). Note the increased monocytic frequency in miR-29b-transduced cells.

Error bars represent SDs. *$p < 0.05$.

See also Figure S5.

focus on miR-125a and miR-29b was that both miRNAs induced strong malignant phenotypes in vivo, and it would be more challenging to revert such strong phenotypes. Because in vivo hematopoietic expansion is often correlated with increased hematopoietic colonies in serial methylcellulose cultures in vitro, we first examined the effect of *TET2* expression on colony formation in the presence of miR-125a or miR-29b. Specifically, wild-type bone marrow cells were transduced with control, miR-125a, or miR-29b (all marked with GFP), in combination with a control or 3′ UTR-less *TET2* cDNA. Consistent with miR-125a and miR-29b being oncogenes, significant increases in secondary colony formation were observed when either miRNA was expressed (Figure S5F). In contrast, *TET2* coexpression potently suppressed miR-125a- and miR-29b-induced secondary colony formation to control levels (Figure S5F). To test the function of *TET2* in suppressing the malignant phenotype in vivo, we next transplanted bone marrow cells transduced with both miRNAs and *TET2* into recipient mice. Expression of *TET2* significantly suppressed the miR-125a- and miR-29b-induced increases in hematopoietic output (reflected by GFP+ percentage) and completely normalized the myeloid-differentiation bias in miR-125a recipients (Figures 5A–5C and 5E–5G). In addition, miR-125a-*TET2*-coexpressing recipients showed the correction of monocytic differentiation bias among myeloid cells (Figure 5D). For miR-29b, myeloid bias index also trended lower with *TET2* coexpression, although the data did not reach statistical

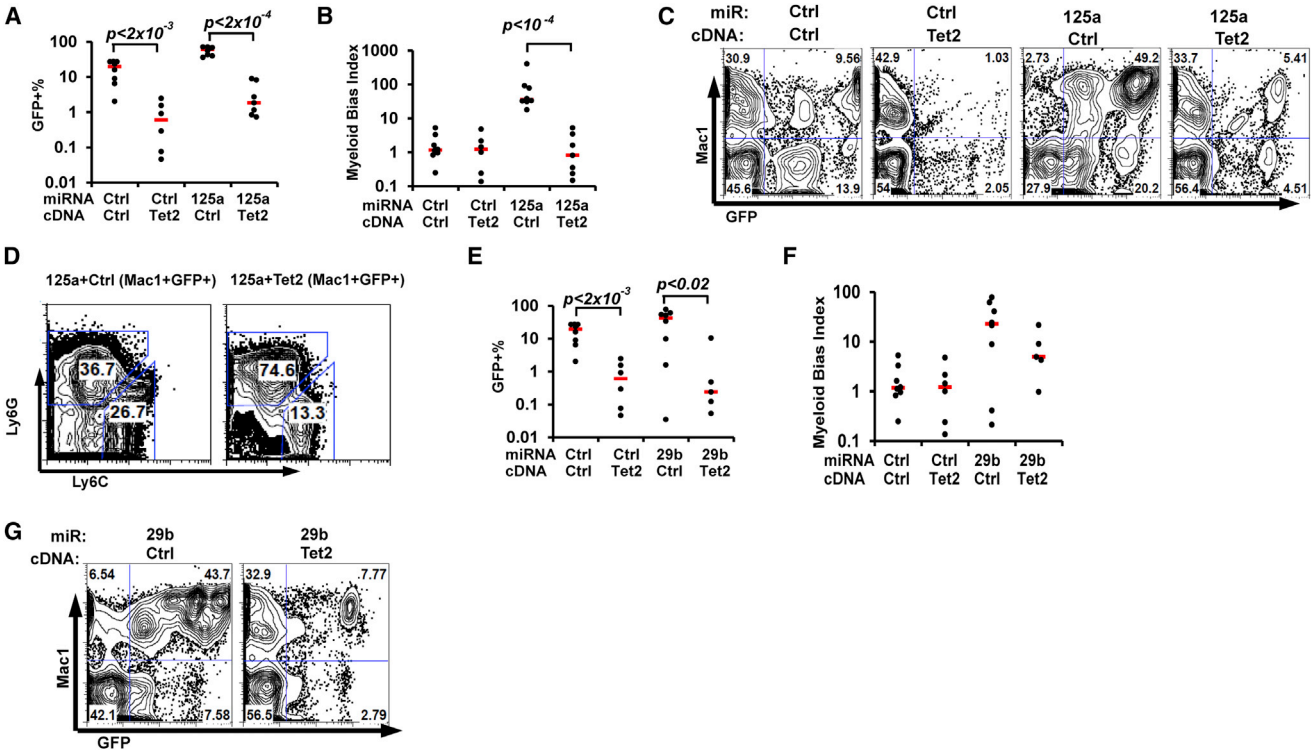


**Figure 5. *TET2* Expression Rescues Malignant Phenotypes by Oncogenic miRNAs**

Bone marrow cells were transduced with Ctrl or miR-125a or miR-29b in combination with a cDNA Ctrl vector or *TET2*. The same number of sorted transduced cells was transplanted into each recipient in each experiment.

(A and B) For miR-125a/*TET2* rescue, GFP+ percentage and myeloid bias index at 3.5 weeks posttransplantation are shown, with each dot representing one recipient.

(C) Representative flow cytometry plots for (A) and (B) are presented.

(D) Bone marrow cells from miR-125a+Ctrl or miR-125a+*TET2* recipients were analyzed for granulocyte (Ly6G+Ly6C−) and monocytes (Ly6G−Ly6C+). Representative flow cytometry plots are shown, after gating on transduced myeloid cell populations (GFP+Mac1+ population). Note that the monocytic bias in the miR-125a+Ctrl recipient was largely corrected by *TET2* expression.

(E and F) For miR-29b/*TET2* rescue, GFP+ percentage and myeloid bias index at 3.5 weeks posttransplantation are shown, with each dot representing one recipient.

(G) Representative flow cytometry plots for (E) and (F) are shown. The short horizontal bars represent median levels. The p values are indicated.

See also Figure S5.

significance (Figure 5F). This incomplete rescue of miR-29b-induced myeloid bias may be due to additional miR-29b targets or suboptimal stoichiometry of *TET2* during rescue. Thus, our data demonstrate the importance of *TET2* targeting in the oncogenic activities of miR-125a and miR-29b and suggest that increasing *TET2* expression could be a potential strategy to combat certain groups of hematopoietic malignancies.

## *TET2*-Targeting miRNAs Are Preferentially Overexpressed in *TET2*-Wild-Type AMLs

We assessed the miRNA-*TET2* mechanism in the pathogenesis of human leukemia. Because decreased *TET2* function could be a result of either genetic *TET2* mutations or elevated expression of its targeting miRNAs, we reasoned that these two mechanisms likely occur independently, rather than redundantly. If true, we would expect *TET2*-targeting miRNAs to be more frequently overexpressed in *TET2*-wild-type leukemia, as compared to those harboring *TET2* mutations. To test this possibility, we profiled miRNA expression for a cohort of 67 cases of cytogenetically normal AMLs, among which 16 patients carry protein sequence-altering *TET2* mutations (Table S3). To assess miRNA overexpression outliers, we used a method similar to COPA (Tomlins et al., 2005) and quantified the frequency of *TET2*-wild-type and *TET2*-mutant samples with outlier overexpression levels, using false discovery rate <0.15 to define significant association (see Experimental Procedures). Among the 17 *TET2*-targeting miRNAs that we measured and passed detection threshold, overexpression of miR-125b, miR-29b, miR-29c, miR-101, and miR-7 was more frequently observed in *TET2*-wild-type cases than *TET2* mutant cases, at two different outlier cutoffs (Figure 6; Table S4). Interestingly, the overexpression spectra of these miRNAs were not fully overlapped in *TET2*-wild-type AMLs (Figure S6E), suggesting that *TET2*-targeting miRNAs are differentially utilized in different AMLs in a largely nonredundant manner. Other *TET2*-targeting miRNAs were significant at a single outlier cutoff (miR-30e), had a single strong expressing sample in the *TET2*-wild-type but not *TET2*-mutant cohort (e.g., miR-520a-5p and miR-202), or were not significant in this cohort (Figures S6A–S6D; Table S4).

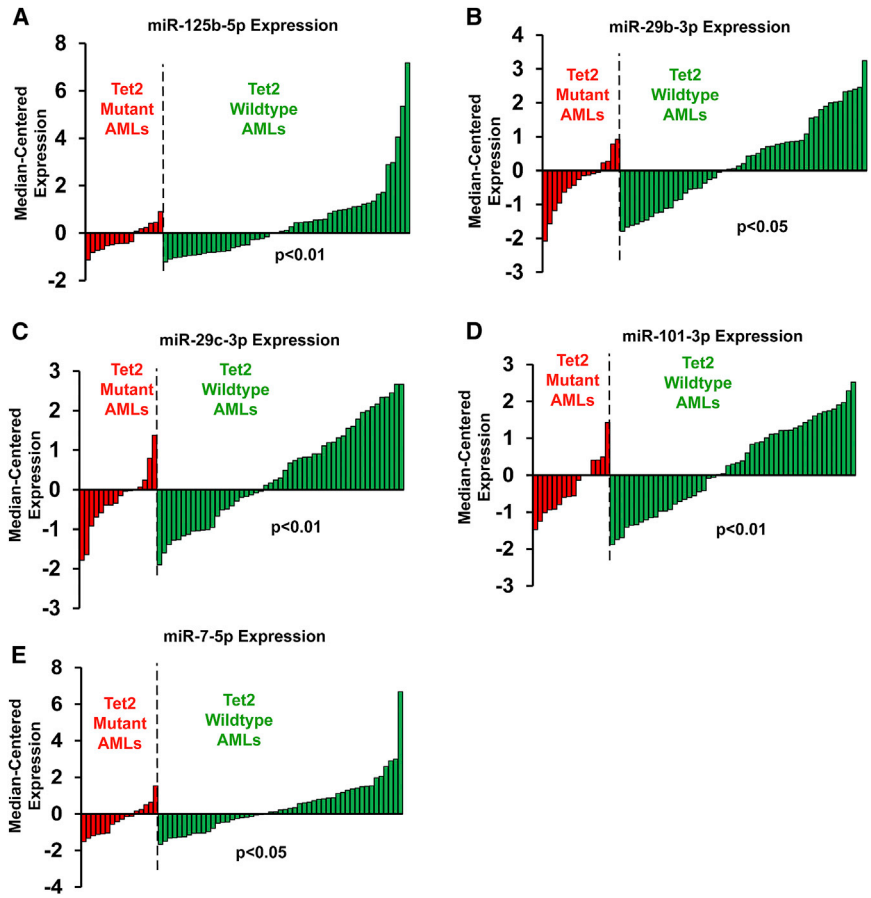


**Figure 6. Preferential Overexpression of *TET2*-Targeting miRNAs in *TET2*-Wild-Type AML**

The expression of *TET2*-targeting miRNAs was measured in a cohort of 67 cytogenetically normal AMLs, among which 16 samples were *TET2* mutant. Expression data were normalized by subtracting cohort median and dividing by median average deviation to reflect outlier expression patterns. The data for miR-125b-5p (A), miR-29b-3p (B), miR-29c-3p (C), miR-101-3p (D), and miR-7-5p (E) are plotted, with higher bars indicating higher expression. The p values reflect the probability of observing more frequent higher expression for the indicated miRNA in *TET2*-wild-type AMLs than *TET2* mutant AMLs. The specific cutoff applied for these p values is examining the top 33% samples with the highest expression of the indicated miRNA. See also Figure S6 and Tables S3, S4, and S5.

An alternative explanation for the preferential overexpression of *TET2*-targeting miRNAs in *TET2*-wild-type leukemia is that *TET2* functionally upregulates these miRNAs. To examine this possibility, we overexpressed *TET2* cDNA in BaF3 and K562 cells and knocked down *TET2* in BaF3 cells (Figures S3B, 2D, and S3D) and then profiled miRNA expression. Results showed that although *TET2* level modulation altered the expression of some miRNAs, the five *TET2*-targeting miRNAs that correlated with *TET2* mutational status were not positively regulated by *TET2* (Table S2). Another possibility is that many other miRNAs can score significantly in this statistical test, and our observation of the association with *TET2*-targeting miRNAs is solely random. To exclude this possibility, we examined >580 miRNAs profiled in this cohort. Excluding the *TET2*-targeting miRNAs, there were 196 miRNAs passing detection threshold, among which 9 miRNAs were similarly associated with *TET2* wild-type status (Table S5). These nine miRNAs include miR-99a, which is located in the same genomic cluster as miR-125b-2 and, thus, coexpressed with the *TET2*-targeting miR-125b (Figure S6F). In addition, three of the miRNAs (miR-18a, miR-18b, and miR-19a) are known to be coexpressed and were correlated with each other in our data set (Table S5). Even without eliminating such influences, our observation of 5 out of 17 (29%) *TET2*-targeting miRNAs scoring in this test is significant (as compared to 9 out of 196, or 4.6%; $p < 0.003$, Fisher's exact test). Taken together, these data support the notion that overexpression of a subset of *TET2*-targeting miRNAs identified in this study can be an important mechanism in human leukemogenesis.

## DISCUSSION

Using a high-throughput reporter screen, our study systematically identified miRNA-mediated regulation of *TET2* through its 3′ UTR and revealed the roles of *TET2*-targeting miRNAs in malignant hematopoiesis. We found that in a cohort of cytogenetically normal human AMLs, multiple *TET2*-targeting miRNAs, including miR-29b, miR-101, miR-125b, miR-29c, and miR-7, were preferentially overexpressed in *TET2*-wild-type specimens than those with *TET2* mutations. These data support a role for miRNA-*TET2* pathway in the pathogenesis of human AML and other malignancies, adding a new layer to the existing paradigms of loss-of-function mutations in *TET2* and gain-of-function mutations in the IDH genes. Our data also argue that in addition to routine genetic mutational analyses on *TET2* and related IDH1/IDH2 genes, which are currently being developed and implemented in clinics, the expression status of our identified *TET2*-targeting miRNAs could be considered as an additional diagnostic parameter to inform the deregulation of the *TET2* pathway. In this regard, measuring *TET2*-targeting miRNAs has advantages over directly measuring TET2 protein or mRNA levels, due to the limited range of differential *TET2* expression and difficulty in its protein measurements. For example, we noticed that the range of differential *TET2* RNA expression in AML samples is ~3-fold (data not shown) and thus is susceptible to interference by measurement noise. TET2-protein measurements suffer from the same restrains, and western-based measurements require a large number of cells. In contrast, *TET2*-targeting miRNAs displayed a much larger dynamic range of expression. For example,

miR-125b expression has a range over 10,000-fold in the same data sets (Figure S4C). Our findings also raise several important questions to be further examined in future diagnostic and prognostic studies. For example, do *TET2*-targeting miRNAs contribute differently in the pathogenesis of single-allelic *TET2* mutants versus biallelic mutants (which our study cohort was not statistically powered to address, given that a much larger cohort will be needed)? In addition, it is important to point out that other *TET2*-targeting miRNAs demonstrated in this study may also have a role in human hematopoietic malignancies, even though they were not significantly associated in this cytogenetically normal AML cohort because they may be involved in other AML types or other hematopoietic malignancies (Shih et al., 2012).

While this work was being revised, it was published that miR-22 targets *TET2* through 3′ UTR and regulates hematopoietic stem cells (Song et al., 2013a, 2013b). While our work systematically complements and extends these findings on *TET2* regulation, it is also interesting to note that we did not detect a repressive effect of miR-22 on *TET2* 3′ UTR (Table S1), even though we confirmed that miR-22 was overexpressed >12-fold (Figure S4G). In our AML cohort, miR-22 did not show significant association with *TET2* mutational status (Table S5). The difference in 3′ UTR data may be due to the use of full-length *TET2* 3′ UTR in our study versus a much shorter 500 bp 3′ UTR fragment (Song et al., 2013b). Because it is recognized that the location of the miRNA binding site within the 3′ UTR and target RNA structure can determine the effectiveness of miRNA binding sites (Bartel, 2009; Long et al., 2007), it raises the possibility that additional mechanisms regulate the presentation of the miR-22 binding site.

Our data also uncovered multiple miRNAs with unrecognized oncogenic potential and revealed *TET2* targeting as a relevant mechanism of previously known oncogenic miRNAs. For example, miR-29b was previously recognized as a tumor suppressor in myeloid leukemia (Garzon et al., 2009a, 2009b), but our study demonstrated an opposite oncogenic role of this miRNA. The differences observed may be related to the level of miR-29b expression or the duration of expression. Interestingly, the miR-29 family and miR-26 family miRNAs regulate *TET1* and *TET3* in addition to *TET2*, suggesting a miRNA-mediated master regulatory program in shaping cellular 5hmC landscape. The in vitro and in vivo data presented here also showed that miRNAs, such as miR-101, miR-29c, miR-26a/26b, and miR-520d, can function as previously unappreciated oncogenes by derailing normal hematopoietic differentiation processes and provided a new molecular mechanism for the known oncogenic miRNAs, including the miR-125 family and miR-29a. It is also interesting to note that although all these miRNAs were capable of targeting *TET2*, their in vivo overexpression phenotypes were variable. One possibility for such differences is the involvement of other targets of the specific miRNAs. For example, miR-125 family miRNAs regulate multiple pathways (Shaham et al., 2012), such as enhancing growth factor signaling and inhibiting apoptosis (Guo et al., 2010, 2012), which may cooperate with *TET2* repression by this miRNA. Similarly, miR-101 can also regulate the PRC2 component EZH2 (Varambally et al., 2008), suggesting a broad effect of this miRNA in regulating multiple enzymes that control the epigenome. Another example is the miR-26 family, which led to transient myeloid differentiation bias but only caused hematopoietic expansion in a small number of mice. Notably, miR-26 has been shown to target cyclin D2 and E2, and inhibit cell cycle in other cancer types (Kota et al., 2009), a mechanism that may modify the *TET2*-targeting effect of this miRNA. Alternatively, the different efficiencies of *TET2* targeting by different miRNAs may themselves contribute to the varying phenotypes. In this regard, it is important to note that minor changes in *TET2* expression cannot only lead to functional consequences in malignant hematopoiesis but also be associated with longer latency or incomplete penetrance. For example, heterozygous *TET2* knockout in mouse, which led to ~50% loss of *TET2* gene expression (Li et al., 2011), results in significant but slower and less-frequent malignant transformation than double-allele knockout (Li et al., 2011; Quivoron et al., 2011; Moran-Crusio et al., 2011). As a third possibility, it is also conceivable that such in vivo phenotype differences were due to different levels of overexpression of *TET2*-targeting miRNAs (Figure S4). It will be interesting to dissect these possibilities in the future.

Finally, our data raise the prospect of enhancing *TET2* expression to combat certain subgroups of hematopoietic malignancies, and implicate modulating *TET2*-targeting miRNAs as a strategy for both solid and hematopoietic cancers. Recently, decreased *TET* gene expression and cellular 5hmC levels have been found as a hallmark of multiple solid cancer types (Yang et al., 2013; Lian et al., 2012; Hsu et al., 2012), whereas elevating *TET1* or *TET2* gene expression has been proposed as a strategy against melanoma and breast cancer (Lian et al., 2012; Hsu et al., 2012). When we expressed *TET2* together with oncogenic miR-29b and miR-125a, we observed strong suppression of miRNA-mediated malignant phenotypes. In the case of miR-125a, *TET2* expression not only suppressed hematopoietic expansion but remarkably also corrected multiple differentiation biases induced by miR-125a. These data suggest that targeting mechanisms that inhibit *TET2* gene expression may be a useful strategy to overcome certain hematopoietic malignancies. Our findings of an extensive network of *TET2*-targeting miRNAs and several pan-TET-inhibitory miRNAs raise the possibility and opportunity for future therapeutic intervention in this pathway.

## EXPERIMENTAL PROCEDURES

### Luciferase Reporter Assay and Analysis

Reporter assays were carried out in 384-well plates. Specifically, 460 miRNA constructs were individually assayed in combination with 3′ UTR luciferase reporters. 293T cells were transfected with 6 ng of 3′ UTR reporter and 54 ng of a miRNA construct in each well. After 2 days, luciferase assays were performed using the Dual-Glo Luciferase kit (Promega). We built three types of control assays into each 384-well plate: one or more CtrlUTR-CtrlMiR assays (a control reporter assayed with a control vector for miRNA expression of the pMIRWAY-puro backbone); two UTR-CtrlMiR assays (*TET2* reporter assayed with a control vector); and multiple CtrlUTR-miR assays (a control reporter assayed with each of the miRNAs on the plate).

Data analysis was performed using custom MATLAB codes. We first took the ratio of renilla luciferase versus firefly luciferase readings (RvF ratios). The RvF ratio of any given well, including controls, was then normalized using the following formula: normalized luciferase activity = ($RvF_{Well}$/mean($RvF_{UTR\text{-}CtrlMiR}$))/(mean($RvF_{CtrlUTR\text{-}miR}$)/mean($RvF_{CtrlUTR\text{-}CtrlMiR}$)). After normalization, the means of all three control assays become 1. We used mean data to identify miRNA-3′ UTR relations that resulted in >25% downregulation. To

identify *TET2*-targeting miRNAs, we excluded constructs for clusters of miRNAs and categorized the same mature miRNA appearing at different genome loci as only one *TET2*-targeting miRNA. See Extended Experimental Procedures for more details.

### Murine Bone Marrow Transplantation and Related Experiments

All mouse experiments were approved by Yale IACUC and followed federal, state, and institutional guidelines. Bone marrow transplantation with single miRNAs cloned into the pMIRWAY-GFP-based vectors was performed as described previously (Guo et al., 2012). These miRNAs include miR-29b-1, miR-125a, miR-26a-1, miR-29c, miR-101-1, miR-767, miR-520d, miR-33, miR-153-2, miR-144, and a vector control.

For *Tet2* rescue experiments, 5-FU-primed bone marrow cells were cotransduced with mouse *Tet2* cDNA (with puromycin marker), or a corresponding vector control together with a specific miRNA expression construct in pMIRWAY-GFP backbone. Transduced cells were cultured, selected with puromycin, and sorted for GFP+ cells for transplantation. A total of 50,000 cells (per mouse) were injected for miR-125a-related rescue and control groups, and 10,000 cells (per mouse) were injected for the miR-29b rescue and control groups.

Assessment of hematopoietic phenotypes was performed as previously described (Guo et al., 2010, 2012; Adams et al., 2012). The myeloid bias index was used to quantify biased differentiation into myeloid lineages, which was calculated by (%GFP+Mac1+)/(%GFP+Mac1−)/((%GFP−Mac1+)/(%GFP−Mac1−)). To examine monocytic differentiation bias, Mac1+ cells from GFP+ fraction (transduced) were gated before examining Ly6C and Ly6G distribution. To examine hematopoietic expansion, peripheral %GFP+/%GFP− ratios were taken and normalized to the ratio at 3.5 weeks posttransplantation. All flow cytometry antibodies were from BD Biosciences or eBioscience.

For assessment of in vivo effect of miRNA overexpression levels or 5hmC levels, Mac1+GFP+ cells was FACS-sorted from recipient mice and subjected to qRT-PCR or 5hmC analysis.

### Clinical Samples and *TET2* Sequence Analysis

All human AML samples were obtained with informed consent and approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (FAHSU). A total of 67 Chinese patients with AML with normal cytogenetics were enrolled between March 2005 and September 2009 at FAHSU, with a median age of 43 years (range 18–76), and a female:male ratio of 28:39. Diagnosis and classification of these patients were defined according to the French-American-British (FAB) and World Health Organization (WHO 2008) classifications. Bone marrow samples were collected at presentation. Mononuclear cells were separated by Ficoll Hypaque, frozen and banked, and subjected to genomic DNA and total RNA preparations. *TET2* mutations were analyzed by PCR amplification of the entire coding region spanning exon 3 to exon 11 followed by direct bidirectional DNA sequencing, as previously described by Delhommeau et al. (2009). For analysis purpose, samples with nonsynonymous coding sequence alterations were classified as *TET2* mutant, whereas those without amino acid-altering coding region changes were defined as *TET2*-wild-type (Table S3).

### Statistical Analysis

The Student's t test was used to assess statistical significance, unless otherwise stated. The false discovery rate was calculated following the Benjamini Hochberg method.

### Additional Procedures

Constructs, computational target analysis, cell culture, western blot and dot blot analyses, quantitative RT-PCR, colony formation assays, and miRNA profiling and data analyses are described in Extended Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.050.

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