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MIR125B1 represses the degradation of the PML-RARA oncoprotein by an autophagy-lysosomal pathway in acute promyelocytic leukemia

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Abbreviations: 3′UTR, 3′-untranslated region; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; ATO, arsenic trioxide

Acute promyelocytic leukemia (APL) is characterized by the t(15;17)-associated *PML-RARA* fusion gene. We have previously found that *MIR125B1* is highly expressed in patients with APL and may be associated with disease pathogenesis; however, the mechanism by which *MIR125B1* exerts its oncogenic potential has not been fully elucidated. Here, we demonstrated that *MIR125B1* abundance correlates with the PML-RARA status. *MIR125B1* overexpression enhanced *PML-RARA* expression and inhibited the ATRA-induced degradation of the PML-RARA oncoprotein. RNA-seq analysis revealed a direct link between the PML-RARA degradation pathway and *MIR125B1*-arrested differentiation. We further demonstrated that the *MIR125B1*-mediated blockade of PML-RARA proteolysis was regulated via an autophagy-lysosomal pathway, contributing to the inhibition of APL differentiation. Furthermore, we identified DRAM2 (DNA-damage regulated autophagy modulator 2), a critical regulator of autophagy, as a novel target that was at least partly responsible for the function of *MIR125B1* involved in autophagy. Importantly, the knockdown phenotypes for *DRAM2* are similar to the effects of overexpressing *MIR125B1* as impairment of PML-RARA degradation, inhibition of autophagy, and myeloid cell differentiation arrest. These effects of *MIR125B1* and its target DRAM2 were further confirmed in an APL mouse model. Thus, *MIR125B1* dysregulation may interfere with the effectiveness of ATRA-mediated differentiation through an autophagy-dependent pathway, representing a novel potential APL therapeutic target.

Introduction

Acute promyelocytic leukemia is a unique subtype of acute myeloid leukemia (AML) that accounts for approximately 10% to 15% of all cases.[1] The molecular feature of APL is an aberrant chromosomal translocation that juxtaposes the *PML* gene with the *RARA* gene,[2] and the subsequent expression of the PML-RARA fusion protein blocks the differentiation of promyelocytes and leads to an accumulation of leukemic promyelocytes. All-trans retinoic acid (ATRA) has successfully been used as a leukemia therapy to target the transcriptional repression mediated by the PML-RARA fusion protein. ATRA has been found to cause disease regression specifically in patients with APL,[3,4] and it induces the degradation of the chimeric protein encoded by the *PML-RARA* oncogene.[5-7]

Understanding the mechanisms involved in APL pathogenesis and its responsiveness to treatment will allow us to identify novel APL treatment targets. It has been unexpectedly found that 2 therapeutic agents, ATRA and arsenic trioxide (ATO), degrade PML-RARA. More recently, studies have demonstrated that autophagy contributes to the therapy-induced degradation of PML-RARA.[7,8] Autophagy is a biological process in which cytoplasmic constituents are sequestered into double-membrane vesicles called autophagosomes and then fuse with lysosomes in which the sequestered contents are degraded.[9,10] Previous studies have also revealed the proteasome system as a primary mechanism involved in the therapy-induced degradation of PML-RARA.[6] However, proteasome and caspase inhibitors do not fully block PML-RARA degradation,[6,11] suggesting that alternative mechanisms may be involved. Moreover, the

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PML-RARA oncoprotein is prone to aggregation, which makes it a preferred substrate for autophagic degradation.[12]

A number of studies have indicated that microRNAs (miRNAs) play crucial roles in fundamental biological processes, including the cell cycle, differentiation, development, and apoptosis.[13-16] It has been demonstrated that miRNAs regulate gene expression by binding to the 3′-untranslated regions (3′UTR) of target mRNAs, which leads to translational suppression. Our group and others have reported the unique miRNA profiles associated with the main AML cytogenetic and molecular subgroups.[17-19] In particular, the t(15;17) translocation leads to elevated *MIR125B1* (microRNA 125b1) expression;[19] however, it is not clear how *MIR125B1* is correlated with PML-RARA status and in which pathways the miRNA may be involved. Although the PML-RARA oncoprotein blocks differentiation and promotes self-renewal in cell lines, this protein causes leukemia in mice only after a long latency period.[20] This finding suggests that PML-RARA may require cooperating events to cause leukemia. Thus, we hypothesized that *MIR125B1* upregulation may be important for the effects of PML-RARA on cell differentiation in APL or may affect the therapy-induced clearance of PML-RARA.

To test our hypothesis, we investigated correlations between *MIR125B1* expression and *PML-RARA* fusion oncogene expression in APL in vivo and in vitro. We found that *MIR125B1* could activate *PML-RARA* expression and repress therapy-induced PML-RARA proteolysis by an autophagy-lysosome pathway. Thus, *MIR125B1* is critical for PML-RARA activities and for cell differentiation, suggesting a novel potential therapeutic target for APL.

Results

PML-RARA promotes *MIR125B1* expression

MIR125B1 overexpression has been described as an important oncogenic event, and consistent abnormalities in *MIR125B1* expression were found in several cases of chromosomal translocation, including t(2;11)(p21;q23) and t(11;14)(q24;q32).[14,21] Our previous study also demonstrates that *MIR125B1* expression is strongly upregulated in patients with APL compared with other AML subtypes and indicated that *MIR125B1* may be involved in APL pathogenesis.[19] Here, we further investigated the expression of *MIR125B1* and *PML-RARA* in the patients before and after therapy to establish the relationship between *MIR125B1* and *PML-RARA*. We found that the *MIR125B1* expression levels correlated well with the level of *PML-RARA* transcripts (**Fig. 1A**, left panel). In patients with t(15;17) primary APL, *MIR125B1* expression was significantly higher than that in complete remission (CR) patients (**Fig. 1A**, right panel). Similarly, in non-CR patients (nonremission, including relapse samples), the *MIR125B1* expression level was higher than that in CR patients. Because therapy-induced treatment of APL led to the clearance of leukemia cells and *PML-RARA* transcripts, we then asked whether the fusion protein is necessary for *MIR125B1* expression or whether *MIR125B1* promotes *PML-RARA* expression. To address this question, we employed a small interfering RNA (siRNA), which specifically targets the breakpoint region of *PML-RARA*, named as si-*PML-RARA* hereafter.[22] Transfection of si-*PML-RARA* resulted in knockdown of *PML-RARA* expression (**Fig. S1A**). Knockdown of *PML-RARA* in NB4 cells led to the downregulation of pri-microRNA (pri-*MIR125B1*) (**Fig. 1B**, top panel) and mature miRNA (*MIR125B1*) (**Fig. 1B**, bottom panel). Next, we employed an additional cell line, U937-PR9, which carries a *PML-RARA* gene that can be induced with $ZnSO_4$ treatment. We first determined the *PML-RARA* fusion transcript expression level upon $ZnSO_4$ treatment and found that the *PML-RARA* transcript was significantly upregulated in cells after 100 μM $ZnSO_4$ stimulation (**Fig. S1B**). The increased *PML-RARA* transcript induced both pri-*MIR125B1* (**Fig. 1C**, left panel) and *MIR125B1* (**Fig. 1C**, right panel) expression (**Fig. 1C**), indicating that *MIR125B1* upregulation may be a consequence of PML-RARA accumulation.

MIR125B1 promotes *PML-RARA* expression and inhibits PML-RARA degradation

In addition to the effects of PML-RARA on *MIR125B1* expression, we then tested whether *MIR125B1* promotes *PML-RARA* expression. For this purpose, NB4 cells were infected with lentiviruses carrying the *MIR125B1* gene (named as NB4-Lv-*MIR125B1* cells hereafter) or a negative control (NB4-Lv-NC cells) to explore the effects of *MIR125B1* on the fusion protein. ATRA treatment was also examined. The results demonstrated that the level of the PML-RARA oncoprotein was increased in cells overexpressing *MIR125B1*, especially when with ATRA treatment (**Fig. 2A**). We also confirmed these results with ATO-treated cells (**Fig. 2B**). Interestingly, we found that stable ectopic expression of *MIR125B1* was able to promote *PML-RARA* expression at the transcriptional level. As shown in **Figure 2C**, *MIR125B1* overexpression increased the expression of *PML-RARA* at time point 0 h. In general, *PML-RARA* increases in the mRNA level after being treated with ATRA,[23] however, this effect was inhibited by *MIR125B1* overexpression. It can be observed that *PML-RARA* mRNA gradually increased in NC group, while decreased in the *MIR125B1* overexpression group, especially at the time point of 48h with ATRA treatment (**Fig. 2C**). In contrast, knockdown of *MIR125B1* by antagomir-125B (a synthetic *MIR125B1* antisense) decreased the expression of *PML-RARA* in primary APL patient bone marrow mononuclear cells, which expressed a high level of endogenous *MIR125B1* (**Fig. S1C**). To exclude the transcriptional impact on *PML-RARA* expression by stable overexpression of *MIR125B1*, we further measured the PML-RARA oncoprotein levels via transiently transfecting cells with *MIR125B1* mimics and treatment with or without ATRA. As shown in **Figure 2D**, when NB4 cells were treated with ATRA shortly after transiently transfecting *MIR125B1*, the PML-RARA level in the NC cells was not significantly changed when compared with that in *MIR125B1* cells prior to treatment (lane 1 and 2 in **Fig. 2D**, and **Fig. S1D**); however, the amount of PML-RARA oncoprotein was increased in cells transfected with *MIR125B1* after ATRA treatment (**Fig. 2D**, lane 3 and 4), suggesting that *MIR125B1* could impair PML-RARA degradation. Moreover, inhibition of endogenous *MIR125B1*

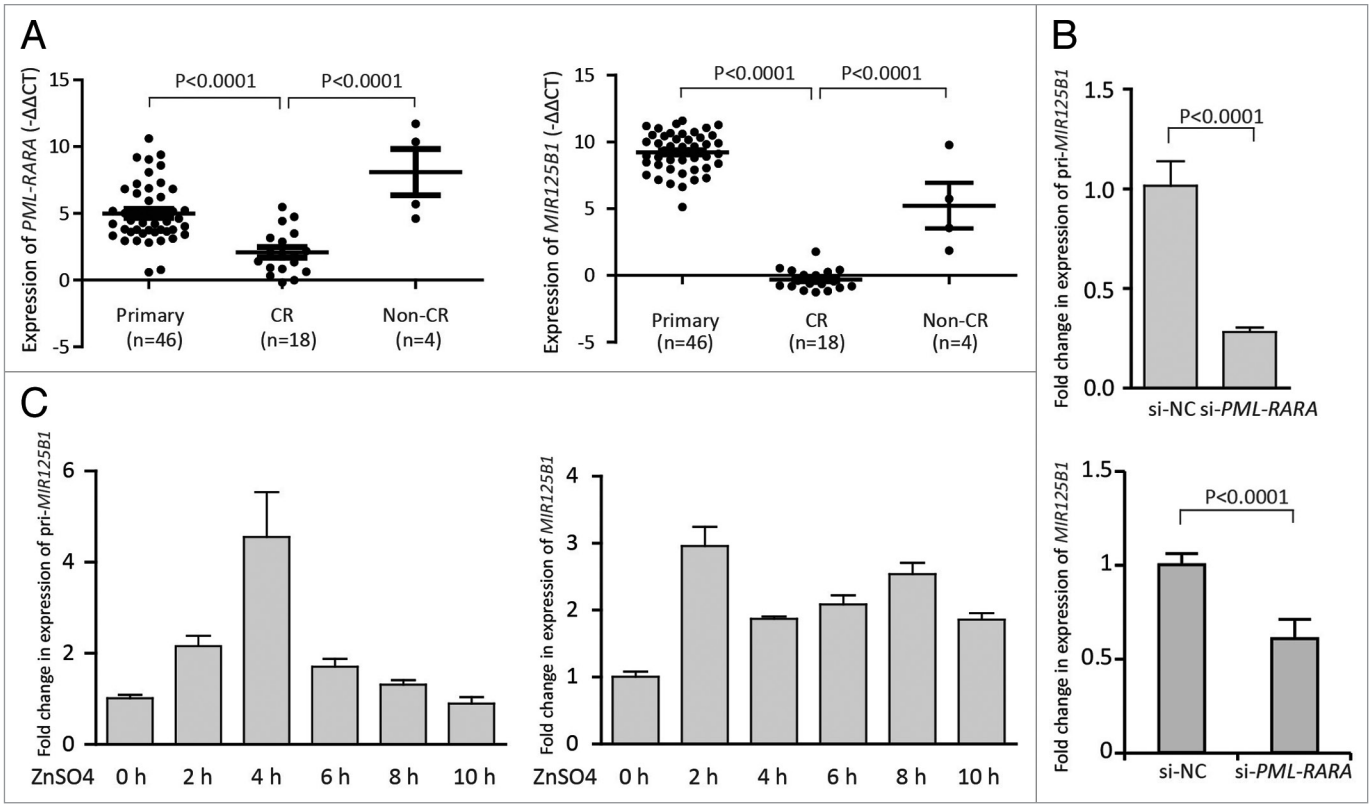

Figure 1. *MIR125B1* is induced by PML-RARA. (**A**) The expression of *PML-RARA* in patients with t(15;17) APL before and after therapy. Left panel, the expression of *PML-RARA* mRNA in patients with t(15;17) APL before and after therapy. Right panel, the expression of *MIR125B1* in patients with t(15;17) APL before and after therapy. This comparison revealed that APL therapy had broadly similar effects on *PML-RARA* and *MIR125B1* expression. (**B**) qRT-PCR analysis of pri-*MIR125B1* and mature *MIR125B1* after knocked down the *PML-RARA*. (**C**) qRT-PCR analysis of pri-*MIR125B1* and mature *MIR125B1* in U937-PR9 cells treated with 100 μM $ZnSO_4$. Experiments were performed in triplicate, and error bars represent SD in all panels. CR: samples in first complete remission; non-CR: noncomplete remission, including relapse samples.

using synthetic *MIR125B1* antisense (anti-*MIR125B1*) facilitated PML-RARA degradation (**Fig. S1E**). These data indicate that *MIR125B1* not only activates *PML-RARA* expression but also inhibits ATRA-induced PML-RARA degradation, showing a close correlation between *MIR125B1* levels and PML-RARA.

Transcriptome analysis indicated that *MIR125B1*-arrested differentiation might partly act through the PML-RARA degradation pathway

Cell differentiation is likely to be a determinant of the clinical response of APL, and *MIR125B1* has been implicated in myeloid cell differentiation arrest.[14] Because of the remarkable association between *MIR125B1* expression and PML-RARA, we further investigated the effects of *MIR125B1* on PML-RARA-regulated genes upon treatment. For this purpose, we performed high throughput RNA-seq (quantification) with RNA from NB4-Lv-*MIR125B1* and NB4-Lv-NC cells treated with or without ATRA to identify genes or pathways that *MIR125B1* may be mediated. We generated an average of 7279023 (range: 7140608- 7454744) reads per sample, and the gene expression profiles derived from RNA-Seq were calculated using the RPKM method.[24] A hierarchical clustering analysis of the differentially expressed genes revealed that the majority of these genes are potentially regulated by *MIR125B1* after treated with ATRA (**Fig. 3A**), including *CEBPB*, *SPI1*, *IFIT3,* and *CEBPE*, which have been reported previously.[25,26] These results are consistent with the differentiation arrest effects of *MIR125B1*.[14] The differential genes are shown in **Table S1**. To confirm the RNA sequencing data, we selected several genes for validation by qRT-PCR (**Fig. S2A**). Next, we compared our mRNA-seq results with recently published data that included potential PML-RARA targets using ChIP-on-chip experiments.[27] We found that there was a significant overlap in the expression of potential PML-RARA target genes that were altered in the presence of *MIR125B1* upon ATRA induction (**Fig. S2B**), indicating that *MIR125B1* blocked PML-RARA-regulated genes which could be relieved upon ATRA treatment. This finding suggests that the effect of *MIR125B1* on APL at least partially occurs via PML-RARA. In addition, using KEGG pathway analysis,[28] several important signal transduction pathways, including the lysosome, MAPK, toll-like, and JAK-STAT pathways (**Tables S2 and S3**), were modulated by ATRA, and the effect of ATRA is impaired in the cells with *MIR125B1* overexpression. Notably, previous studies have indicated an important role for lysosomes or the lysosome-dependent pathway in PML-RARA degradation and APL cell differentiation after therapeutic treatment.[7,29] Collectively, these results suggest that PML-RARA degradation may be involved in

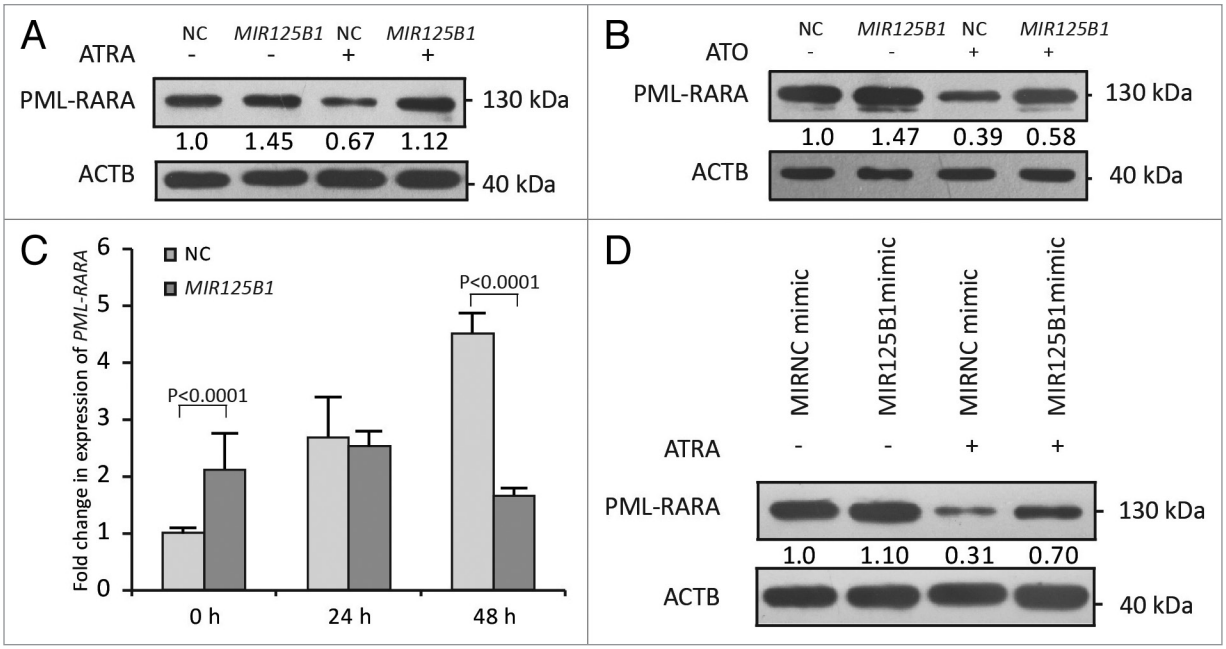

Figure 2. *MIR125B1* increased the PML-RARA oncoprotein level. (**A**) Western blot of NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with or without ATRA (1 μM) for 48 h. (**B**) Western blot of NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with or without ATO (1 μM) for 24 h. (**C**) *MIR125B1* increases the *PML-RARA* mRNA level upon ATRA treatment. Gene expression was normalized relative to *ACTB* (n = 3 independently obtained biological samples). (**D**) Immunoblot analyses of PML-RARA after transient transfection with *MIR125B1*, followed by treatment with ATRA (1 μM, 48 h) or not. Cells lysates were prepared for western blotting with antibodies against RARA. ACTB expression served as a loading control.

MIR125B1-mediated cell differentiation arrest, and *MIR125B1* expression may have a direct bearing on PML-RARA during differentiation therapy.

MIR125B1 inhibits the ATRA-induced clearance of PML-RARA by blocking the autophagy-lysosomal pathway

The above findings suggest that *MIR125B1* may regulate the lysosomal pathway in ATRA-induced PML-RARA oncoprotein degradation and cell differentiation. Recent studies indicated a close link between the lysosomal pathway and autophagic degradation and demonstrated the importance of autophagy in APL differentiation and PML-RARA oncoprotein degradation.[7,8] To better understand the role of autophagy in PML-RARA degradation, we first examined the expression of PML-RARA when degradation was blocked by 2 inhibitors chloroquine or bafilomycin A_1. Both inhibitors interrupt the autophagosome-lysosome fusion step.[10] Consistent with these previous studies, chloroquine and bafilomycin A_1 significantly blocked ATRA-induced degradation of PML-RARA (**Fig. S3A and S3B**). Additionally, inhibition of autophagy by knockdown of *ATG5* decreased degradation of PML-RARA (**Fig. S3C**). We therefore asked whether *MIR125B1* could impair the degradation of PML-RARA via the autophagy-lysosomal pathway. To address this question, we first investigated the expression of a number of genes in the autophagy and lysosomal pathways in our RNA-seq data set. We found that lysosomal and autophagic genes were modulated by ATRA but the modulations were impaired in the presence of *MIR125B1* (**Fig. 3B and C**). To further explore whether *MIR125B1* inhibits the ATRA-induced clearance of PML-RARA through the autophagy-lysosomal pathway, we next examined the effects of *MIR125B1* on ATRA-induced autophagy and lysosomal biogenesis. Ultrastructural analysis (**Fig. 4A**) and immunofluorescence (**Fig. 4B**) revealed that the ability of ATRA to induce autophagy was considerably reduced in the presence of *MIR125B1*. Accordingly, the expansion of the lysosomal compartment by ATRA was also impaired in NB4 cells that stably overexpressed *MIR125B1* (**Fig. 5A**), indicating *MIR125B1* involvement in lysosomal biogenesis. Furthermore, western blotting was used to detect LC3, whose cleaved and lipidated form (LC3-II) is widely regarded as an autophagosome marker. LC3-II levels were increased in cells treated with ATRA but repressed in cells overexpressing *MIR125B1* (**Fig. 5B**). We further observed an increased autophagic flux in cells transfected with a synthetic *MIR125B1* antisense (anti-*MIR125B1*) (**Fig. S3D**). To further validate the link between *MIR125B1* and the autophagy pathway, cells were treated with an autophagy inducer (rapamycin) in the presence or absence of ATRA. We found that PML-RARA expression was markedly decreased in NB4-Lv-*MIR125B1* cells after cotreatment with rapamycin and ATRA (**Fig. 5C**). Taken together, our findings suggest that *MIR125B1* inhibits PML-RARA proteolysis via the autophagy-lysosomal pathway.

MIR125B1 inhibits degradation of PML-RARA fusion protein by targeting DRAM2, a critical regulator of autophagy

We then explored the molecular mechanism of *MIR125B1* in autophagy. TargetScan was used to generate a list of *MIR125B1* target candidates. Several genes (*DRAM2*, *ATG4D*, *UVRAG*) associated with autophagy were found to have *MIR125B1*-binding sites in their 3′UTRs (**Fig. 6A**). Dual-luciferase reporter analysis revealed that *MIR125B1* directly suppressed the

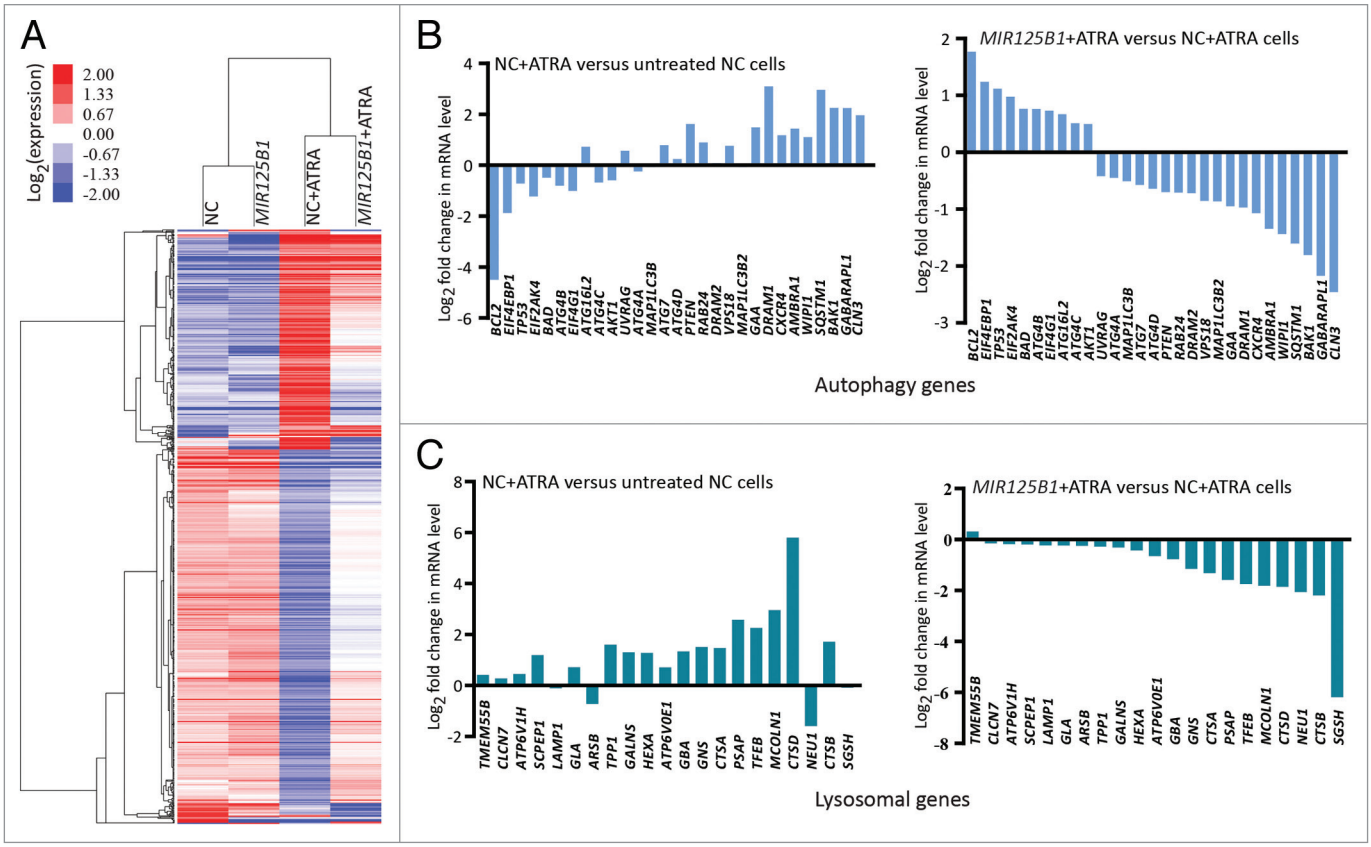

Figure 3. *MIR125B1* attenuated the expression of a subset of PML-RARA target genes involved in myeloid differentiation. (**A**) Heatmap displaying the hierarchical clustering of genes in NB4-Lv-NC and NB4-Lv-*MIR125B1* cells in response to ATRA treatment. The color intensity represents the degree of expression. A red-blue color scale was used to reflect standardized gene expression, with red representing a high expression and blue representing a low expression (scale shown in the upper left). A cutoff value of FDR ≤ 0.001 and a $\log_2$-fold change ratio ≥ 1 change relative to the untreated groups was selected. The identities of these genes are listed in **Table S1**. (**B and C**) Enforced *MIR125B1* expression in NB4 cells repress the modification of a vast cast of lysosome and autophagy genes to ATRA stimuli, Related to (**A**) expression profiling of all genes whose expression is specifically altered in NB4 cells.

luciferase activity of *DRAM2*, *ATG4D*, and *UVRAG* (**Fig. 6B**). Notably, both *MIR125B1* and the binding site in the *DRAM2* are highly conserved across species, and DRAM2 was recently found to be involved in autophagy.[30] To further confirm that the DRAM2 protein is suppressed by *MIR125B1*, we performed both *MIR125B1* overexpression and knockdown experiments in NB4 cells and examined the expression of *DRAM2*. As shown in **Figure 6C**, *MIR125B1* overexpression caused an apparent decrease in the amount of DRAM2 in NB4 cells (upper panel), while inhibition of endogenous *MIR125B1* using a synthetic *MIR125B1* inhibitor increased DRAM2 expression (bottom panel). To gain further insight into whether *MIR125B1* suppresses autophagy by regulating DRAM2, we examined the function of DRAM2 in ATRA induction. We employed an siRNA (si-*DRAM2*) to knock down DRAM2 and then used immunoblotting assay to confirm that DRAM2 expression was effectively silenced (**Fig. S3E**). We next investigated the function of DRAM2. When treated with ATRA, DRAM2 knockdown resulted in increasing amounts of PML-RARA oncoprotein (**Fig. 6D**, left panel), inhibition of autophagy (**Fig. 6D**, right panel and **Fig. S4A**), and myeloid cell differentiation arrest (**Fig. 6E**). We have also employed a second siRNA sequence against *DRAM2* to confirm the functions of DRAM2 in APL cell differentiation and autophagy (**Fig. S4B–S4D**). These results showed that knockdown of DRAM2 phenocopied the effects of overexpressing *MIR125B1*, further validating that DRAM2 is a functional target of *MIR125B1*. All together, this suggested that *MIR125B1* may suppress the autophagy-lysosomal pathway, at least in part, by repressing DRAM2 and subsequently impairment of PML-RARA proteolysis and myeloid cell differentiation.

***MIR125B1* overexpression inhibits PML-RARA proteolysis and leukemic cell differentiation in a mouse model**

The observations above indicate that *MIR125B1* is associated with *PML-RARA* expression and may be involved in the development and treatment of APL. To further understand the function of *MIR125B1* in APL pathogenesis and cell differentiation therapy, a human APL-ascites SCID mouse model was employed to study the response of *MIR125B1* to therapy. A diagram illustrating the experimental design used for the APL-ascites NOD-SCID mouse model is shown in **Figure 7A**. When the NOD-SCID mice developed an ascites burden, the ascitic fluid was withdrawn, and the ascitic cells carrying the *MIR125B1*, a miRNA negative control, or NB4 cells alone (called A-NB4-*MIR125B1*, A-NB4-NC and A-NB4, respectively) were

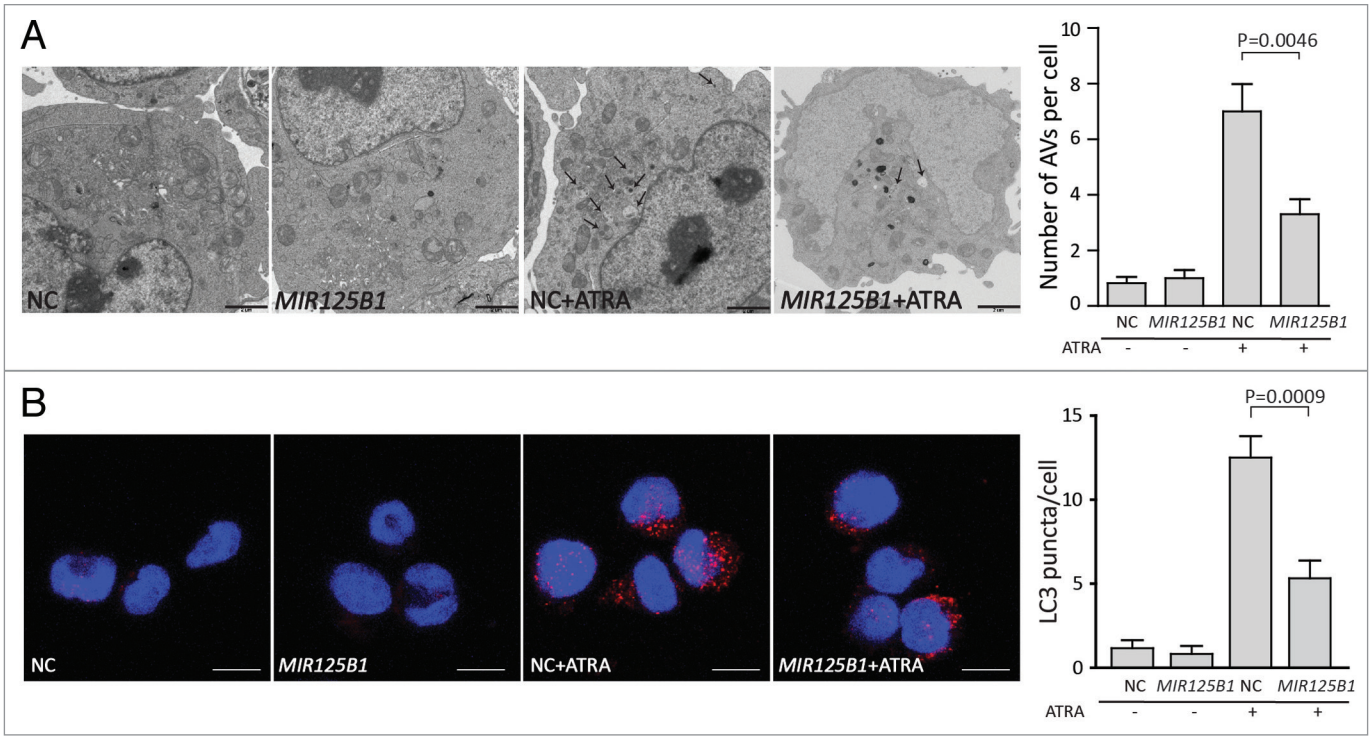

Figure 4. *MIR125B1* impaired the autophagy-lysosomal-dependent degradation of PML-RARA. (**A**) The ultrastructure of NB4-Lv-NC and NB4-Lv-*MIR125B1* cells was observed by electron microscopy after ATRA treatment. The number of autophagosomes observed by transmission electron microscopy was calculated (right panel, n = 10 cells). NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with ATRA (1 μM, 48 h) or not. (**B**) Confocal microscopy after staining with an antibody directed against LC3. NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with ATRA (1 μM, 48 h) or not. A representative image of 3 independent experiments is shown. Scale bar: 10 μm.

cultured in RPMI 1640 medium and treated with 1 μM ATRA. Cell differentiation was detected by FACS analysis (**Fig. S5A**) and Wright-Giemsa staining (**Fig. 7B**). A-NB4-*MIR125B1* cells demonstrated less differentiation than the other cell types. In addition, we measured cell proliferation at 24, 48, and 72 h using a CCK-8 assay and found that the proliferation of A-NB4-*MIR125B1* cells was stimulated by *MIR125B1* overexpression (**Fig. S5B**). Overexpression of *MIR125B1* resulted in inhibition of autophagy and PML-RARA proteolysis (**Fig. 7C**; **Fig. S5C**). We also generated a transplantation model with DRAM2 knockdown cells. As shown in **Figure 7C**, silencing DRAM2 expression by infecting with lentivirus expressing shRNAs targeting *DRAM2* impaired PML-RARA degradation in vivo. These results indicate that *MIR125B1* overexpression impairs degradation of PML-RARA via the autophagy-lysosomal pathway in SCID-APL cells undergoing differentiation therapy (**Fig. 7E**).

Discussion

In this study, we found that *MIR125B1* abundance correlated with PML-RARA status in patients with APL and activated *PML-RARA* expression. Furthermore, we provided the in vitro and in vivo evidence that the degradation of PML-RARA via the autophagy-lysosomal pathway is impaired by *MIR125B1* and that this impairment subsequently arrests cell differentiation.

PML-RARA is the initiating factor for APL development and blocks promyelocyte differentiation. However, leukemia development in transgenic mice expressing PML-RARA occurs after a long latency period,[31] which strongly suggests that additional genetic events collaborate with PML-RARA to block differentiation and stimulate a number of APL features. Previous studies have demonstrated that *MIR125B1* is able to confer a competitive advantage on engrafting hematopoietic cells,[32] and *MIR125B1* overexpression is a consistent abnormality in several patients with chromosomal translocations.[14,21] In particular, *MIR125B1* is highly expressed in patients with APL, suggesting that it could be a primary oncogenic event in APL leukemogenesis. However, the underlying molecular mechanisms of *MIR125B1* in APL remain largely unknown. The observations in this study indicated that loss of PML-RARA decreased *MIR125B1* expression. Interestingly, *MIR125B1* also increased *PML-RARA* expression, suggesting that PML-RARA and *MIR125B1* might form a positive feedback loop. In line with the increased *PML-RARA* expression by *MIR125B1*, we found that a stable ectopic expression of *MIR125B1* affected some of the mRNA transcript levels of hypothetical target genes involved in PML-RARA regulation (**Fig. S5D**). Thus, it is possible that *MIR125B1* regulates the expression of PML-RARA through altered interaction of the *PML-RARA* promoter with transcription factors affected by target genes of *MIR125B1* (**Fig. S6**). Given that a high level of PML-RARA appears to be required during the initiation stages

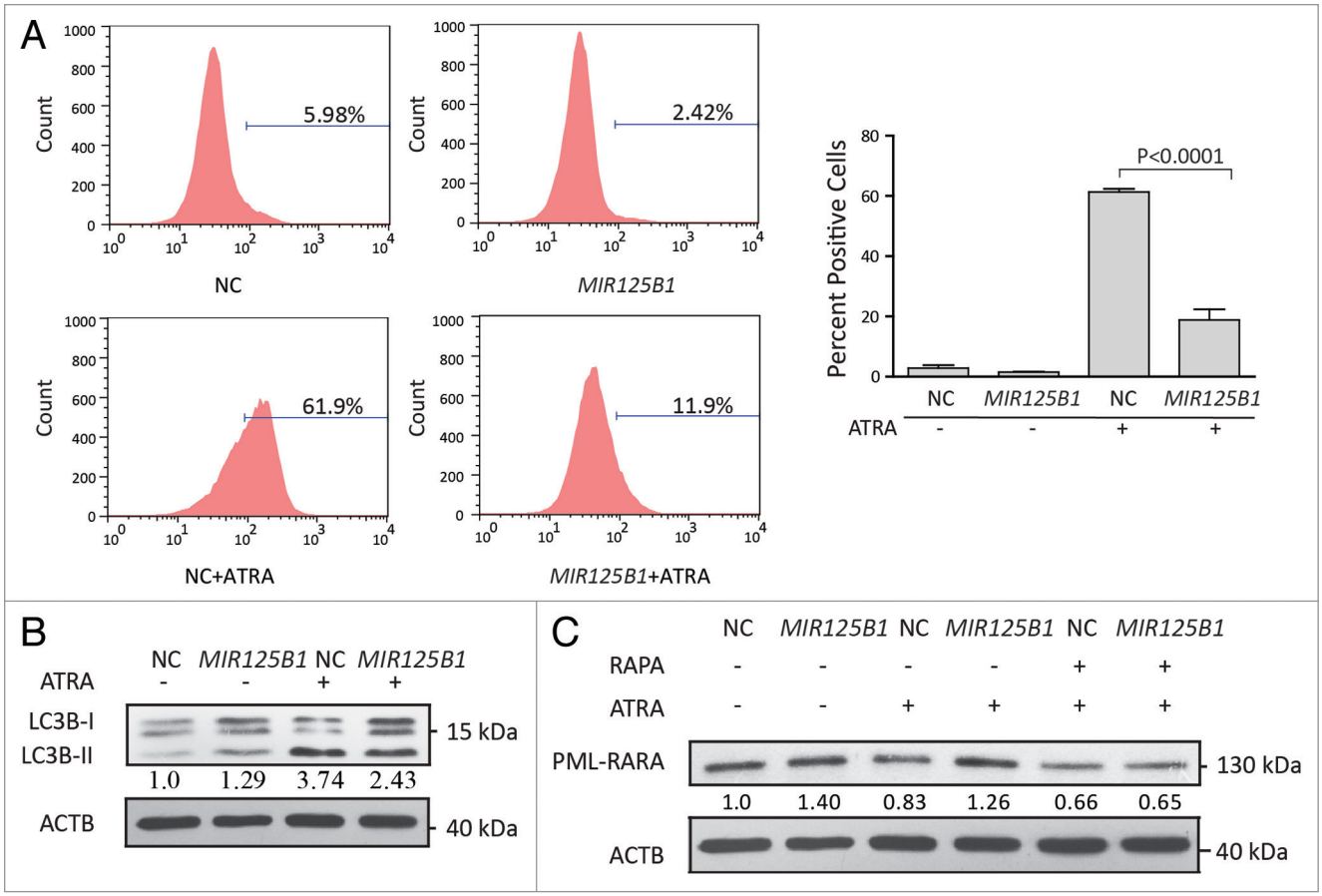

Figure 5. Effect of *MIR125B1* overexpression on autophagy. (**A**) Fluorescence-activated cell sorting (FACS) analysis after staining with the lysosome-specific dye LysoTracker Red. The analysis was performed in triplicate, and 30,000 cells were analyzed. The bars indicate the proportion of cells with fluorescence intensity greater than the indicated threshold. (**B**) Western blot analyses of LC3 in NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with ATRA. ACTB was used as a loading control. LC3-II/ACTB densitometric ratios were recorded. (**C**) Western blot of NB4-Lv-NC and NB4-Lv-*MIR125B1* cells treated with 1 μM ATRA or in combination with rapamycin (100 nM) for 24 h. ACTB was used as a loading control. PML-RARA/ACTB densitometric ratios were recorded.

of cancer,[5,33] the strong *MIR125B1* upregulation may promote the initiation of oncogenesis by PML-RARA. This finding is the first evidence that an miRNA cooperates with a fusion protein and has a critical role in leukemia development and response to treatment. However, whether this cooperation is essential for APL initiation requires further investigation.

It has been reported that the treatment of cultured APL cells with ATRA triggers the differentiation of malignant promyelocytes via transcriptional regulation.[34] Similarly, in our study, numerous differentially expressed genes after ATRA treatment were initially repressed by PML-RARA; this repression can be relieved by ATRA and restored by *MIR125B1*. The involvement of PML-RARA target genes, together with enrichment for genes belonging to the PML-RARA degradation pathway, led to the assumption that there is a direct link between *MIR125B1*-mediated differentiation arrest and PML-RARA degradation. These findings are of particular interest because *MIR125B1* and PML-RARA have been closely linked with APL.

It has been demonstrated that ATRA exerts its therapeutic effect by promoting the degradation of the PML-RARA oncogenic protein.[6-8] Therapy-induced PML-RARA degradation allows the transcriptional derepression of PML-RARA target genes, and PML-RARA knockdown results in spontaneous differentiation in APL models.[22] Moreover, the inhibition of PML-RARA degradation leads to a differentiation block.[7,8] We conclude that PML-RARA destruction is not merely a consequence of differentiation, but it plays an important role in the induction of differentiation. Indeed, other studies have found that PML-RARA remains a potent repressor even in the presence of ATRA.[23,35] In line with these observations, the *MIR125B1*-mediated inhibition of transcriptional derepression occur simultaneously with high PML-RARA oncoprotein levels and a differentiation block, and the degradation impairment contributes to differentiation arrest. Although our data suggest a role for PML-RARA degradation in the effects of *MIR125B1* on cell differentiation, we cannot exclude that *MIR125B1* upregulates *PML-RARA* at the transcriptional level and leads to an increase in the amount of fusion protein, which may also contribute to the function of *MIR125B1* during differentiation.

The degradation of PML-RARA has been shown to be involved in the activity of the ubiquitin-proteasome and autophagy-lysosomal pathways.[6-8] However, the PML-RARA

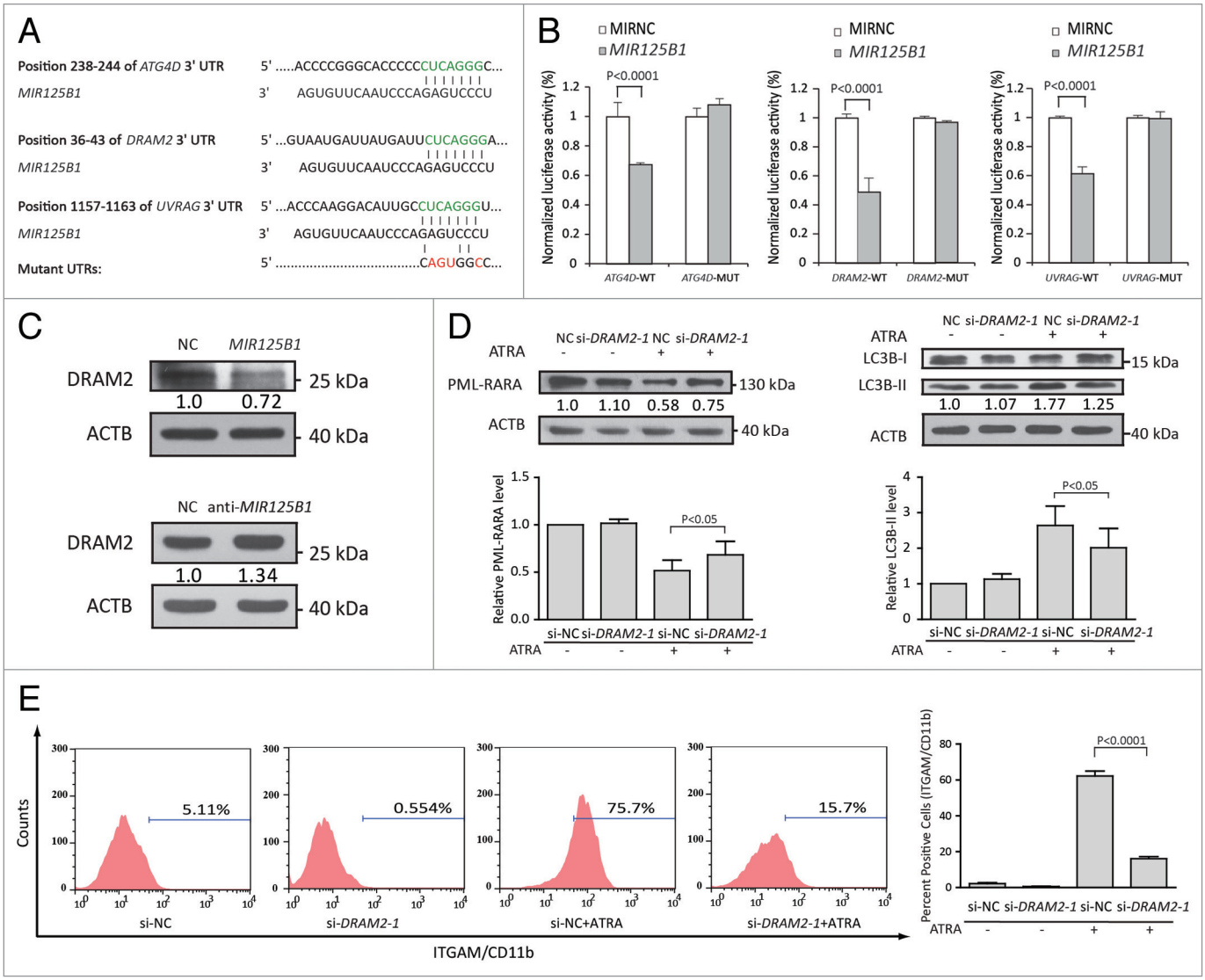

Figure 6. *MIR125B1* directly targeted autophagy genes involved in autophagy, PML-RARA degradation, and differentiation. (**A**) Schematic representation of the interaction of miRNA with the 3′UTR of its corresponding targets. The miRNA response elements (MRE) in the 3′UTR of human were predicted by TargetScan and in green. Each predicted MRE 3′UTR was inserted into a psiCHECK-2 vector immediately downstream from the Renilla luciferase gene. (**B**) Luciferase reporter assays analyzing the putative targets of *MIR125B1*. NC or *MIR125B1* duplexes were cotransfected with psiCHECK-2 plasmids with the 3′UTR of target genes-wt or the target genes-mut. The firefly luciferase activity of each sample was normalized to the Renilla luciferase activity (n = 3 independent experiments performed in triplicate). (**C**) Western blot analysis of DRAM2 in NB4 cells transduced with lentiviral vectors expressing *MIR125B1* (top panel) or shRNAs targeting *MIR125B1* (bottom panel). (**D**) Western blot analyses of PML-RARA and LC3 in DRAM2-knockdown NB4 cells, followed by treatment with ATRA. LC3-II/ACTB densitometric ratios were recorded. Quantification of data from 3 independent experiments (bottom panel). (**E**) Flow cytometry analysis of ITGAM/CD11b cell-surface expression in NB4 cells transfected with si-DRAM2 or si-NC, followed by treatment with or without ATRA. Values are derived from n = 3 independent experiments data are reported as mean ± SD.

oncoprotein is prone to aggregation, indicating that it may represent a preferable substrate for autophagic degradation. In this study, we provided evidence that *MIR125B1* inhibited the autophagy-lysosomal pathway induced by ATRA. Importantly, autophagy appears to rely on the cooperation of autophagosomes and lysosomes. Indeed, lysosomal and autophagic genes were regulated by *MIR125B1*. Bioinformatic analysis was also used to address the important role of the lysosome in ATRA-mediated differentiation of APL. The repression of the autophagy-lysosome system imposed by *MIR125B1* was demonstrated by the decreased number of autophagosomes and this was accompanied by inhibition of lysosomal and autophagic genes (**Fig. 3B and C**). Thus, it is suggested that impairment of PML-RARA degradation partially occurs through abrogation of the autophagic-lysosomal system. Furthermore, we identified DRAM2 as a novel target that was at least partly responsible for the function of *MIR125B1* in autophagy. Importantly, the knockdown phenotype for DRAM2 is similar to the phenotype of overexpressing *MIR125B1*, including impairment of PML-RARA degradation, inhibition of autophagy, and myeloid cell

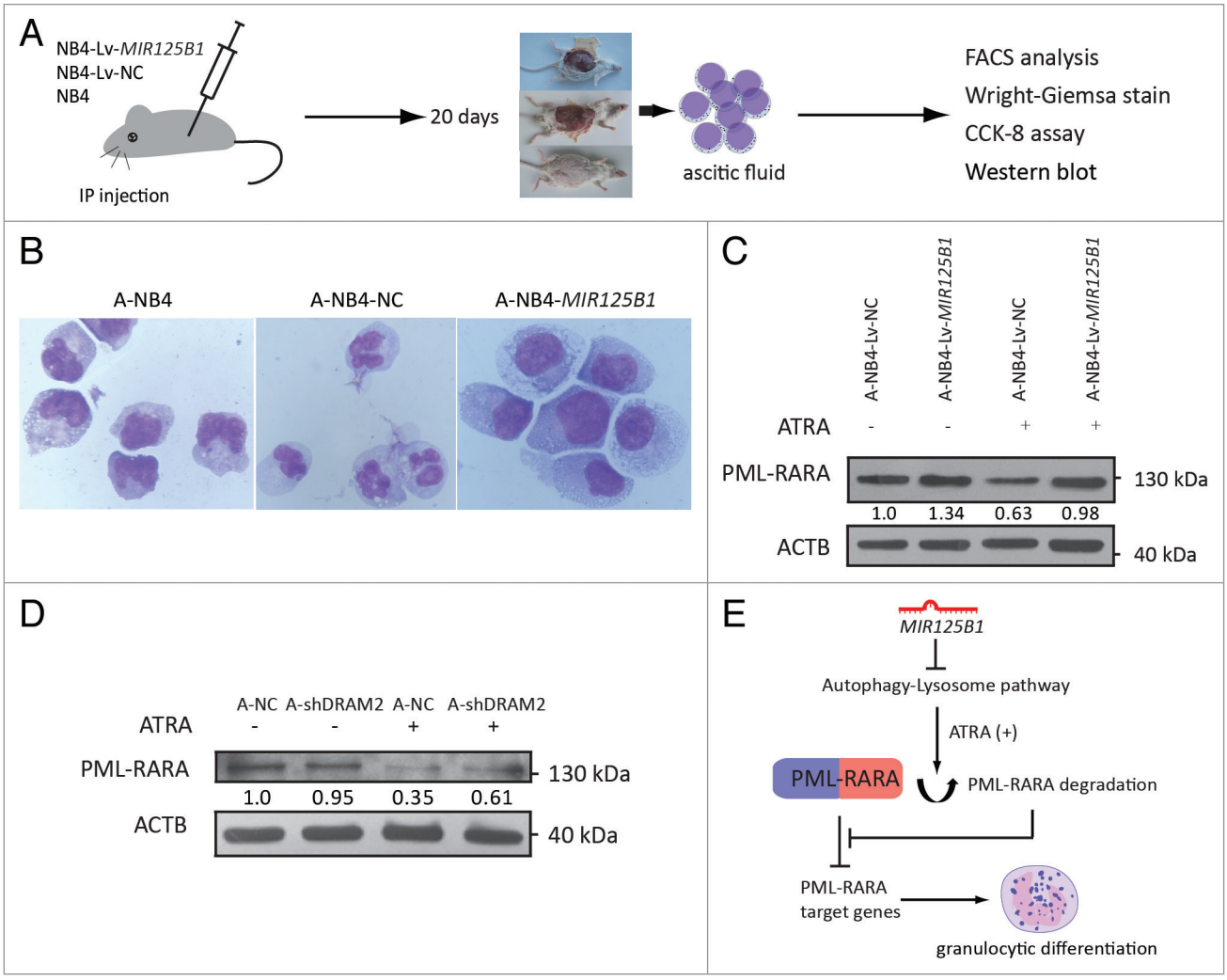

Figure 7. Effects of *MIR125B1* overexpression in a human APL-ascites SCID mouse model. (**A**) Diagram illustrating the experimental design of the mice xenograft experiment. IP, intraperitoneal transplantation. (**B**) Wright-Giemsa staining was performed to evaluate cell differentiation upon 72 h of ATRA treatment. (**C**) Western blot analyses of PML-RARA in A-NB4-Lv-NC and A-NB4-Lv-*MIR125B1* cells treated with ATRA. A-NB4: ascitic cells developed from the injection of NB4 cells; A-NB4-NC: ascitic cells developed from the injection of NB4-Lv-NC cells; A-NB4-*MIR125B1*: ascitic cells developed from the injection of NB4-Lv-*MIR125B1* cells. (**D**) Western blot analyses of PML-RARA in A-shDRAM2-NB4 and A-shNC-NB4 cells. A-shNC-NB4, ascitic cells developed from the injection of shNC-NB4 cells; A-shDRAM2-NB4, ascitic cells developed from the injection of shDRAM2-NB4 cells. ACTB was used as a loading control. (**E**) A proposed model for *MIR125B1* and PML-RARA in APL. See Discussion for details.

differentiation arrest. Although there are conflicting reports as to the role of DRAM2 in autophagy,[30,36] our results support the finding that DRAM2 is involved in autophagy induction. The discrepancy in these studies may be caused by differences in cell type, stimuli, and autophagy monitoring systems.

In summary, we provided evidence for a direct link between *MIR125B1* and PML-RARA in t(15;17) APL. *MIR125B1* overexpression can significantly increase the expression of the APL fusion oncogene *PML-RARA* and repress the therapy-induced proteolysis of PML-RARA by an autophagy-lysosomal pathway. *MIR125B1* therefore inhibits the ATRA-induced transcription of PML-RARA target genes that were initially repressed by PML-RARA, resulting in cell differentiation arrest. The oncogenic activity of *MIR125B1* in the treatment of t(15;17) APL was also demonstrated in vivo. Moreover, the data presented in this study suggested that the expression levels of *MIR125B1* are associated with the therapeutic status of the disease.

Materials and Methods

Patients and samples

A total of 80 bone marrow samples including 46 APL samples at diagnosis, 22 APL samples after therapy, and 12 normal donors, from the First and Second Affiliated Hospital of Sun Yat-sen University were enrolled in this study. Patients' characteristics are summarized in **Table 1**. Written informed consent was obtained from the parents and guardians. This study was approved by the ethics committee of the affiliated hospitals of Sun Yat-sen University.

Cell lines and cell cultures

NB4 cells were cultured in RPMI 1640 (HyClone, SH30027) containing 10% fetal bovine serum (HyClone, SV30160). The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. All drugs were purchased from Sigma-Aldrich and used at the following final concentrations: 1 μM ATRA (Sigma-Aldrich, R2625, stock 10 mM in EtOH) and 100 nM rapamycin (Sigma-Aldrich, R8781, stock 100 mM in dimethyl sulfoxide). Cell differentiation was assessed by measuring the surface ITGAM/CD11b (eBioscience, 11-0113) and FUT4/CD15 (eBioscience, 46-0159) antigen expression by flow cytometry analysis or cell morphology changes using Wright-Giemsa staining.[14]

Lysosomal staining and FACS analysis

Cells were maintained in 50 nM of the acidotropic dye LysoTracker Red DND-99 (Molecular Probes, L7528) for 60 min. The red lysosomal fluorescence of 30,000 cells per sample was determined by flow cytometry using a BD FACSAria cytometer (BD Biosciences, San Jose, CA, USA).

RNA isolation and RNA-SEQ (quantification)

The total RNA was extracted from samples with TRIzol (Invitrogen, 15596) according to the manufacturer's instructions. cDNA libraries were prepared as previously described.[37] The library products were then ready for sequencing analysis via an IlluminaHiSeq™ 2000 (San Diego, CA, USA). Results from reads that could be uniquely mapped to a gene were used to calculate the expression level. The numbers of gene reads were further measured by assessing the number of uniquely mapped reads per kilobase of exon region per million mappable reads (RPKM) using Chen's method,[38] which is based on the Audic method[39] for analyzing differential expression. We identified differentially expressed genes between samples using the following criteria: FDR ≤ 0.001 and a $\log_2$-fold change ratio ≥ 1.

Quantitative real-time PCR analysis

qRT-PCR was performed to detect mature miRNAs expression. Briefly, RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega, M1705) and amplified with specific miRNA RT primers and PCR amplification primers. *U6* served as internal control. For *PML-RARA*, primers were designed as previously reported.[40] PML breakpoints were rapidly assigned by qRT-PCR using a common *PML-RARA* reverse primer in conjunction with PML intron 3 (bcr3) breakpoint-specific forward primers. *ACTB* was used as internal controls. The oligonucleotide sequences are available in **Table S4**.

Transfection

Cells were transfected using the Neon® Transfection System (Invitrogen) with 100 pmol of oligonucleotides in 10 μl reactions. The hsa-*MIR125B1* miRNA mimics (Ambion, AM17100) and a mimics negative control (Ambion, AM17111) were purchased from Ambion. The *MIR125B1* inhibitor (RiboBio, anti-*MIR125B1*, miR10004669-1-2) and inhibitor negative control (RiboBio, miR02201-1-5) were purchased from RiboBio. The antagomir (chemically engineered oligonucleotides anti-miRNAs that could be easily transfected into cells and inhibited endogenous miRNA) against human *MIR125B1* (RiboBio, miR30000423-1-10) and antagomir negative control (RiboBio, miR03201-1-10) were obtained from RiboBio. The sequences of small interfering RNA (siRNA) that specifically targets the breakpoint region of *PML-RARA* were designed as previously described.[22] The nucleotide sequence for *DRAM2* siRNA was 5′ GUGCUUCACA UGAUCACUAC UG 3′ (si-*DRAM2-1*) and 5′ GAACACGGCU ACUUUCCAG 3′ (si-DRAM2-2). The following siRNA sequences targeting the *DRAM2* were designed and expressed as shRNA: si-*DRAM2*-1 5′ GUGCUUCACA UGAUCACUAC UG 3′. The nucleotide sequence for *ATG5* siRNA was 5′CAUCUGAGCU ACCCGGAUA3′.

Table 1. APL patients' characteristics[a]

APL Primary (n = 46)	Characteristics	Median (range)	No. (%)
	Cytogenetics		
	t(15;17)		
	Age at diagnosis, y	8.11 (0–14)	46 (100)
	Sex		
	Male		19 (41.3)
	Female		27 (56.7)
	WBC count, × 10^9/L	15.67	
	Less than 10		30 (65.2)
	10–50		9 (19.6)
	50 or higher		5 (10.9)
	N/A		2 (4.3)
APL after therapy (n = 22)	CR		18 (81.8)
	NR		2 (9.1)
	Relapse		2 (9.1)

[a]WBC, white blood cell; CR, samples in first complete remission; relapse, samples at relapse. NR, Non-remission; N/A, not available. CR and Relapse samples are from patients of 46 primary patients with 3 y of clinical follow-up.

Western blot

Protein extracts were boiled in RIPA buffer (Beyotime, P0013) and separated in a sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel. The proteins were then transferred to a polyvinylidene fluoride membrane (Millipore, HVPPEAC12) and probed with anti-RARA (C-20; Santa Cruz Biotechnology, sc-551), anti-MTOR (Cell Signaling Technology, 2983), anti-LC3B (Sigma-Aldrich, L7543), anti-DRAM2 (Sigma-Aldrich, HPA018036) and anti-ACTB (Sigma-Aldrich, A2103) antibodies.

Animal models

NB4 cells were transfected with Lv-*MIR125B1* (lentivirus vector expressing *MIR125B1*) or Lv-NC (lentivirus vector expressing miRNA negative control) according to the recommended protocol (GeneChem). The transfected cells (NB4-Lv-*MIR125B1* and NB4-Lv-NC) were sorted by flow cytometry (FACStar+ instrument, BD Biosciences, San Jose, CA, USA), and the GFP-positive cells were used for in vivo experiments.

Five-wk-old NOD-SCID mice were maintained under specific pathogen-free conditions in the Laboratory Animal Center of Sun Yat-sen University, and all experiments were performed in accordance with our Institutional Animal Guidelines. The human APL-ascites SCID mouse model was used as previously described with modifications.[41] The tumor xenografts were established by single intraperitoneal transplantation in NOD-SCID mice with 1×10^6 NB4 cells in 0.1 ml RPMI-1640 medium.

Immunofluorescence

Cells were fixed in 4% formaldehyde for 30 min at room temperature prior to cell permeabilization with 0.1% Triton X-100 (4 °C, 10 min). The cells were blocked for 2 h with 2.5% BSA in PBS (Beyotime, ST023 and C0221A). After blocking, they cells were then incubated for 20 h with an anti-LC3 antibody, washed 3 times for 5 min with PBS, and then incubated for 1 h with Alexafluor 594-conjugated (Invitrogen, A21207) secondary antibodies. Fluorescence signals were analyzed using a Leica TCS SP5 II confocal microscope (Leica Corp., Wetzlar, Hesse, Germany).

Transmission electron microscopy analysis

Cells were washed with PBS and fixed in 3% glutaraldehyde dissolved in 0.1 mol/l phosphate buffer (pH 7.4) for 30 min at room temperature. The cells were then postfixed for 1 h in 1% OsO_4 (Sigma-Aldrich, O5500). After dehydration, the cells were embedded in Epon 812 (SPI Supplies, 02635-AB) and polymerized at 60 °C for 24 h. EM images were acquired from thin sections using a JEM1400 electron microscope (JEOL, Akishima, Tokyo, Japan). Images were digitally acquired from a randomly selected pool under each condition. At least 10 cells per group were analyzed.

Statistical analysis

Data were expressed as the mean ± SD of 3 independent experiments. The significance of the differences between groups was determined by a 2-tailed Student *t* test. A P value < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/29592

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