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Overexpression of RBM4 promotes acute myeloid leukemia cell differentiation by regulating alternative splicing of TFEB

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Alternative splicing is an efficient and ubiquitous transcriptional regulatory mechanism that expands the coding capacity of the genome and is associated with the occurrence and progression of cancer. The differentiation-promoting regimen is a potential therapeutic approach in cancer treatment. In this study, we screened NPMc-positive and NPMc-negative acute myeloid leukemia (AML) samples from the Cancer Genome Atlas, focusing on the splicing factor RNA-binding motif protein 4 (RBM4) and its splicing mechanism on the target gene transcription factor EB (*TFEB*), which are most relevant to the prognosis of AML. We also investigated the impact of the *TFEB*-dominant spliceosome on autophagy and differentiation of THP-1 and K562 cells. The results showed that RBM4 recognized the CU-rich sequence in intron 8 of *TFEB*, increasing the production of the *TFEB*-L spliceosome, which promoted autophagy. Overexpression of RBM4 increased autophagy and promoted cell differentiation. The combination of TFEB-L with the therapeutic drug rapamycin further promoted the differentiation of leukemia cell lines and primary leukemia cells in AML patients. This study suggested that overexpression of RBM4 could promote cell differentiation by promoting the production of the TFEB-dominant spliceosome, demonstrating the potential of the TFEB-dominant spliceosome combined with chemotherapy drugs to promote leukemia cell differentiation and improve patient prognosis.

Autophagy plays a central role in the development and progression of acute myeloid leukemia (AML) and chemotherapy resistance (1–3). Autophagy is not only a cyclic mechanism that assists cells in coping with nutritional stress but also regulates cell differentiation, cell death, and cell cycle (4). In fact, research has shown that when autophagy is activated in acute lymphocytic leukemia, it can effectively inhibit the cycle arrest of leukemia bone marrow cells, thereby improving treatment efficacy (5, 6). Differentiation therapy is less toxic than classical cytotoxic therapy in AML (7). Therefore, autophagy has great potential for application in the anticancer treatment of AML.

In previous bioinformatics analysis, we obtained RNA splicing patterns from the Cancer Genome Atlas (TCGA) SpliceSeq database and expression profile data and relevant clinical information from the UCSC Xena database for AML. The results showed that RNA-binding motif protein 4 (RBM4) is closely related to the survival of AML patients. RBM4 is a splicing regulator that preferentially binds CU-rich elements to regulate alternative splicing (AS) processes (8). In addition, RBM4 plays an important role in controlling apoptosis, proliferation, and migration in various tumor types, including lung cancer, ovarian cancer, and prostate cancer (9). Imbalanced splicing of pre-mRNA characterizes tumor malignancies. RBM4 accumulation correlates with SRPK1 upregulation in breast cancer. RBM4 binds CU-rich elements in MCL-1, modulating exon exclusion. This upregulates IR-B and MCL-1S, reducing apoptotic resistance in breast cancer cells, implicating the SRPK1-RBM4 network in tumorigenesis *via* altered apoptotic sensitivity (10). However, Chang *et al.* found that RBM4 affects HIF-1α exon 14 in a CU element–dependent manner and overexpression of RBM4 enhances the inclusion of HIF-1α exon 14 in A549 cells (11). In addition to providing mechanistic insights of cancer-related splicing dysregulation, Wang's study establishes RBM4 as a tumor suppressor with therapeutic potential and clinical values as a prognostic factor (12). The downregulation of RBM4 expression has been linked to unfavorable overall survival rates in patients with lung cancer, breast cancer, and ovarian cancer (13). Therefore, overexpression of RBM4 is beneficial for improving tumor prognosis. RNA splicing patterns suggest that transcription factor EB (*TFEB*) is a significant target gene of RBM4, and its expression and activity are modulated by RBM4 (14).

TFEB is a transcription factor belonging to the MiTF/TFE family, which also includes MITF, TFE3, and TFEC. TFEB plays a key role in various cellular processes, especially in lysosome biogenesis and autophagy regulation (15). In response to intracellular amino acid fluctuations and mammalian target of rapamycin (mTOR) pathway signaling, TFEB dynamically shifts between cytoplasm and nucleus.

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Amino acid abundance activates mTORC1, phosphorylating TFEB and retaining it in the cytoplasm. Conversely, amino acid scarcity or stress deactivates mTORC1, allowing TFEB dephosphorylation and nuclear translocation. In the nucleus, TFEB promotes autophagy, lysosomal biogenesis, and catabolic processes, enabling cellular adaptation to changing conditions. In the cytoplasm, TFEB binds to 14-3-3 proteins and remains inactive; in the nucleus, TFEB activates the transcription of its target genes, thereby regulating processes such as lysosome generation, autophagy, and lipid metabolism (16). TFEB plays an important role in autophagy during all-trans retinoic acid (ATRA)-induced myeloid differentiation, and autophagy induction enhances the differentiation of acute promyelocytic leukemia cells (17). During the process of myeloid differentiation in acute promyelocytic leukemia (APL) induced by ATRA, TFEB emerges as a crucial player in autophagy. Autophagy, a cellular degradation process that involves the sequestration and breakdown of damaged or unnecessary components, is significantly enhanced by TFEB's activity. Specifically, the induction of autophagy by TFEB not only facilitates the clearance of debris but also potentiates the differentiation of APL cells towards a more mature, functional myeloid lineage. Therefore, the expression and function of TFEB in promoting autophagy are essential for the successful differentiation of APL cells under ATRA treatment. Different splice variants of TFEB have different functions, expressions, and locations, which affect the transcriptional regulatory activity of TFEB. Although TFEB plays an important role in cell biology, there is still relatively little information about the specific functions and mechanisms of its splice variants, and they have not yet been fully understood.

In this study, we explored the regulatory mechanism of RBM4 on TFEB AS, as well as the localization of the two splice variants in cells and their effects on cell proliferation and differentiation, with a view to further understanding the functions and potential clinical applications of TFEB.

Results

Splicing factors closely related to survival in AML

TCGA SpliceSeq (https://bioinformatics.mdanderson.org/TCGASpliceSeq/) is a database for studying mRNA splicing patterns in TCGA Project RNASeq data. We screened the percentage of samples with percent spliced in value $> 75\%$ to obtain the splicing patterns of protein-related genes in leukemia patients. Expression profiling data and relevant clinical information (overall survival > 30) for leukemia were obtained using UCSC Xena (http://xena.ucsc.edu/) and integrated with variable splicing data for screening, resulting in the selection of 129 leukemia samples to be analyzed for subsequent studies. To determine which splicing factors are associated with survival, we performed survival analyses of splice factors based on gene expression. The results showed that six splice factors were significantly associated with overall survival, among which low expression of the splicing factors RBM4, HNRNPA2B1, and NOVA2 was associated with poor prognosis (Fig. 1, *A–C*), whereas high expression of the splicing factors SRSF1 (14), HNRNPF and KHDRBS2 was associated with poor prognosis (Fig. 1, *D–E*). In addition, the correlation between percent spliced in values of prognosis-related AS events and the expression of prognosis-related splicing factors was investigated using the Spearman test. Among them, six survival-associated splicing factors were significantly

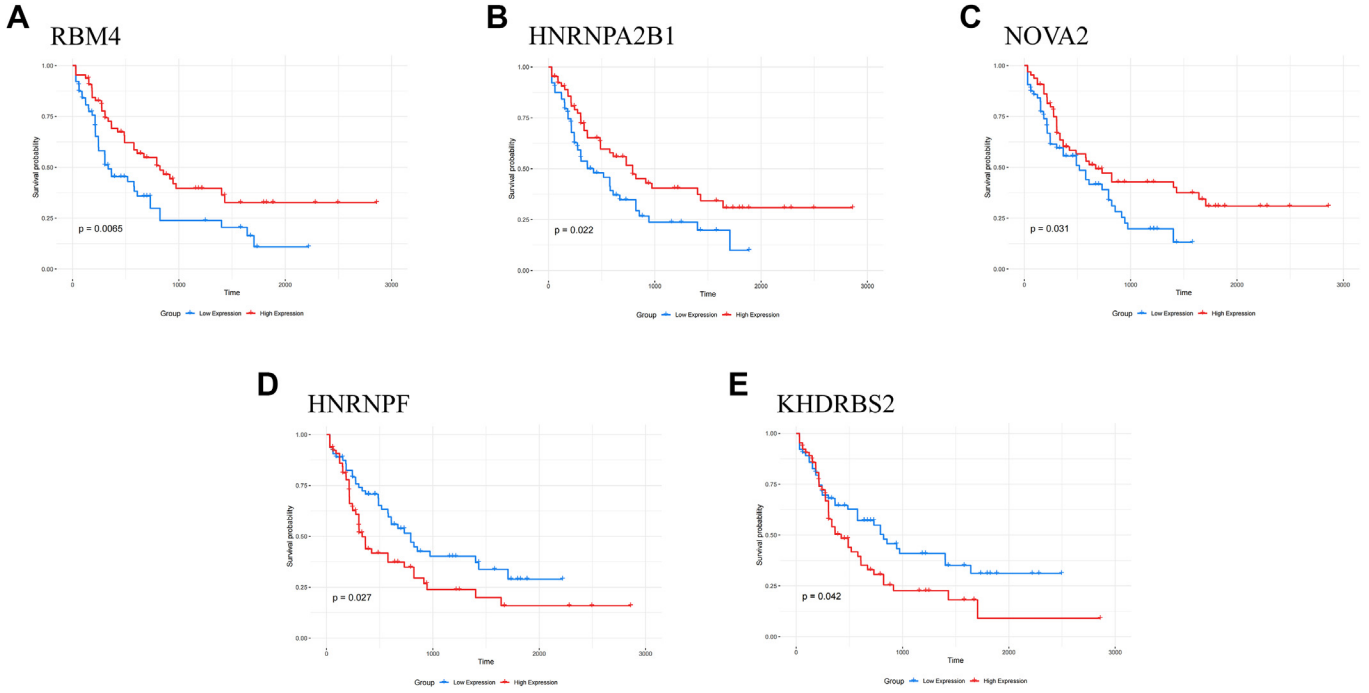

Figure 1. The relationship between splice factors expression and clinical outcome in patients with acute myeloid leukemia. *A–E*, the relationship between the expression of RBM4, HNRNPA2B1, NOVA2, HNRNPF, KHDRBS2, and AML survival. RBM4, RNA-binding motif protein 4.

correlated with 33 genes involved in prognosis-related AS events (14). To elucidate the relationship between autophagy and differentiation in leukemia cells, this study focused on the relationship RBM4 and TFEB in AML.

RBM4 reprograms the splicing profiles of TFEB in a CU element–dependent manner

The interaction between the splicing factor RBM4 and the CU element has frequently been shown to modulate variable splicing. To investigate the regulatory mechanism of *TFEB* exon 8, we constructed a *TFEB* miniGene. Concurrently, we recognized three CU elements located in the intron downstream of exon 8 of *TFEB*. Consequently, we conducted precise point mutations on these three CU elements, resulting in the successful creation of 3 C/T > G nucleotide-substituted mutants (Fig. 2).

After overexpression of RBM4 in K562 and THP-1 cells, the levels of p62 and LC3 increased at both mRNA and protein levels compared to the control group, suggesting an enhanced autophagy capacity in the cells (Fig. 3, *A–D*). To further explore whether RBM4 induces cell differentiation, flow cytometry was used to detect the expression level of CD11b in cells after overexpressing RBM4. The results showed that compared with the control group, the expression of CD11b was enhanced after overexpression of RBM4 in K562 and THP-1 cells, indicating that overexpression of RBM4 promoted cell differentiation (Fig. 3, *E* and *F*).

Meanwhile, overexpression of RBM4 in K562 and THP-1 cells led to an increase in exon 8 inclusion, as demonstrated in Figure 4, *A* and *B*. RNAi was utilized to elucidate the putative role of endogenous RBM4 in the modulation of exon 8 splicing. The effect of RBM4 on *TFEB* exon 8 splicing was assayed by measuring the ratio of exon 8 splicing isoforms (*TFEB*-L/*TFEB*-S) using RT-PCR. The results, as demonstrated in Figure 4*C*, indicated that RNAi-mediated knockdown of RBM4 led to a reduction in the inclusion of *TFEB* exon 8 and a subsequent decrease in the *TFEB*-L/*TFEB*-S ratio compared to the negative control.

Based on the above results, we further investigated the AS mechanism of RBM4 on the target gene *TFEB* using the constructed miniGene. Following the cotransfection of the RBM4 expression vector with either WT or mutant genes into 293T cells was utilized to assess the expression levels of *TFEB*-L and *TFEB*-S. The obtained results indicated that, in comparison to WT miniGenes, the cotransfection of the CU2 (TTCCTTCT) mutant led to a notable reduction in the inclusion of *TFEB* exon 8 and a corresponding decrease in the *TFEB*-L/*TFEB*-S ratio (Fig. 4D). The experimental findings suggest that RBM4 specifically interacts with the CU2 sequence (TTCCTTCT) located 69 nt to 76 nt downstream of *TFEB* exon 8 to regulate the inclusion of *TFEB* exon 8.

TFEB-L enhances the differentiation of AML cell by promoting autophagy

To generate stable cell lines expressing either TFEB-L or TFEB-S, lentiviral vectors encoding these isoforms were constructed and used to infect 293T, K562, and THP-1 cells.

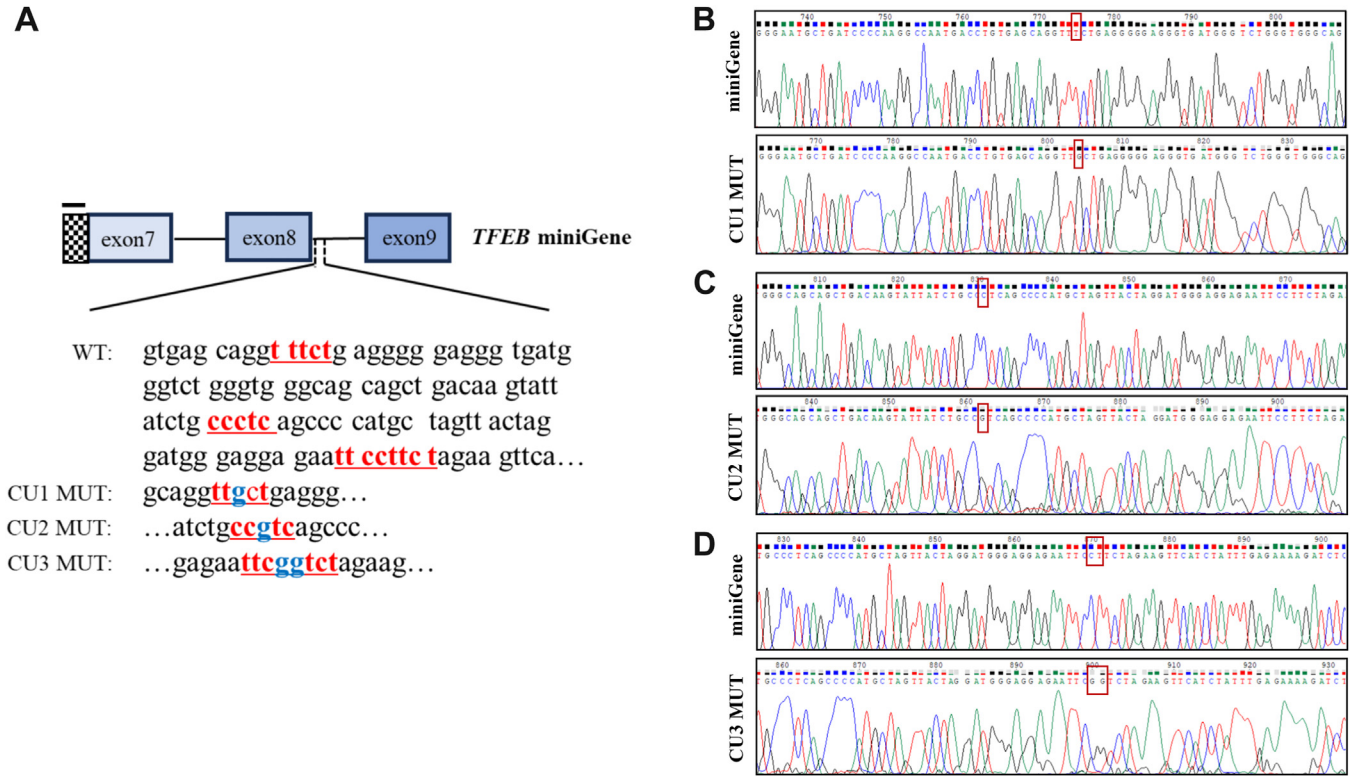

Figure 2. The putative CU sequences and DNA sequencing diagram of the CU sequence in intron 8 of the *TFEB* minigene. *A*, schematic diagram of CU-rich sequences and mutation sites within 120 bp in intron 8. *B*, CU1 sequencing map, MUT: +13 nt C > G. *C*, CU2 sequencing map, MUT: +69 nt T > G. *D*, CU3 sequencing map, MUT: +107nt108 nt CT>GG. TFEB, transcription factor EB.

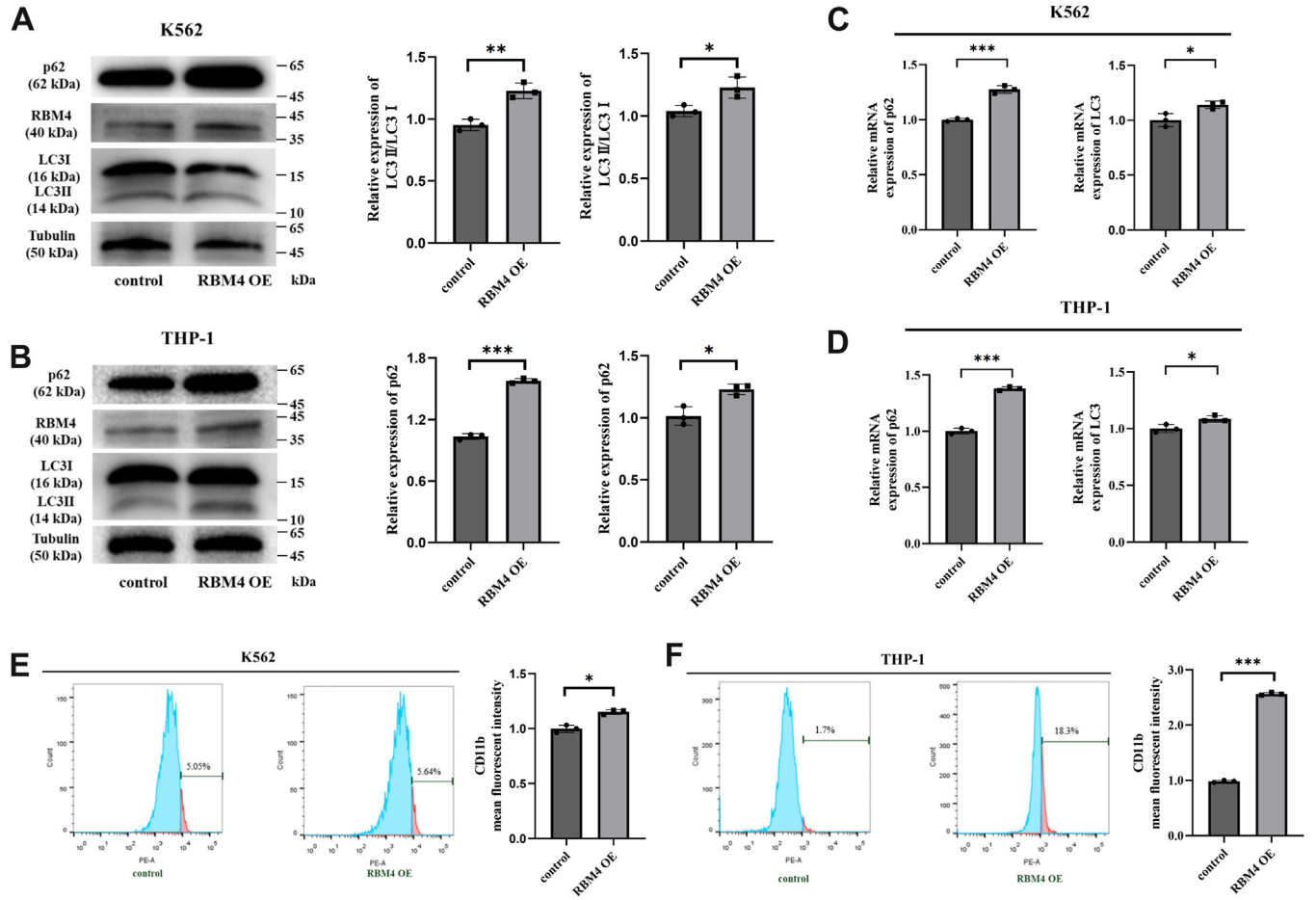

Figure 3. The effects of RBM4 overexpression on autophagy and differentiation in cell lines. *A-B*, the expression levels of p62 and LC3 were detected by Western blot after overexpression RBM4 in K562 and THP-1 cells. *C-D*, the expression levels of p62 and LC3 were detected by qRT-PCR after overexpression RBM4 in K562 and THP-1 cells. *E-F*, the expression levels of CD11b was detected by flow cytometry after overexpression RBM4 in K562 and THP-1 cells. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). qRT-PCR, real-time quantitative PCR; RBM4, RNA-binding motif protein 4.

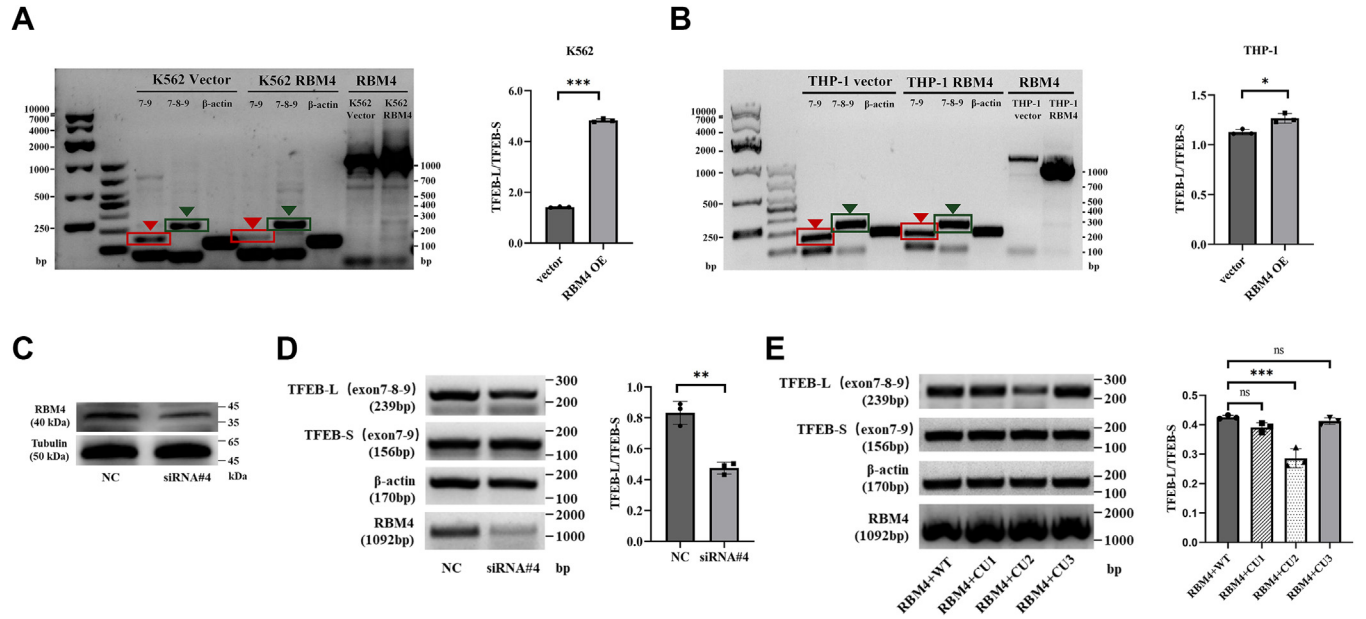

Figure 4. RBM4 regulates alternative splicing of *TFEB* by binding to the CU sequence in intron 8. *A*, the expression levels of *TFEB*-L and *TFEB*-S after overexpression RBM4 in K562 cells. *B*, the expression levels of *TFEB*-L and *TFEB*-S after overexpression RBM4 in THP-1 cells. *C*, the expression level of RBM4 was detected by Western blot in HeLa cells. *D*, the expression levels of *TFEB*-L and *TFEB*-S detected by RT-PCR after knocking down endogenous RBM4 in HeLa cells. *E*, expression levels of *TFEB*-L and *TFEB*-S after cotransfection of RBM4 vector with WT or mutant of TFEB minigene. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). RBM4, RNA-binding motif protein 4; TFEB, transcription factor EB.

Stable clones expressing either TFEB-L or TFEB-S were selected using G418 antibiotic treatment. The expression levels of autophagy-related genes were assayed using real-time quantitative PCR (qRT-PCR). Compared to cells stably expressing TFEB-L, the expression of LC3 and p62 was suppressed in cells stably expressing TFEB-S (Fig. 5, *A–C*). Similar results were observed for changes in LC3 and p62 protein levels, which were detected by Western blot (Fig. 5, *E* and *F*).

To assess the ability of TFEB-L and TFEB-S to promote autophagy, 293T cells stably expressing TFEB-L and TFEB-S were transfected with an LC3 expression vector fused to GFP. Autophagosome formation was observed using confocal

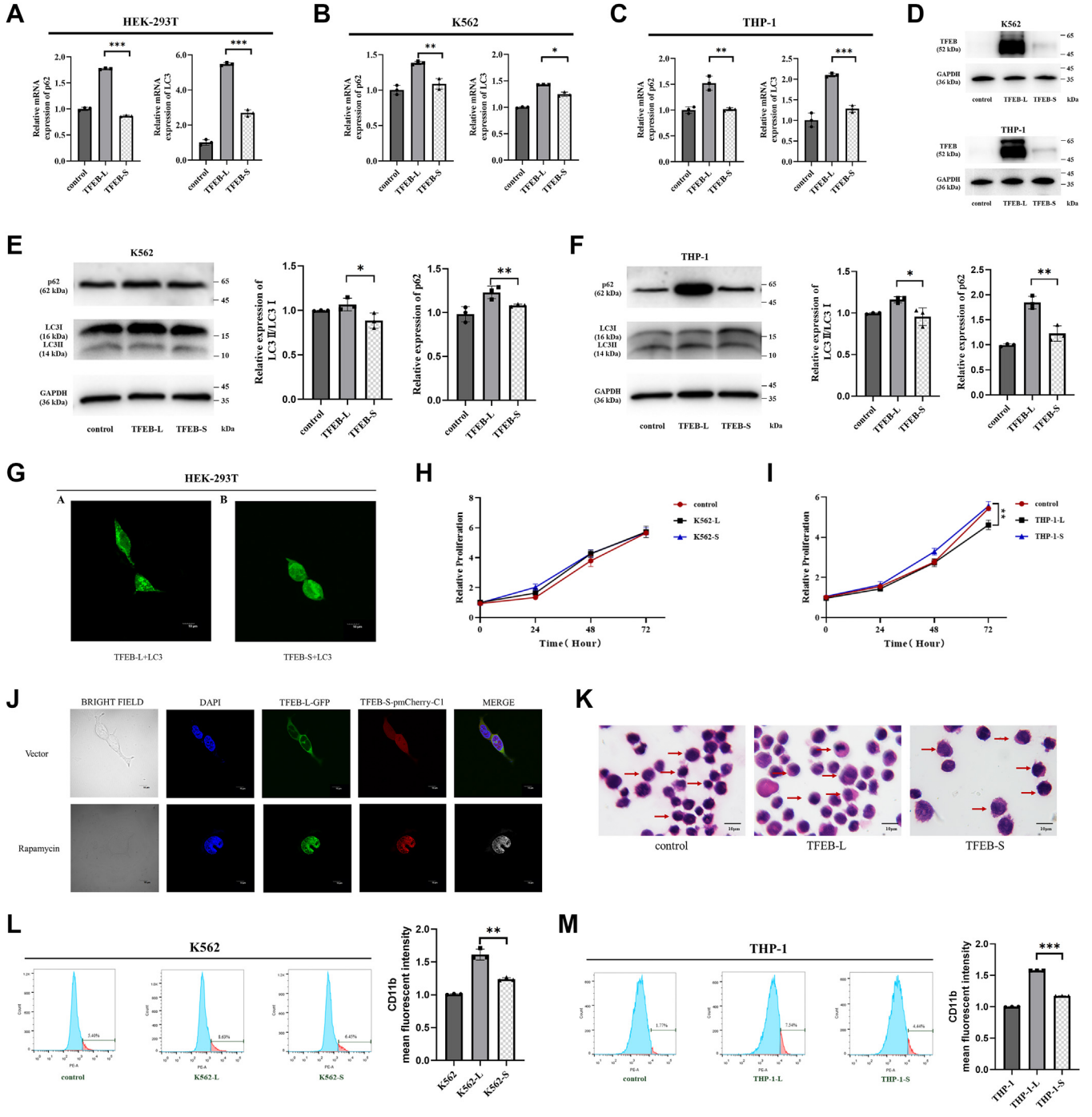

Figure 5. TFEB-L enhances the differentiation of acute myeloid leukemia cells by promoting autophagy. *A-C*, the expression levels of p62 and LC3 were detected by qRT-PCR in HEK-293T, K562, and THP-1 cells with high expression of TFEB-L or TFEB-S. *D*, the expression levels were detected by Western blot after lentiviral vector transduction of TFEB-L or TFEB-S into K562 and THP-1 cells. *E-F*, the protein levels of p62 and LC3 were detected by Western blot in K562 and THP-1 cells with high expression of TFEB-L or TFEB-S. *G*, the formation of autophagic vesicle was observed in HEK-293T cells after cotransfected of LC3 with TFEB-L or TFEB-S under confocal microscopy. *H-I*, cell proliferation in K562 and THP-1 after overexpression of TFEB-L or TFEB-S. *J*, nuclear translocation of TFEB-L and TFEB-S before and after rapamycin treatment. *K*, morphological observation of THP-1 cells after overexpression of TFEB-L or TFEB-S. *L-M*, the expression levels of CD11b was detected by flow cytometry in K562 and THP-1 cells after overexpression of TFEB-L or TFEB-S. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). qRT-PCR, real-time quantitative PCR; TFEB, transcription factor EB.

fluorescence microscopy. As shown in Figure 5*G*, significant induction of autophagosome formation was observed in the TFEB-L–transfected group. Compared to the control group, the cell proliferation of stable expression of TFEB-L was inhibited when assayed by the cell counting kit-8 (CCK-8) method (Fig. 5, *H* and *I*).

To further determine the concentration of rapamycin that promotes TFEB nuclear translocation, 293T cells was treated with gradient concentrations of rapamycin. Finally, it was observed that at concentrations of 1.526 nM [L.L.] or higher, TFEB-L with GFP green fluorescence and TFEB-S with mCherry red fluorescence colocated in the nucleus. Therefore, 1.526 nM [L.L.] rapamycin was determined to be the optimal concentration for subsequent experiments (Fig. 5*J*). The results indicate that rapamycin promotes the entry of TFEB-L and TFEB-S into the nucleus.

In order to investigate the differentiation of leukemia cells, we used morphological analysis and flow cytometry to detect the effect of TFEB-L and TFEB-S on cell differentiation. THP-1 cells stably expressing TFEB-L and TFEB-S were stained with H&E, and the morphological changes of the cells in different groups were observed under a microscope. As shown in Figure 5, *K*, compared to the control group, the TFEB-L group exhibited a significant decrease in the ratio of cell nucleus volume to cytoplasm. The cell nuclei appeared renal-shaped, tilted toward one side, with concave and lobulated lobes. Flow cytometry analysis revealed an increase in the positivity of surface antigen CD11b (PE) in the TFEB-L stable cell lines (Fig. 5, *L* and *M*). These results effectively demonstrate that TFEB-L serves as a dominant splice isoform that promotes autophagy and differentiation in leukemia cells.

TFEB-L combined with rapamycin has a synergistic effect on leukemia cell differentiation

We combined TFEB-L spliceosome with rapamycin, an autophagy activator, to investigate their potential synergistic effect in promoting leukemia cell differentiation. CCK-8 assay showed that the proliferation of leukemia cell line THP-1 was inhibited with increase of rapamycin concentration over time (Fig. 6*A*). After 24 h of combined treatment of TFEB-L and rapamycin on THP-1 and K562 cells, flow cytometry was used to detect the expression of CD11b on the cell surface. As shown in Figure 6, *B* and *C*, compared with the control group, TFEB-L combined with rapamycin promoted the differentiation ability of THP-1 and K562 cells.

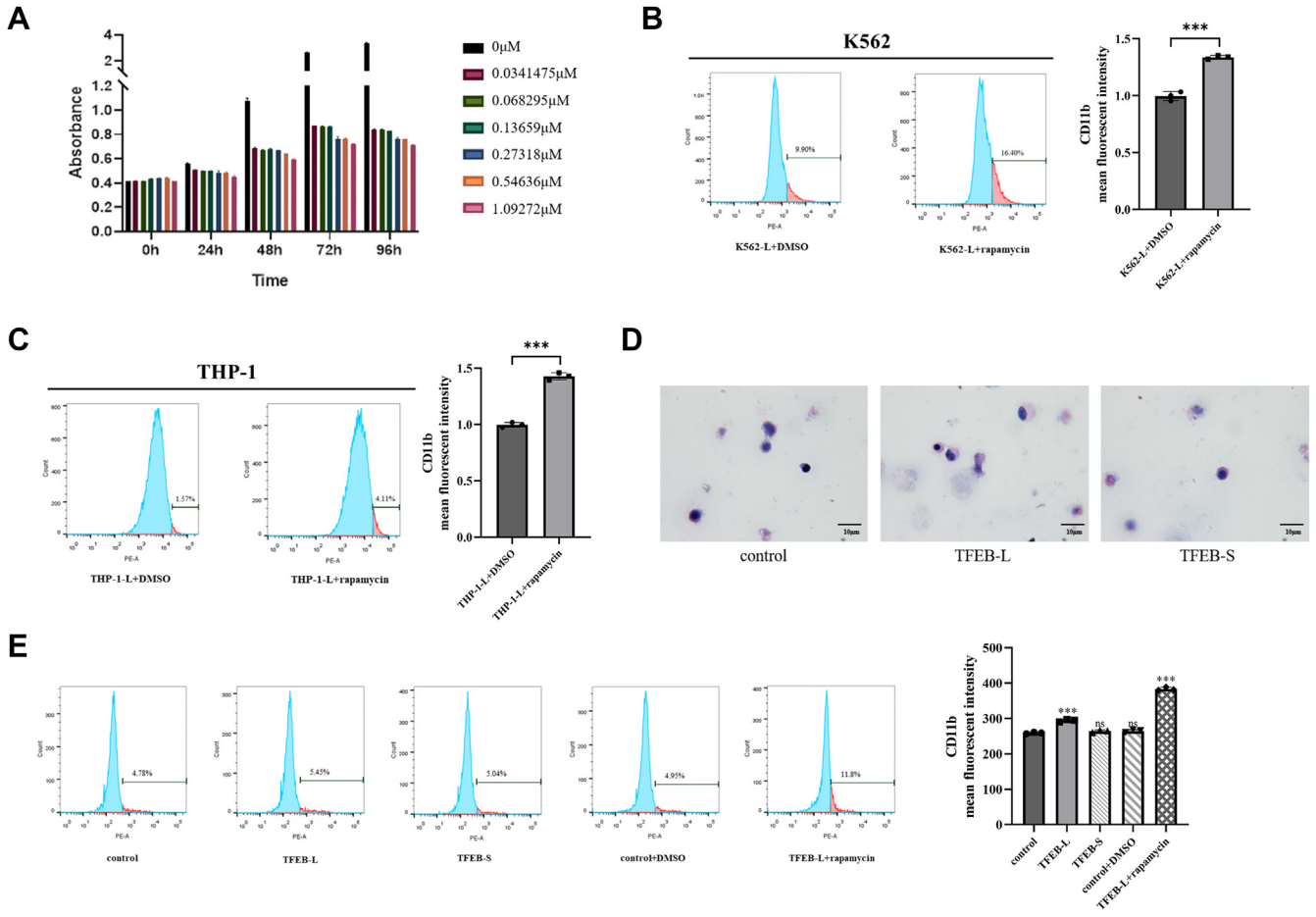

Figure 6. TFEB-L combined with rapamycin has a synergistic effect on leukemia cell differentiation. *A*, the inhibitory effect of different time points and gradient concentrations of rapamycin on THP-1 cell lines. *B-C*, the expression level of CD11b after overexpression of TFEB-L combined with rapamycin treatment in K562 and THP-1 cells. *D*, morphological observation of primary leukemia cells after overexpression of TFEB-L or TFEB-S. *E*, the expression levels of CD11b in primary leukemia cells. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). TFEB, transcription factor EB; RBM4.

Finally, we collected bone marrow samples from three patients with AML and treated them with TFEB-L and rapamycin *in vitro*. Flow cytometry was used to detect cell differentiation. The results showed that after cotreatment with TFEB-L and rapamycin, the differentiation ability of primary leukemia cells was further enhanced (Fig. 6, *D* and *E*), which was consistent with the *in vitro* experimental results obtained in the THP-1 cell line. These results suggest that the combined application of TFEB-L and rapamycin promote the differentiation of primary leukemia cells.

Discussion

The results of bioinformatics analysis indicated that the abnormal AS of TFEB was closely associated with RBM4. By examining the molecular mechanism through which RBM4 regulates AS of TFEB, we aimed to investigate how autophagy could be activated by modulating distinct spliceosomes of TFEB, thereby enhancing cell differentiation and improving the prognosis of AML when RBM4 is overexpressed. Our findings demonstrated that elevated expression of RBM4 potentiated the expression of TFEB-L spliceosomes, subsequently facilitating cell autophagy and differentiation. In turn, this provided a partial explanation for the more favorable prognosis observed among AML patients with high RBM4 expression in bioinformatics analysis.

TFEB serves as a crucial transcription factor in regulating cellular autophagy, a vital degradation process within cells. This process involves the transportation of damaged proteins or organelles to lysosomes for degradation, thereby maintaining cellular homeostasis (18). TFEB directly binds to the promoter regions of autophagy-related genes, such as LC3 and ATG5, and activates their expression (19). This activation promotes the occurrence of cellular autophagy. The activity of TFEB is modulated by various signaling pathways, including the mTOR pathway. When mTOR activity is elevated, it suppresses the nuclear translocation of TFEB, thus inhibiting cellular autophagy (20). Conversely, when mTOR activity diminishes, TFEB can enter the nucleus and activate the expression of autophagy-related genes.

The TFEB-L isoform serves as a proautophagy subtype, whereas the TFEB-S variant, resulting from AS between exon 7 and exon 9, lacks the helix-ring-helix and leucine zipper domain. Consequently, TFEB-S acts as a negative regulator of TFEB-L, suppressing its autophagy pathway. Our findings align with prior studies (21), further confirming the reliability and validity of our research outcomes. Various environmental stimuli, including starvation, bacterial infection, proinflammatory factors, endoplasmic reticulum stress, oxidative stress, and mitochondrial damage, induce the nuclear translocation of TFEB, enhancing its transcriptional activity (22–24). This nuclear localization coordinates the expression of lysosome-related genes, regulating transcription of genes involved in autophagosome formation and lysosomal activity.

Recent studies have shown that autophagy also plays an important role in the process of cell differentiation. During the process of cell differentiation, autophagy can help clear unwanted or harmful substances within the cell, providing a more stable and conducive environment for newly generated cells to differentiate. In addition, autophagy also affects the direction of cell differentiation by regulating intracellular signaling pathways (25, 26). Due to its ability to promote autophagy, TFEB also affects the process of cell differentiation to a certain extent. By regulating the activity of TFEB, the level of intracellular autophagy can be affected, thereby changing the direction and efficiency of cell differentiation (27). In this study, we overexpressed TFEB-L and found that it promoted cell differentiation both *in vivo* and *in vitro* (Figs. 5 and 6), this finding further validates that TFEB-L promotes cell differentiation by modulating autophagy.

AML is a malignant disease affecting myeloid hematopoietic stem cells. Although significant progress has been made in its treatment in the past decades, it still faces many challenges (28–30). Some drugs such as retinoic acid and differentiation inducers induce leukemia cell differentiation by regulating intracellular signaling pathways. These drugs can restore normal hematopoietic function in leukemia cells and reduce their malignant behavior (6, 31, 32). Therefore, in-depth study of the molecular mechanism of cell differentiation is expected to provide new ideas and methods for the treatment of AML. In this study, leukemia cell lines THP-1 or primary leukemia cells was treated with rapamycin and TFEB-L in combination, and the results showed that the cells underwent differentiation and cell proliferation was inhibited (Fig. 6). As a self-renewal mechanism in cells, autophagy affects the fate of tumor cells by regulating the process of cell differentiation. Further research on the relationship between autophagy and tumor cell differentiation is expected to open up new avenues for tumor treatment (33, 34).

In summary, the treatment of AML still faces many challenges and requires continuous exploration of new treatment strategies. The combination of RBM4 or TFEB-L with conventional chemotherapy drugs is expected to be a potentially effective treatment strategy to improve the prognosis of AML patients.

Experimental procedures

Cells and reagents

HEK-293T, HeLa, K562, and THP-1 cell lines were purchased from National Infrastructure of Cell Line Resource. HEK-293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin G/streptomycin. K562 and THP-1 cells were cultured in RPMI 1640 medium containing 10% (v/v) FBS and 1% (v/v) penicillin G/streptomycin. All cells were cultured at 37 °Cin a humidified 5% CO_2 atmosphere.

Construction overexpression and knockdown (siRNA) vector of RBM4

Total RNA was extracted from the peripheral blood leucocytes of healthy donor and reverse-transcribed into complementary DNA (cDNA). Human RBM4 CDS was amplified using specific primers with enzyme cleavage sites, and cloned

into the pLV-C-GFPSpark lentiviral control plasmid to construct an RBM4 expression vector. To knock down *RBM4* expression, siRNAs targeting the human *RBM4* gene were synthesized in RiboBip (RiboBio). Cells were seeded in 6-well cell culture plates, and once cells reached a confluence of 50% to 70%, they were transfected with *RBM4* plasmid. Meanwhile, HeLa cells were transfected with siRNAs, cultures for 24 h, and then the expression levels of RBM4 in the cells were detected by qRT-PCR.

RT-PCR and real-time quantitative RT-PCR

Total RNA was extracted using Trizol (Takara), and then dissolved in enzyme-free water. One microgram of total RNA was reverse-transcribed using PrimeScriptTM RT Master Mix (Takara). After incubating at 37 °C for 15 min, the reactions were terminated by heating at 85 °C for 5 s.

The RT-PCR amplification system includes Premix Taq 10 μl, primer (F and R) 0.3 μl each, cDNA 50 ng, ddH2O to make up 20 μl. After mixing the above system, perform PCR amplification with the following conditions: predenaturation at 95 °C for 2 min; 94 °C for 30 s, 52 °C for TFEB-L or 59 °C for TFEB-S for 30 s, if required by the primer design) for 30 s, 72 °C for 1 min, 28 cycles for TFEB-L, and TFEB-S or 20 cycles for β-actin. The PCR product is analyzed by gel electrophoresis and the Image J software to determine the relative expression level of the amplified product.

The qRT-PCR amplification system comprises 2 × SYBR Green Premix Ex Taq II (10 μl); primers (F and R), each at 0.3 μl; 50 × Rox Reference Dye II (0.4 μl); cDNA (50 ng), which is synthesized from mRNA *via* the process of reverse transcription; and ddH_2O to make up to a total volume of 20 μl. The thermal cycling conditions are as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s. All qRT-PCR analysis was conducted on an Applied Biosystems (Thermo Fisher Scientific) instrument. Relative gene expression levels were determined using the comparative $2^{-\triangle\triangle Ct}$ method. The specific primer sequences are listed in Table. 1.

Cell proliferation and differentiation

Cells were seeded in 96-well plates at a density of 3000 cells per well. After incubating for 0, 24, 48, and 72 h, 10 μl of CCK-8 solution was added to each well. The cells were then incubated at 37 °C in the dark for 4 h, and the absorbance at 450 nm and 650 nm was measured using a spectrophotometer (Thermo Fisher Scientific) to assess cell proliferation.

Cells were collected from both the treatment group and the control group, with a total of 1×10^6 cells from each group, respectively. Wash the cells once with PBS solution, resuspend them in 100 μl of PBS, and then add 5 μl of phycoerythrin-labeled CD11b antibody (Beckman). Incubate the cells at 4 °C in the dark for 30 min. After washing once with PBS, add 400 μl of PBS and detect cell differentiation using upstream cytometry.

Western blotting

Cells were collected and lysed using radio immunoprecipitation assay lysis buffer (BOSTER), which was supplemented with protease inhibitors and phosphatase inhibitors. The protein samples (50 μg) were then loaded onto a 10% to 15% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 5% skimmed milk powder for 2 h and then incubated overnight with one of the following primary antibodies: anti-LC3, anti-p62, or anti-GAPDH (Abmart). The membrane was washed three times with TBST (0.1% Tween-20) and then incubated with the secondary antibody (Goat anti-Rabbit IgG antibody 1:10,000, BOSTER) for 2 h. The immunoblots were then developed using enhanced chemiluminescence (BOSTER).

Construction and site-specific mutagenesis of TFEB miniGene vector

TFEB miniGene includes exon 7-intron 7-exon 8-intron 8-exon 9 of human *TFEB* gene. The miniGene was initially constructed on the PMV expression vector by the Beijing Institute of Genomics (BGI), and subsequently cloned into the pLV-C-GFP Spark lentiviral control plasmid (Sino Biological) for further experiments. The CU-rich sequence on intron 8 of *TFEB* miniGene was subjected to site-directed mutagenesis using the M5 Site-directed Mutagenesis Kit (Mei5 Biotechnology) with specific primers following the manufacturer's instructions (Table 1). All mutations were subsequently confirmed *via* DNA sequencing.

Table 1
Primer sequences

Gene	Forward	Reverse
RBM4-CDS	CCGGAATTCGCCACCATGGTGAAGCTGTTCATCGGAAAC	CGGGGTACCAAAGGCTGAGTACCGCGCC
LC3	CCTGGACAAGACCAAGTTTTTG	GTAGACCATATAGAGGAAGCCG
P62	GCAGAAGAGCAGCAGCGTCAG	CTTGTGCAGCGGCCATGAGAG
TFEB-L	ATCACAACTTAATTGAAAGGAGACG	CAGAGCTGCTTGTTGGTCATC
TFEB-S	TCACAACTTAAGGACGTGCGC	TTTTGCAGGTCCTTCTGCATC
TFEB-CDS	CCGGAATTCGCCACCATGGCGTCACGCATAGGGTTGC	CGGGGTACCCAGCACATCGCCCTCCTCCATG
β-actin	ACTCTTCCAGCCTTCCTTCC	TCTCCTTCTGCATCCTGTCG
RBM4	GCTGGTTTCTGTGCGGGCTC	ATGTGCACAAAGCCGTAATTCTTAATG
T732G	GGTTGCTGAGGGGGAGGGT	CCCCCTCAGCAACCTGCTCACAGGTCATTG
C788G	CTGCCGTCAGCCCCATGCTAGTTAC	GGGCTGACGGCAGATAATACTTGTCAGCTGC
CT827GG	GAGAATTCGGTCTAGAAGTTCATCTATTTGAGAAAAGA	TAGACCGAATTCTCCTCCCATCCTAG

RBM4, RNA-binding motif protein 4; TFEB, transcription factor EB.

Sensitivity testing of THP-1 cells to rapamycin

CCK-8 assay was used to determine the sensitivity of the THP-1 cell line to the drug rapamycin (MCE). Five thousand cells were seeded into 96-well plates and the drug was added. After treating the cells with rapamycin of gradient concentration for 0 h, 24 h, 48 h, 72 h, and 96 h, 10 μl of CCK8 reagent was added to each well and incubated in the dark at 37 °C for 2 h. Subsequently, the *A* values at 450 nm and 650 nm were measured using a microplate reader. Using GraphPad Prism software version 9.0 (https://www.graphpad.com/), dose–response curves were then plotted.

Subcellular localization

The HEK-293T cells were transfected with either the TFEB-L or TFEB-S expression plasmids. After 24 h of transfection, the cells were treated with the optimal concentration of rapamycin for 6 h. To visualize the nuclei, Hoechst 33342 staining was performed. Confocal images of TFEB nuclear translocation were captured before and after drug treatment in HEK293T cells using an Olympus FV3000 laser scanning microscope.

Morphological assays of cell differentiation

Cells (1×10^6) are collected at 1000 rpm, centrifuged for 5 min, and washed twice with PBS. After the slides are smeared and allowed to stand for 2 min, they are dried for methanol fixation. Three to five drops of hematoxylin are added, and the slides are stained for 2 min. The dye is then gently rinsed with running water, and an equal volume of eosin is added. The slides are gently shaken to stain them evenly, and the staining process lasts for 1 min and 30 s. Finally, any remaining liquid is washed off with running water, the slides are allowed to dry, and the stained cells are observed under a microscope. The images are saved for further analysis.

Isolation and culture of primary leukemia cells

The bone marrow from three patients with AML (non-M3 AML) is collected and placed in sterile tubes containing heparin. The lymphocyte isolation solution is then added to the samples to separated leukemia cells. The bone marrow is added to the lymphocyte separation solution and centrifuged at 3000 r/min for 30 min. The intermediate cell layer is carefully aspirated using a pipette and placed in another dry centrifuge tube. It is then rinsed twice with PBS solution and centrifuged at 1000 r/min for 10 min to collect the leukemia cells. Leukemia cells were cultured in RPMI 1640 medium containing 10% (v/v) FBS and 1% (v/v) penicillin G/streptomycin at 37 °Cin a humidified 5% CO_2 atmosphere.

Ethical considerations

The human studies reported in this manuscript followed the Declaration of Helsinki ethics and was approved by Shanxi Medical University's Ethics Committee (2021SLL043). All participants signed informed consent.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 (https://www.ibm.com/spss) software. The *t* test was applied to compare the means of continuous data between two groups, considering the SDs within each group. The one-way ANOVA was utilized for the comparison of continuous data between three or more groups, and the least significant difference method was employed for pairwise comparison between groups. $p < 0.05$ was considered statistically significant.

Data availability

All the data described in the article are located within the article.

Author contributions—Y. D. writing–review and editing; Y. D. writing–original draft; S. L., J. W., and K. Y. data curation; J. X. software; Q. W. and L. L. conceptualization; J. L., J. H., and X. C. methodology; Y. T. visualization; H. W. supervision.

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Abbreviations—The abbreviations used are: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; CCK-8, cell counting kit-8; cDNA, complementary DNA; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; qRT-PCR, real-time quantitative PCR; RBM4, RNA-binding motif protein 4; TCGA, The Cancer Genome Atlas; TFEB, transcription factor EB.

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