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ARRB1-promoted NOTCH1 degradation is suppressed by oncomiR miR-223 in T cell acute lymphoblastic leukemia

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Abstract

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Conflict of Interest Disclosures

All the authors declare no conflicts of interest.

T cell acute lymphoblastic leukemia (T-ALL) is a type of aggressive leukemia with inferior prognosis. While activating mutations of *NOTCH1* are observed in most T-ALL cases, these mutations alone are not sufficient to drive the full development of T-ALL. β-Arrestins (ARRBs) are versatile and multifunctional adapter proteins that regulate diverse cellular functions, including promoting the development of cancer. However, the role of ARRBs in T-ALL have largely remained elusive. In this study, we showed that ARRB1 is expressed at low levels in assayed T-ALL clinical samples and cell lines. Exogenous ARRB1 expression inhibited T-ALL proliferation and improved the survival of T-ALL xenograft animals. ARRB1 facilitated NOTCH1 ubiquitination and degradation through interactions with NOTCH1 and DTX1. Mechanistically, the oncogenic microRNA (oncomiR) miR-223 targets the 3'-UTR of ARRB1 (BUTR) and inhibits its expression in T-ALL. Furthermore, overexpression of the ARRB1-derived miR-223 sponge suppressed T-ALL cell proliferation and induce apoptosis. Collectively, these results demonstrate that ARRB1 acts as a tumor suppressor in T-ALL by promoting NOTCH1 degradation, which is inhibited by elevated miR-223, suggesting that ARRB1 may serve as a valid drug target in the development of novel T-ALL therapeutics.

Introduction

Clinically characterized by high white blood cell counts, hepatosplenomegaly, an increased risk of central nervous system infiltration and high relapse rates, T-ALL is associated with inferior prognosis. Although the success rates for acute lymphoblastic leukemia (ALL) treatment have markedly improved, the 5 year event-free survival rate of T-ALL is approximately 80%, significantly lower than that of B cell acute lymphoblastic leukemia (B-ALL; ref. 1,2). Thus, there is an urgent clinical need to develop novel and efficacious therapeutics for T-ALL, which can be greatly facilitated by understanding the molecular mechanisms underlying leukemogenesis.

The constitutive activation of NOTCH1 is the most prominent oncogenic pathway, presenting in nearly 70% of T-ALL patients (3,4). The NOTCH1 pathway is activated by the ligand-mediated proteolytic release and translocation of intracellular NOTCH1 (ICN1) to the nucleus, where it regulates the expression of target genes. NOTCH1 deprivation during hematopoiesis leads to an absence of T cells in the thymus (5). In contrast, the overexpression of ICN1 in hematopoietic stem cells (HSCs) induces extrathymic T-cell development (6,7), even T-ALL transformation (8). Two categories of NOTCH1 mutations are typically identified in T-ALL patients. The more common NOTCH1 mutations (40–45% of tumors) occur in the heterodimerization domain (HD; ref. 3,4), while the other type of mutations (30% of tumors) occur in the C-terminal PEST domain (9). Nonetheless, NOTCH1 mutations alone are not sufficient to drive the development of full-blown leukemogenesis, suggesting that additional genetic and/or epigenetic alterations may be required for T-ALL development and progression (10).

As members of the β-arrestin (ARRB) protein family, β-arrestin1 (ARRB1) was originally identified as a molecule involved in the desensitization and endocytosis of G protein coupled receptors (GPCRs; ref. 11–13). Although the functions of these proteins are not completely understood, ARRBs are versatile and multifunctional adapter proteins that regulate a diverse

array of cellular functions (14–18). ARRB1 also serves as an E3 ligase adaptor for its substrates to mediate ubiquitination (19–23). We previously showed that ARRB1 is abundantly expressed in leukemia-initiating cells and can sustain the renewal capacity and senescence of cells, leading to the expansion of B cells to form B-ALL (24,25). However, little is known regarding the potential role of ARRB1 in T-ALL development and progression.

In this study, we investigated the role of ARRB1 in T-ALL progression. We showed that ARRB1 inhibits the progression of T-ALL cells by serving as a scaffold and interacting with NOTCH1 and DTX1 to facilitate the ubiquitination and degradation of NOTCH1. Moreover, the exogenous expression of miR-223 was shown to lead to a significant decrease in ARRB1 expression in T-ALL cells, which can be rescued by an miR-223 sponge. The data suggest that ARRB1 may serve as a valid drug target for the development of novel and efficacious therapeutics for T-ALL treatment.

Materials and Methods

Cell culture and chemicals

HEK-293T and human T-ALL cell lines, including Molt4, CCRF-CEM, and Sup-T1 were obtained from ATCC (Manassas, VA). Jurkat, Cutll1 and Molt3 T-ALL lines were kindly provided by Dr. Panagiotis Ntziachristos (26). All T-ALL cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen, USA), L-glutamine and penicillin/streptomycin, while HEK293T cells were maintained in complete DMEM. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All cell lines were obtained more than 6 months prior to experiments and were passaged for less than 3 months after thawing. All cell lines were cultured according to the manufacturer's instructions and confirmed as Mycoplasma negative by PCR methods. Cellular experiments were performed within 20 passages after thawing. The information of the T-ALL lines is provided in Supplementary Table 1.

T-ALL clinical samples

The enrollment and human subject protection plans for the T-ALL patients involved in this study were approved by the Ethics Committee of Chongqing Medical University, Chongqing, China. Prior to the collection and use of the clinical samples, patients and their guardians were provided with detailed information about the benefits and risks of the study. The written informed consent forms were signed by the guardians during their hospitalization at the Children's Hospital of Chongqing Medical University according to the Declaration of Helsinki. The completed consent forms were kept on file. All essential patient identifiers were removed to protect the patients' privacy. Primary leukemic cells were isolated from bone marrow aspirates of freshly diagnosed T-ALL patients prior to chemotherapy by gradient centrifugation. A normal peripheral T-cell population was enriched from age-matched healthy donors using an EasySep™ Human T-Cell Isolation kit (Stem Cell Tech, USA). The accession number for the RNA-seq data of T-ALL patients is GEO Submission GSE141140. The clinical characteristics and genetic alterations of the used samples are listed in Supplementary Table 2.

Establishment of a xenograft mouse model of human T-ALL

The use and care of the experimental animals was approved by the Ethics Committee of Chongqing Medical University. Briefly, young NOD/SCID mice (6–8-weeks old, male), which were pretreated with anti-CD122 (kindly provided by Dr. Dengli Hong; ref. 27), were subjected to a sub-lethal dose irradiation (300 cGy) at the Radiation Facility of the Army Medical University, Chongqing, China. Subsequently, 3×10^5 T-ALL cells per mouse were tail-vein injected into the irradiated NOD/SCID mice. The injected mice were observed for survival for up to 60 days after injection. For optical imaging, mice were intraperitoneally (i.p.) injected with D-luciferin sodium salt (Gold Biotechnology, St. Louis, MO) at 100 mg/kg in 0.1 mL of PBS. Pseudo-images were obtained by superimposing the emitted light over gray-scale images of the mice.

Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated in three independent experiments. The data are presented as the means ± SD. Statistical significances between the treatments and control groups were determined by one-way analysis of variance and Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Exogenous ARRB1 expression inhibits the progression of T-ALL in vivo and in vitro

To elucidate the function of ARRB1 in T cell acute lymphoblastic leukemia (T-ALL) cells, we constructed retroviral vectors to overexpress ARRB1 (β1) as well as two shRNAs targeting ARRB1, sh-ARRB1 #1 and #2 (sh-1 and sh-2), which co-express GFP and firefly luciferase. After transducing the T-ALL cell lines with the retroviral vectors, we injected them into the immune-deficient mice to assess the function of ARRB1 in T-ALL cells *in vivo*. Surprisingly, unlike the previously reported tumor-promoting activity of ARRB1 in B-ALL cells (24,25), the bioluminescence imaging results showed a slight tumor burden in mice grafted with ARRB1-overexpressing T-ALL cells compared with that observed in the scrambled vector-expressing T-ALL cells. Moreover, we also observed greater organ infiltration in the mice xenografted with ARRB1 knockdown T-ALL cells (Figure 1A). Histological images consistently showed a significantly lower tumor burden in the organs of mice injected with ARRB1 overexpressing T-ALL cells comparing with the control group, and these mice also exhibited a higher survival rate (Figure 1A–D; Supplementary Figure 1A–B). In contrast, silencing ARRB1 in T-ALL cells led to a significant accumulation of tumor cells in mouse organs and a decreased survival rate compared with that observed for the parental control group (Figure 1A–D; Supplementary Figure 1A–B). The CCK8 assay results demonstrated that ARRB1 overexpression led to a significant decrease in cell proliferative activity in all tested T-ALL lines (Figure 1E; Supplementary Figure 1C). Consequently, while the rate of GFP^+ cells observed in the parental Molt3 cells was stable, GFP expression in the Molt3 cells overexpressing ARRB1 was gradually lost over subsequent passages (Supplementary Figure 1D). The cell cycle and apoptosis assay results indicated that ARRB1 overexpression in T-ALL cells arrested the cells in the G_0 phase (Figure 1F) and induced apparent apoptosis (Figure 1G–H; Supplementary Figure 1E–F).

Collectively, these data indicate the ARRB1 suppresses T-ALL tumor progression *in vivo* and *in vitro*.

ARRB1 expression is decreased in T-ALL cells

Considering that ARRB1 may serves as an important tumor suppressor of T-ALL progression, we next assessed the complete transcriptomic profiles of a panel of clinical primary T-ALL samples (n=13) and peripheral T cells from healthy donors (n=4) by performing RNA-Seq analysis (Supplementary Table 2). While numerous genes, including cancer associated pathways such as NOTCH1, PI3K, RAS, WNT and TP53, exhibited aberrant expression patterns in T-ALL cells, we observed the abnormal expression of several T-ALL specific oncogenes, such as TAL1, LYL1, and LMO1/2 (Figure 2A; Supplementary Figure 2A–B; Supplementary Table 3). Furthermore, the clustering analysis results indicated an overall trend of a lower level of ARRB1 expression in the T-ALL samples than was observed in the T cell controls (Figure 2A).

To confirm the RNA-Seq results, we analyzed the GEO databases and discovered that the expression of ARRB1 was generally downregulated in T-ALL cells compared with that observed in normal bone marrow cells (Figure 2B). Furthermore, we enrolled additional T-ALL patients (n=17) and analyzed ARRB1 expression by qPCR analysis. The results indicated that ARRB1 expression was significantly lower in 13 of the 17 analyzed T-ALL samples compared with that observed in the normal T cell controls (Figure 2C; Supplementary Figure 2C). Accordingly, the ARRB1 protein levels were lower than those observed in the control samples (Figure 2D; Supplementary Figure 2D). Hierarchical clustering analysis failed to distinguish between the ARRB1 expression levels and the patients bearing NOTCH1/FBXW7 mutations (Supplementary Table 2; Supplementary Figure 2E–G). Taken together, these results strongly suggest that ARRB1 expression is downregulated in T-ALL.

ARRB1 suppresses the expression of T-ALL-associated oncogenes

To elucidate the functional role of ARRB1-induced suppression of T-ALL progression, we examined the transcriptomic changes of individual Jurkat cells with altered ARRB1 expression through an RNA-Seq analysis. The expression heat map shows the genes that were positively and negatively associated with ARRB1 (Figure 3A). Genes there were positively associated with ARRB1 included those involved in the response to GPCR desensitization and signal conduction, such as PPARA, GRK4 and DNM1. Interestingly, genes that were negatively associated with ARRB1 included several T-ALL related oncogenes, such as TAL1 and JAK2, as well as the NOTCH1 target genes HES1 and HES2 (Figure 3A; Supplementary Table 4).

We verified the RNA-Seq data in three T-ALL lines (Jurkat, Molt4 and Cutll1) by qPCR (Figure 3B–D) and observed that HES1 and HES2 were consistently and negatively regulated by ARRB1 overexpression in the three tested lines, whereas silencing ARRB1 led to increased HES1 and HES2 expression in T-ALL cells (Figure 3B–D). The inverse relationship between ARRB1 expression and that of HES1 and TAL1 was confirmed at the protein level (Figure 3E). Collectively, these results strongly suggest that ARRB1 may exert

its tumor suppressive function by downregulating T-ALL specific oncogenic events, especially those associated with the NOTCH1 signaling pathway.

ARRB1 effectively promotes ICN1 degradation in T-ALL cells

To further assess how ARRB1 regulates NOTCH1 signaling in T-ALL cells, we first tested whether ARRB1 expression affects NOTCH1 expression and observed that either the overexpression or silencing ARRB1 expression in Jurkat and Molt4 cells did not significantly impact the levels of NOTCH1 mRNA (Figure 4A) or intracellular NOTCH1 (ICN1) protein (Figure 4B; Supplementary Figure 3A) under conventional assay conditions. As ARRB1 was reported to be involved in the NOTCH1 cleavage and the release of the ICN1 domain by participating in the assembly of the γ-secretase complex (28), we tested the effect of ARRB1 on the formation of ICN1 in T-ALL cells using a γ-secretase inhibitor and showed that the ICN1 levels were not significantly affected by the ARRB1 expression status (Supplementary Figure 3B).

ARRB1 was reported to mediate the interaction between E3 ubiquitin ligases DTX1 or NEDD4 and their substrate NOTCH1 to promote the ubiquitination and degradation of NOTCH1 in *Drosophila* and mice (21,23). To more rigorously assess the dynamic status of ICN1 degradation, we blocked protein synthesis using cycloheximide. The results showed that ICN1 protein levels significantly decreased 2 hours after treatment in T-ALL cells overexpressing ARRB1 (β1) compared with that observed in the control cells, whereas silencing ARRB1 (using sh-1 and sh-2) led to relatively stable ICN1 protein levels in both cell lines (Figure 4C; Supplementary Figure 3C), suggesting that ARRB1 overexpression may promote the ICN1 degradation. To further confirm the effect of ARRB1 on ICN1 degradation, we additionally blocked protein degradation using the proteasome inhibitor MG-132, after stimulating cells with cycloheximide. We observed that the ARRB1-mediated degradation of ICN1 was diminished and that the ICN1 protein level became similar to that of the control and ARRB1-silenced groups at the tested time points (Figure 4D). Thus, these data demonstrate that ARRB1 promotes the degradation of ICN1 in T-ALL cells.

ARRB1 facilitates the ubiquitination of ICN1 in T-ALL cells

To investigate whether ARRB1 regulates NOTCH1 degradation through its ubiquitination, we performed ICN1 pull-down assays using ARRB1-overexpressing or ARRB1-silenced T-ALL cells and Western blotted with an ubiquitin antibody. We found a significant increase in ICN1 ubiquitination in all T-ALL cells overexpressing ARRB1 compared to that observed in the control or ARRB1 knockdown groups (Figure 5A; Supplementary Figure 3D). Furthermore, immunofluorescence staining analysis revealed that ARRB1 and NOTCH1 co-localized in the cytoplasm of Jurkat cells after EDTA stimulation (ref. 29; Figure 5B).

The results of co-immunoprecipitation (Co-IP) experiments showed that ARRB1 directly interacted with ICN1 in Jurkat cells, suggesting that ARRB1 may directly regulate the ubiquitination of ICN1 protein (Figure 5C). After screening candidate E3 ligases of ICN1, we observed that DTX1, which can catalyze the ubiquitination and subsequent degradation of ICN1 in *Drosophila* and mammalian cells (30), but not FBXW7, NEDD4 or ITCH was able to bind to ARRB1 in Jurkat cells (Figure 5D; Supplementary Figure 4A–C).

To further investigate the functional role of the ARRB1-DTX1 complex in promoting the ubiquitination of ICN1, we transfected HEK293T cells with exogenous His-ICN1 alone or in combination with HA-ARRB1 and/or Flag-DTX1. The Co-IP results showed that neither ARRB1 nor DTX1 alone was sufficient to catalyze the ubiquitination of ICN1 protein, whereas in combination they were able to induce a high level of ICN1 ubiquitination (Figure 5E). Furthermore, by silencing either ARRB1 or DTX1 we demonstrated that ARRB1 and DTX1 acted synergistically to promote the ICN1 ubiquitination in HEK293T cells (Figure 5F). Taken together, these results demonstrate that ARRB1 can synergize with DTX1 to catalyze the ubiquitination of ICN1 for targeted proteasomal degradation.

As a NOTCH1 target gene, DTX1 was shown to redirect lymphoid progenitor cells to B cells by antagonizing NOTCH1 signaling during lymphogenesis (31,32), as well as serving as a suppressor by mediating the ubiquitination and subsequent degradation of NOTCH1 in sarcoma cells (33). However, it is not clear if DTX1 plays a role in T-ALL. By mining the GEO database, we observed that DTX1 expression was not significantly affected in T-ALL (Supplementary Figure 5A). Consistent with these results, we did not observe any differences in DTX1 expression between our clinical samples and the normal controls (Supplementary Figure 5B). We further knocked down DTX1 expression in Jurkat cells or overexpressed ARRB1 (Supplementary Figure 5C–D) and observed that silencing the DTX1 expression in ARRB1 overexpression T-ALL cells could overcome the ARRB1-mediated growth inhibition of T-ALL cells (Supplementary Figure 5E), suggesting that the ARRB1-mediated inhibition of NOTCH1 and T-ALL depends on DTX1.

ARRB1 is directly targeted at its 3’-UTR by oncomiR miR-223 in T-ALL cells

The next question we asked is why ARRB1 is poorly expressed in T-ALL. While ARRB1 expression can be regulated at the genomic level, an alternative possibility is that ARRB1 may be epigenetically regulated, as it was reported that epigenetic aberrations are prevalent in T-ALL (4,26,34–36). Thus, we speculated that ARRB1 may be downregulated by noncoding RNAs, especially miRNAs, which can reduce the expression of their target genes by promoting mRNA degradation or inhibiting translation (37). Our RNA-Seq results for the T-ALL clinical samples indicated that miR-223 was the most highly expressed miRNA in the analyzed T-ALL samples (Figure 6A). By searching several miRNA target site prediction bioinformatic programs, we further showed that miR-223–3p can target the 3’-UTR of *ARRB1* (referred to hereafter as BUTR) (Figure 6A). Interestingly, miR-223 is considered a hematopoietic-specific oncomiR.

To determine if miR-223 directly targets *ARRB1*, we constructed a reporter by subcloning the 402-bp BUTR fragment into the 3’ end of the GLuc coding region, referred to hereafter as GLuc-BUTR (Figure 6A–B). As a control, we scrambled the *ARRB1* binding site and made a control reporter (GLuc-SCR) (Figure 6A–B). We observed that miR-223 was able to inhibit GLuc-BUTR reporter activity, whereas this inhibition was not observed for the control reporter (Figure 6B). Furthermore, we established a Jurkat line that stably overexpresses miR-223 and showed that the expression of ARRB1 and other known miR-223 target genes was effectively downregulated in these cells compared to those transfected with the miR-223 scrambled (scr) sequence control (Figure 6C), which was

confirmed in other T-ALL lines and HEK293T cells (Figure 6D). In contrast, the expression of a miR-223 sponge (AM223) in Jurkat cells expressing miR-223 was shown to rescue the expression of ARRB1 and other target genes (Figure 6E), suggesting that miR-223 may target ARRB1 with high specificity. We further confirmed the inhibitory effect of miR-223 on ARRB1 expression at the protein level in T-ALL cells (Figure 6F). Taken together, these results strongly suggest that miR-223 may potently and specifically target ARRB1 in T-ALL cells.

Exogenous expression of the ARRB1 3'-UTR containing the miR-223 binding site (BUTR) is incompatible with T-ALL cell survival

We next tested whether the ARRB1 3'-UTR containing the miR-223 binding site (BUTR) could act as a miR-223 sponge and negatively regulate the proliferation of T-ALL cells by constructing retroviral (RV) vectors that co-express mRFP and BUTR or the scrambled SCR control (Figure 7A). Using these vectors, we observed that the overexpression of BUTR but not the control SCR significantly inhibited Jurkat cell proliferation, which was rescued by the simultaneous expression of miR-223 (Figure 7B).

We further examined if overexpression of the miR-223 sponge BUTR would cause apoptosis in T-ALL cells and showed that BUTR overexpression in Jurkat and CCRF-CEM cells induced apparent apoptosis, as indicated by the presence of cleaved caspases 3 (Figure 7C; Supplementary Figure 6). Consistent with results of the apoptosis assay, the overexpression of BUTR in Jurkat cells effectively arrested cells in the G_0/G_1 phases and reduced the cell numbers in the G_2/S phases (Figure 7D).

We further examined the specificity of the ARRB1-derived anti-miR-223 sponge and observed that the overexpression of BUTR effectively rescued ARRB1 expression, and to lesser extent FBXW7 expression, without significant rescue of the additional miR-223 target genes RHOB and HLF (Figure 7E). The western blotting results also revealed that BUTR rescued the ARRB1 protein levels while also suppressing the level of ICN1 and its target gene DTX1 (Figure 7F). Collectively, these results demonstrate that the exogenous expression of the miR-223 sponge BUTR may be incompatible with the survival of T-ALL cells. These results also suggest that ARRB1-based antagonism of miR-223 may be further explored as a novel T-ALL therapeutic.

Discussion

Although β-arrestins (ARRBs) were originally identified for their ability to block agonist coupling to GPCRs, increasing evidence suggests that ARRBs may have diverse biological functions independent of G protein activation (11–13). By serving as multiprotein scaffolds to bring components of various signaling pathways into close proximity, ARRBs have been shown to participate in signaling activities transduced by ERK, JNK, p38-MAPK, AKT, PI3 kinase, and RhoA (14–18,38,39). ARRBs can be translocated to the nucleus and regulate gene expression (12). Furthermore, ARRB1 can serve as an E3 ligase adaptor for its substrates, such as NOTCH1, to mediate ubiquitination through its interaction with DTX1 (19–22). Nonetheless, the diverse functions mediated by ARRBs remain incompletely understood.

We previously showed that the abundant expression of ARRB1 in leukemia-initiating cells can sustain the renewal capacity and senescence of cells, leading to the expansion of B cells to form B-ALL (24,25). Collectively, the results of the vast majority of recent studies indicate that ARRB-dependent signaling contributes to cancer phenotypes in specific malignancies. In this study we uncovered a tumor-suppressing role for ARRB1 in T-ALL, which was supported by the expression data from clinical samples and the results of *in vitro* and *in vivo* analyses. Furthermore, we identified a novel underlying mechanism through which ARRB1 expression is targeted by the oncomiR miR-223 in T-ALL cells.

Consistent with our findings, the results of a recent study by Francesca Ferrandino *et al* (40) also suggested a tumor-suppressing role of ARRB1 during T-ALL initiation in NOTCH3 transgenic mice, although through a different mechanism. In the transgenic mice, NOTCH3 upregulates the CXCR4 receptor on the surfaces of CD4 and CD8 double positive thymocytes by catalyzing the phosphorylation of ARRB1 and inhibiting the internalization of CXCR4. Consequently, NOTCH3 and CXCR4 positive thymocytes escape the thymus and ultimately colonize bone marrow to degenerate into T-ALL. Taken together, these results and our current findings reveal a previously unrecognized tumor-suppression function of ARRB1 in T-ALL, indicating that further investigation is thus highly warranted.

MicroRNAs (miRNAs) are a class of small noncoding RNAs consisting of 18–25 nucleotides. By pairing the seed sequence with their target mRNAs, miRNAs regulate target gene expression and tune biological processes (41). The dysregulation of miRNAs contributes to the initiation and maintenance of cancer (42). As an oncomiR, miR-223 has been reported to play important roles in inflammation, infection and cancer development (43–45). We observed that miR-223 was abundantly expressed in most T-ALL patient samples, suggesting that it has a potentially important role in T-ALL. Interestingly, ectopic miR-223 was previously shown to cooperate with ICN1 to initiate the leukemogenesis of a T lineage in a xenograft mouse model (36). More recently, miR-223 was identified as the most dynamically regulated miRNA by the T-ALL specific oncogenic transcription factor TAL1 (46). Aberrant miR-223 upregulation by TAL1 is crucial for the optimal growth of TAL1-positive T-ALL cells, and miR-223 overexpression led to a marked downregulation of FBXW7 protein expression, suggesting that TAL1-mediated upregulation of miR-223 may promote the malignant T-ALL phenotype by repressing FBXW7 tumor suppressor (46). Interestingly, another recent study reported that the promoter region of miR-223 contains a binding site for RBPJ, a coactivator of NOTCH1, and that NOTCH1-mediated activation of miR-223 represses the tumor suppressor FBXW7 in T-ALL cell lines (47), suggesting there may be a positive feedback regulatory circuitry between NOTCH and miR-223. In this study, we further demonstrated that ARRB1 can promote NOTCH1 degradation in T-ALL, which is effectively inhibited by miR-223.

In summary, in this study, we investigated whether ARRB1 plays a role in T-ALL development and/or progression. We conducted an RNA-Seq analysis of T-ALL clinical samples and observed that ARRB1 was poorly expressed in T-ALL clinical samples and cell lines. Exogenous ARRB1 expression inhibited T-ALL proliferation and drastically improved the survival rate of xenograft animals. In addition, we observed that the tumor suppression activity was at least in part attributable to ARRB1-mediated NOTCH1 degradation. Further

analysis revealed that the oncomiR miR-223 targets the 3'-UTR of ARRB1 (aka, BUTR) to inhibit ARRB1 expression in T-ALL. Furthermore, we demonstrated that the overexpression of the ARRB1-derived miR-223 sponge BUTR was incompatible with cell proliferation and induced apoptosis in T-ALL cells. Thus, our results demonstrate a tumor-suppressing role for ARRB1 in T-ALL, which is effectively antagonized by miR-223. It is conceivable that miR-223 may serve as a valid drug target for the development of novel and efficacious therapeutics for T-ALL treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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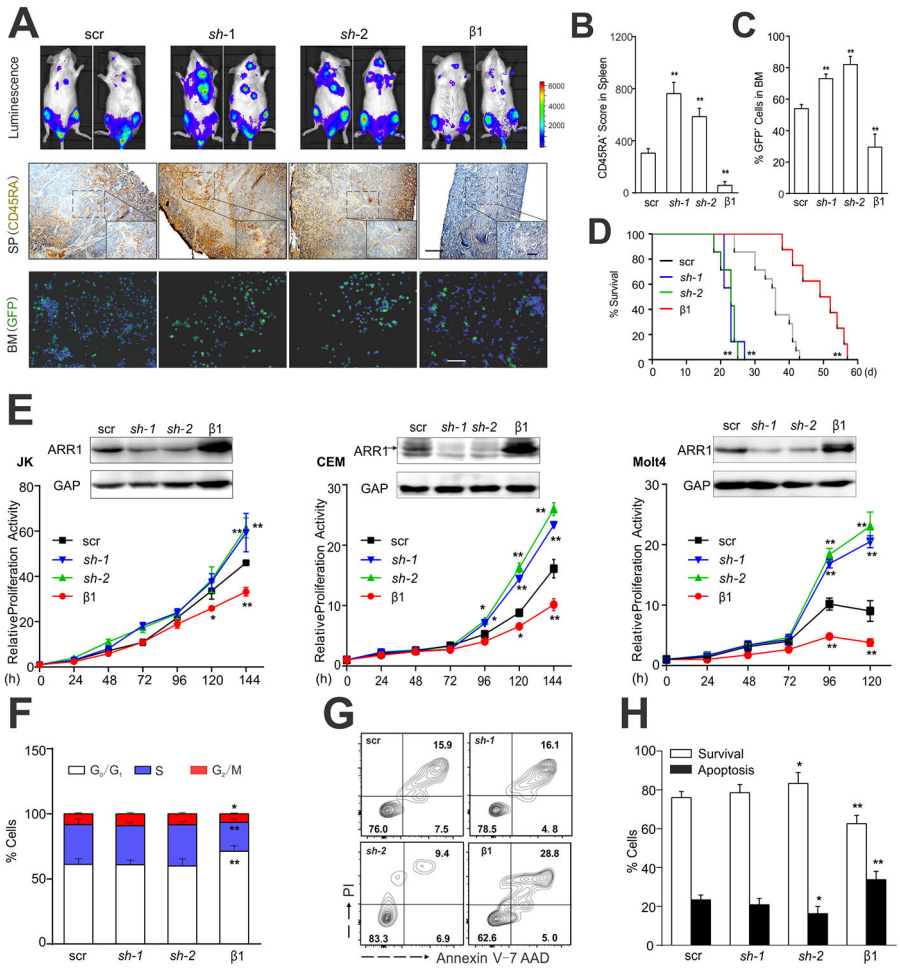

Figure 1. ARRB1 inhibits the tumor progression of human T-ALL cells.

(A) GFP- and firefly luciferase-tagged Jurkat cells with ARRB1 overexpression (β1) or knockdown (sh-1 and sh-2) were tail-vein injected into irradiated NOD/SCID mice (n=5 per group). Twenty-one days after injection, bioluminescence images were obtained after administrating the mice D-Luciferin sodium salt. Subsequently, the mice were sacrificed, the spleens were spliced and stained with an anti-CD45RA antibody, and the bone marrow of the femurs was flushed out and subjected to anti-GFP immunostaining to assess the leukemia burden. Black scale bar, 500 μm; white scale bar, 100 μm. **(B)** The histogram shows the scores for the $CD45RA^+$ cells in the spleens. Image Pro Plus was used to calculate the scores, and three samples were counted for each group. **(C)** The histogram shows the rate of the GFP^+ cells in bone marrow. Three samples were counted for each group. **(D)** The survival curve of the mice (n=9 per group) tail-vein injected with Jurkat cells that were transduced with retroviruses expressing shRNAs targeting ARRB1, full-length ARRB1 or scrambled sequence expressing vector (scr). **(E)** CCK8 assay. The effect of ARRB1 overexpression or silencing on the proliferation of the T-ALL cell lines Jurkat, Molt4, and CCRF-CEM. "**" $p<0.01$ compared with that of the controls. For the western blot, ARRB1 and GAPDH are denoted as ARR1 and GAP, respectively. **(F) & (G)** Cell cycle and apoptosis analysis. **(H)** The histogram shows the rate of survival and apoptosis cells in each

group. The oligonucleotide used in this assay is listed in Supplementary Table 5. “*” $p<0.05$; “**” $p<0.01$

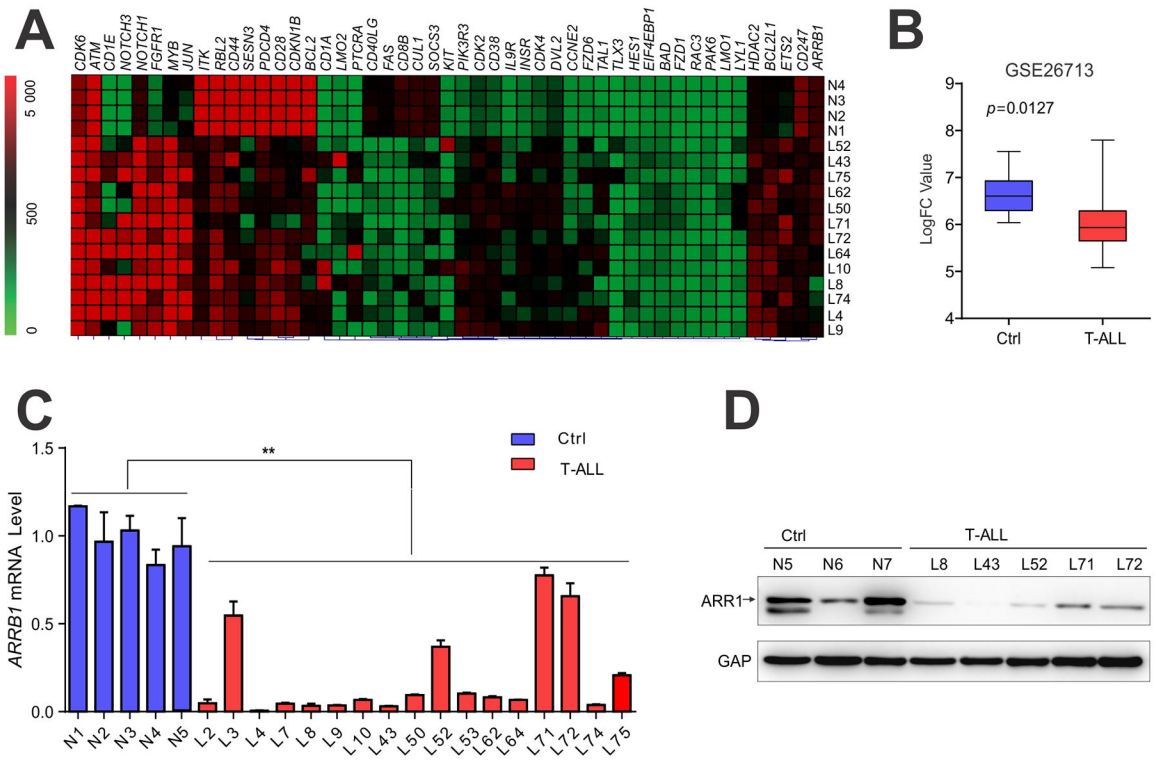

Figure 2. ARRB1 is poorly expressed in human T-ALL.

(A) Heat map of the transcriptomic RNA-Seq results obtained from the bone marrow samples derived from the 13 T-ALL patients and 4 healthy T-cell donors. **(B)** The expression of ARRB1 detected in GSE26713 deposited in the GEO database (Ctrl, n=7; T-ALL, n=117). **(C)** qPCR analysis of ARRB1 expression in the selected T-ALL samples and healthy donors. "**" $p < 0.01$ *vs.* healthy donors as a group. **(D)** Western blotting analysis of ARRB1 expression in the selected T-ALL samples and healthy donors. "N#", healthy donor number; "L#", T-ALL patient number, as indicating in Supplementary Table 2.

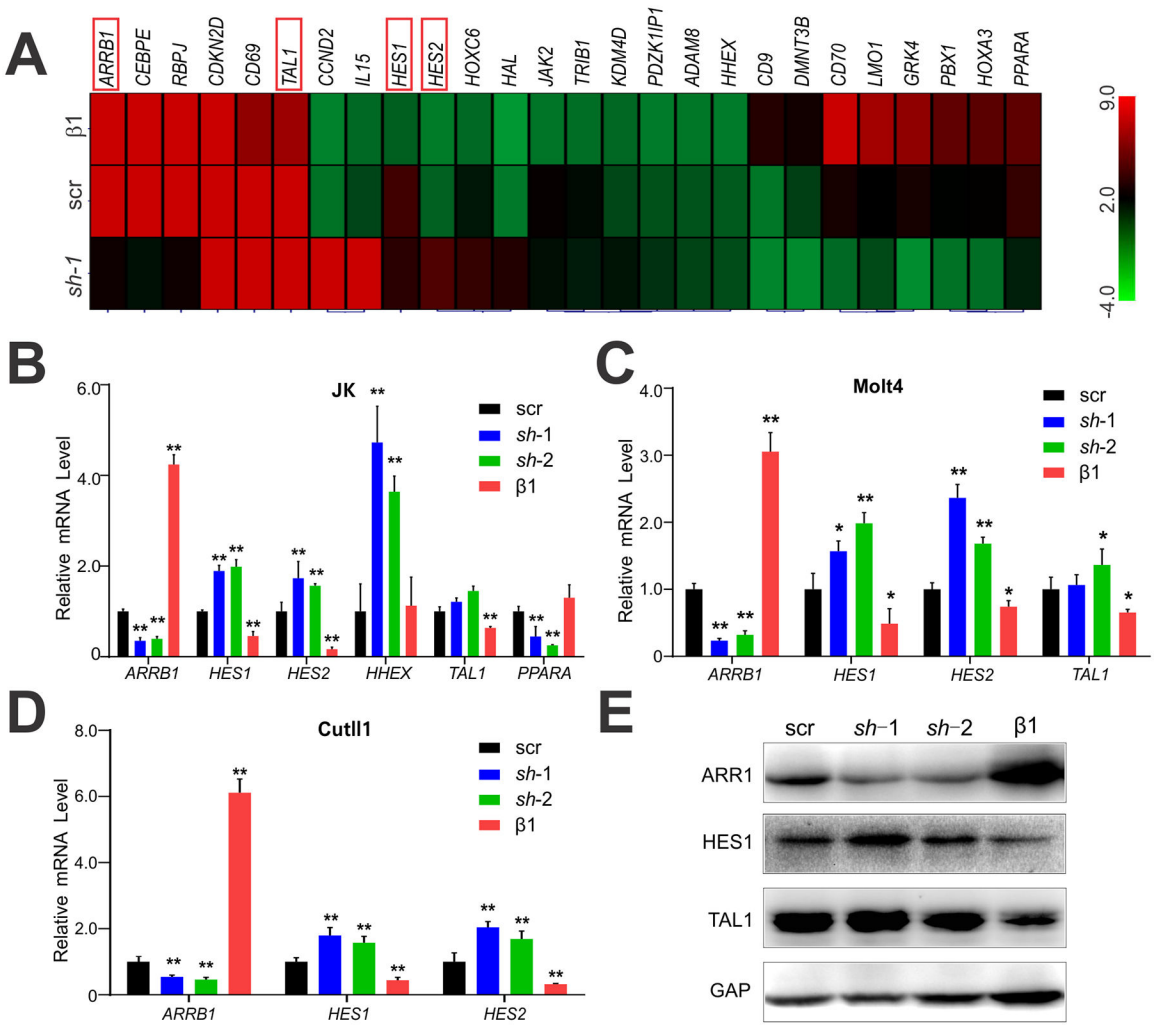

Figure 3. ARRB1 expression is inversely correlated with the expression of TAL1 and NOTCH1 signaling components.

(A) Heat map of the RNA-Seq results obtained using Jurkat cells either overexpressing ARRB1 (β1) or silencing ARRB1 (*sh*-1), compared with the scrambled control (scr). The TAL1 and NOTCH1 targets HES1 & HES2 are boxed. qPCR confirmation of the expression of TAL1 and NOTCH signaling components in Jurkat **(B)**, Molt4 **(C)** and Cutll1 **(D)** cells. "*" $p<0.05$; "**" $p<0.01$. **(E)** Western blotting analysis of the expression of HES1 and TAL1 as well as ARRB1 in Jurkat cells overexpressing ARRB1 (β1) or silencing ARRB1 (*sh*-1 and *sh*-2). The data represent one of three experiments.

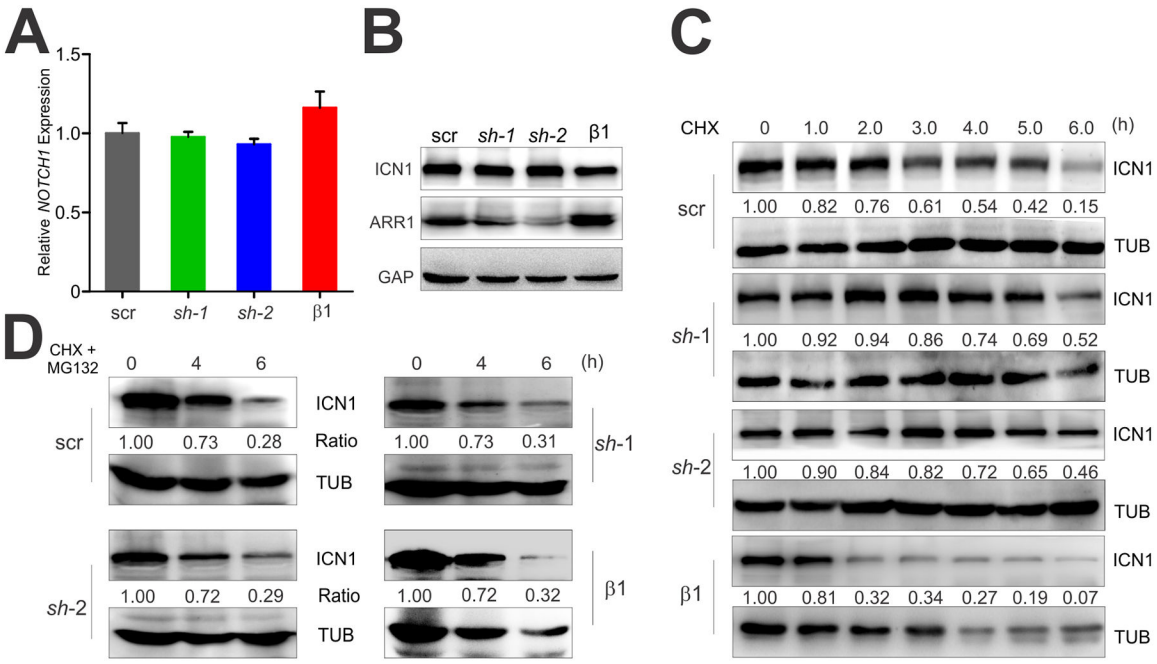

Figure 4. ARRB1 promotes the degradation of ICN1 in T-ALL cells.

The effects of overexpressing ARRB1 (β1) or silencing ARRB1 (sh-1 and sh-2) in Jurkat cells on NOTCH1 expression or ICN1 protein levels were assessed by qPCR **(A)** and western blotting **(B)**. **(C)** and **(D)** The stability of ICN1 protein in Jurkat cells with overexpressed or silenced ARRB1 expression, as determined by western blotting after cyclohexamide (CHX; 100 mg/ml) treatment, without **(C)** or with the proteasome inhibitor MG-132 (**D**). The relative expression of ICN1 to β-TUBULIN (TUB) at each time point was normalized to that observed at 0 hour. β-TUBULIN or GAPDH was used as an internal control. “scr”, scrambled. The data represent one of three experiments.

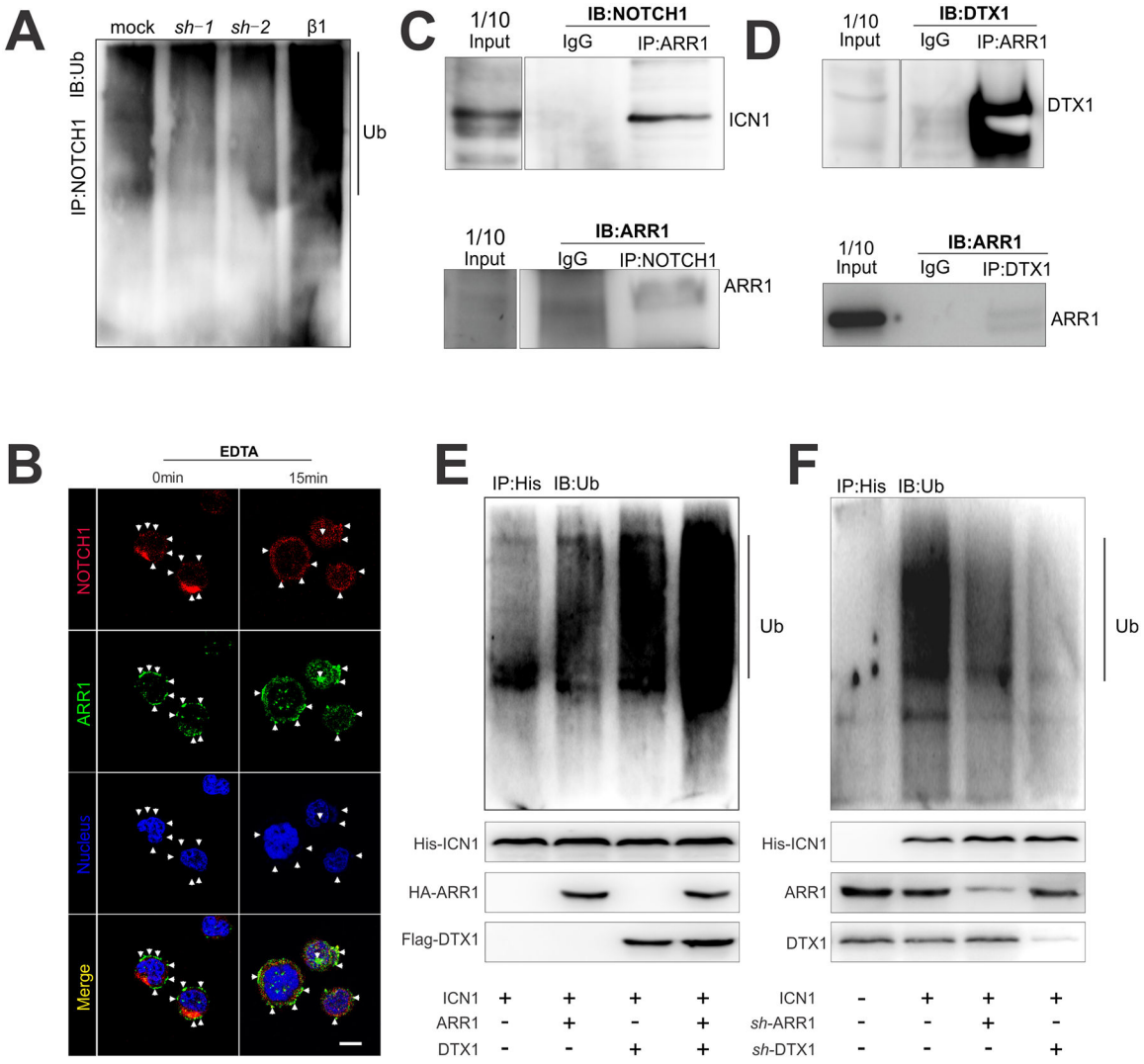

Figure 5. ARRB1 promotes ICN1 ubiquitination in T-ALL cells by serving as a scaffold.

(A) Ubiquitination of the ICN1 protein. ARRB1-overexpressing or knockdown Jurkat cells were treated with MG-132 and immunoprecipitated with a NOTCH1 antibody, followed by immunoblotting against ubiquitin. **(B)** Co-localization of NOTCH1 and ARRB1. Jurkat cells were treated with EDTA at the indicated time points and then subjected to immunofluorescence staining with NOTCH1 or ARRB1 antibodies. Fluorescence signals were detected under a confocal microscope. The arrow indicates the location of an ARRB1 cluster in cells. Scale bar, 10 μm. **(C)** ARRB1 and NOTCH1 proteins were reciprocally immunoprecipitated in Jurkat cells. **(D)** ARRB1 and DTX1 proteins were reciprocally immunoprecipitated in Jurkat cells. IgG served as negative control for the IP procedure. **(E)** Interactions among NOTCH1, ARRB1 and DTX1 and NOTCH1 ubiquitination in a reconstituted system. HEK293T cells were co-transfected with His-ICN1 and HA-ARRB1 and/or Flag-DTX1 for 24 h. The transfected cells were treated with MG-132 for 4 h, lysed and subjected to pulldown assays with Ni-NTA Magnetic Agarose followed by immunoblotting with anti-ubiquitination antibody. **(F)** Silencing ARRB1 or DTX1 leads to a decrease in NOTCH1 ubiquitination. HEK293T cells were co-transfected with His-ICN1

and sh-ARRB1 or sh-DTX1 for 24 h. The transfected cells were treated with MG-132 for 4 h, lysed and subjected to pulldown assays with Ni-NTA Magnetic Agarose, followed by immunoblotting with an anti-ubiquitination antibody. “IP”, immunoprecipitation antibody; “IB”, immunoblotting antibody. All the antibodies used are listed in Supplementary Table 6. The data represent one of three experiments.

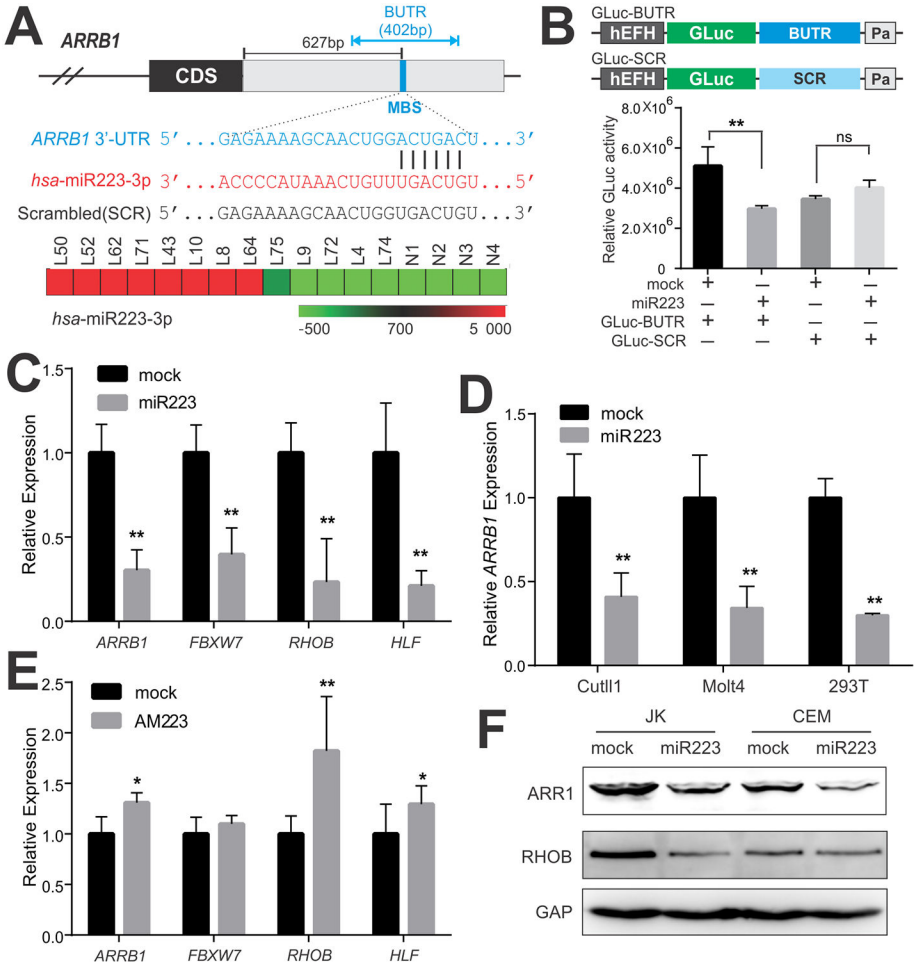

Figure 6. OncomiR-223 suppresses ARRB1 expression in T-ALL cells.

(A) The location and sequence of the predicted binding site of *hsa*-miR-223–3p in the 3'-UTR of human ARRB1 mRNA. The potential binding site with 6-base pairs (miR-223 binding site or MBS) is located 627 nt downstream of the stop codon of the ARRB1 transcript. A 402-bp fragment of the ARRB1 3-UTR (i.e., 200 bp up- and downstream of the binding site) was designated as BUTR, which was used for reporter and anti-miR-223 assays. A scrambled mutant (SCR) was also designed by completely changing the nucleotides at the miR-223 binding sites. The heat map shows the miR-223 expression as the highest expressed miRNAs in the T-ALL clinical samples. **(B)** Gaussia luciferase reporter assay. BUTR and the control SCR fragments were placed downstream of the GLuc coding sequence, resulting in GLuc-BUTR and GLuc-SCR reporters. The reporters were co-transfected into HEK-293 cells with a pri-miR-223 expression vector or a miR-223 scrambled sequence expression vector (scr). GLuc activities were assayed 48 h after transfection. "**" $p<0.01$. **(C)** qPCR analysis of miR-223 target gene expression, including ARRB1, in Jurkat cells. **(D)** Cutll1, Molt4 and HEK293T cells were infected with miR-223 expression or control (scr) retroviruses for 48 h. Total RNA was isolated from the infected cells and subjected to qPCR analysis. "**" $p<0.01$. **(E)** miR-223 antagonist rescues the expression of miR-223 target genes. Jurkat cells were infected with anti-miR-223 (AM223)

expression or control (scr) retroviruses for 48 h. Total RNA was isolated from the infected cells and subjected to qPCR analysis. "*" $p<0.05$; "**" $p<0.01$. **(F)** ARRB1 protein levels are downregulated by miR-223. Jurkat and CEM cells were infected with miR-223 expression or control (scr) retroviruses for 48 h. Total cell lysates were subjected to western blotting with ARRB1, RHOB or GAPDH antibodies. The data represent one of three experiments.

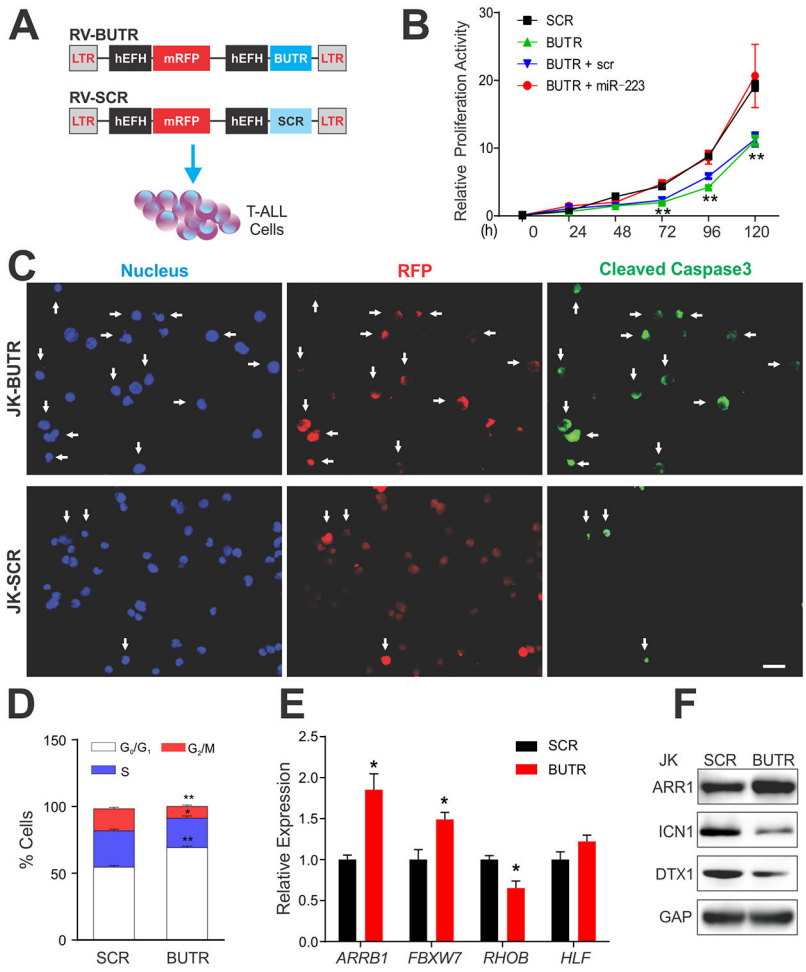

Figure 7. Suppression of miR-223 function effectively inhibits T-ALL cell proliferation and induces apoptosis.

(A) Schematic representation of the miR-223 sponge vector RV-BUTR, which expresses a 402-bp fragment encompassing the miR-223 binding site in the ARRB1 3'-UTR, and the binding site scrambled control RV-SCR. **(B)** BUTR overexpression inhibits T-ALL cell proliferation, which can be overcome by exogenous miR-223. Jurkat cells were infected with RV-BUTR or RV-SCR alone or co-infected with miR-223 or the scrambled vector (scr). The infected cells were subjected to a CCK8 cell proliferation assay. "**" $p<0.01$ vs. SCR control group. **(C)** BUTR overexpression induces the production of cleaved caspase-3. Jurkat cells were infected with RV-BUTR or RV-SCR for 48 h and then subjected to immunofluorescence staining with a cleaved caspase-3 antibody. The RFP and immunostaining signals were recorded under a fluorescence microscope. Cells staining positive for cleaved caspase-3 are indicated with arrows. Representative images are shown. Scale bar, 25 μm. **(D)** Cell cycle analysis. Jurkat cells were infected with RV-BUTR or RV-SCR for 48 h and then subjected to cell cycle analysis. "**" $p<0.01$ *vs.* the SCR control group. **(E)** BUTR overexpression restores the expression of miR-223 target genes. Jurkat cells were infected with RV-BUTR or RV-SCR for 48 h. Total RNA was isolated and subjected to qPCR analysis to assess the expression of miR-223 target genes, including *ARRB1*, *FBXW7* and *RHOB*. "*" $p<0.05$ vs. the SCR control group. **(F)** Jurkat cells were

infected with RV-BUTR or RV-SCR for 48 h. Total cell lysates were subjected to western blotting with ARRB1, NOTCH1, DTX1 or GAPDH antibodies. The data represent one of three experiments.