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The deregulated expression of miR-125b in acute myeloid leukemia is dependent on the transcription factor C/EBPα

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MicroRNAs (miRNA) are small-noncoding RNAs of 21 nucleotides (nt) that regulate the expression of several genes.[1,2] Transcribed as-primary miRNAs are processed in the nucleus into 70–80 nt, hairpin-shaped precursors, called pre-miRNAs.[1,2] They are then exported in the cytoplasm and further processed into mature miRNAs (21 nt), and incorporated in the RNA-induced silencing complex.[1,2]

The miR-125b is upregulated in many neoplastic blood disorders, including acute myeloid leukemia (AML).[3–6] Enforced constitutive overexpression of miR-125b in mice induces myeloid leukemia.[7] It has been indicated that miR-125b in a myeloid context, might act as an oncomiR able to transform cells by targeting multiple genes involved in apoptosis, cell cycle and differentiation (Tili *et al.*[6] and references therein). Relevant to myeloid leukemia, C/EBPα is frequently mutated in AML, but surprisingly, none of the observed mutations result in full ablation of the gene.[8,9] This indicates that activity of C/EBPα is required for

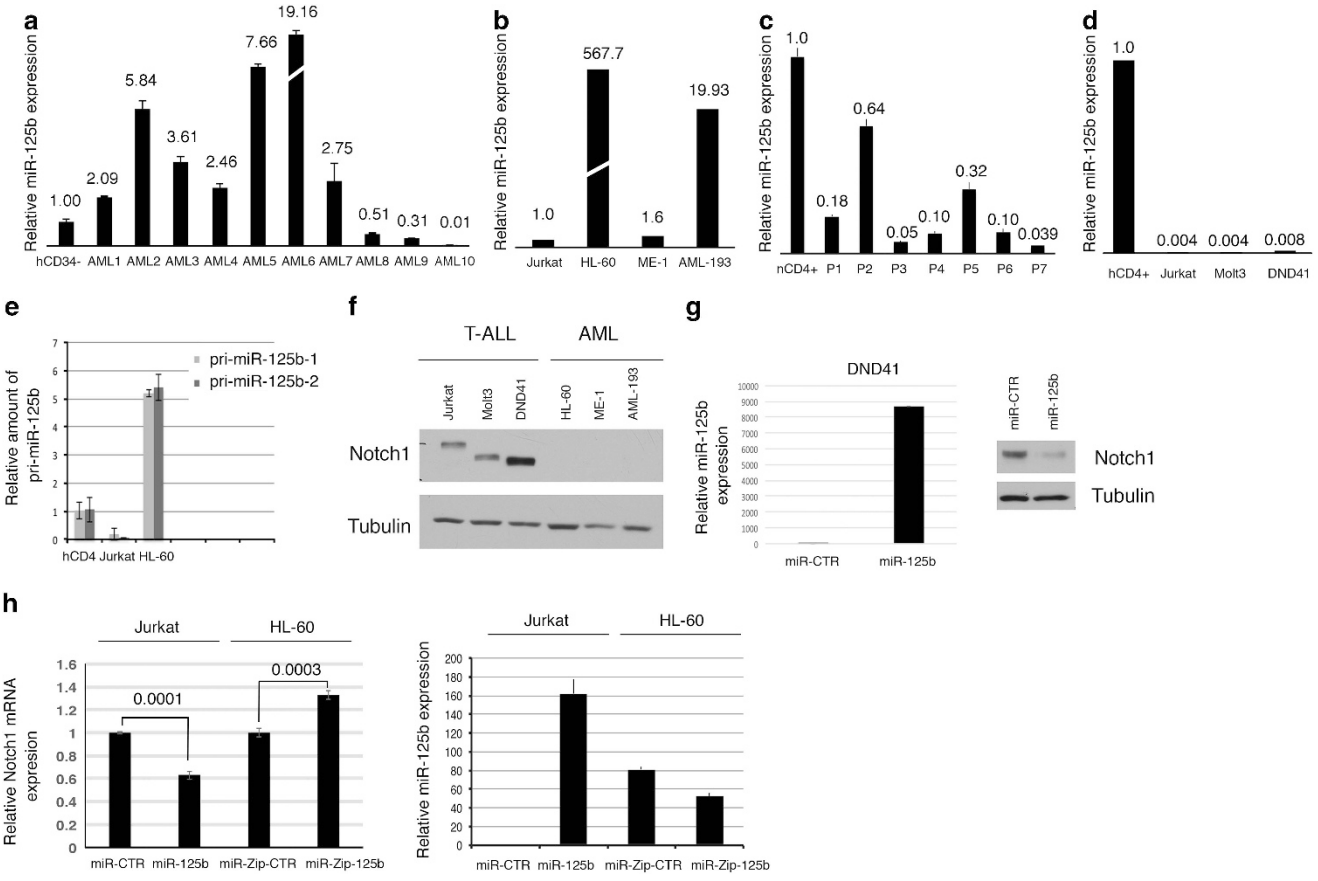

Figure 1. miR-125b is upregulated in AML patient samples. (**a**) qRT-PCR for miR-125 was carried out using bone marrow cells derived from AML patient samples ($n = 10$ and healthy donors CD34 − cells). Values were normalized to U6 and further to the expression level of three healthy donors. (**b**) qRT-PCR for miR-125b was carried out using AML-derived cell lines, values are expressed as fold increase over Jurkat cells. Values were normalized to U6. (**c**) qRT-PCR for miR-125 was carried out using PBC cells derived from T-ALL patient samples ($n = 7$ and healthy donors CD4+ cells). Values were normalized to U6 and further to the expression level of three healthy donors. (**d**) qRT-PCR for miR-125b was carried out using T-ALL-derived cell lines, values are expressed as fold increase over CD4+ healthy donor-derived cells. Values were normalized to U6. (**e**) pri-miR-125b-1 and two transcripts were determined by qRT-PCR in the indicated cells. (**f**) Western blot analysis of Notch1 expression in whole cell extract in the indicated cell lines. Tubulin is shown as a loading control. (**g**) DND41 cell line were transfected with either miR-ctr or mature miR-125b, and mature miR-125b expression was analyzed by qRT-PCR (left). miR-ctr and miR-125b transfected cells were analyzed by western blot with the indicated antibodies. (**h**) Left panel, T-ALL- (Jurkat) and AML (HL-60)-derived cell lines were infected with either miR-ctr/miR-125b or MiRZIP-ctr/MiRZIP125b, and Notch1 mRNA expression was analyzed. Right panel, miR-125b expression was evaluated in the samples shown in the left panel. All results were expressed as means ± s.d., and *P*-values are indicated.

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AMLs, thus in addition to work as a tumor suppressor C/EBPα appears to be required for the development of at least some AML subtypes.[8–10] We previously showed that the manifestation of Hailey–Hailey disease, a rare skin disorder, was in part dependent on Notch1 downmodulation mediated by miR-125b upregulation.[11,12] Notably, although the involvement of Notch signaling as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL) is well characterized, Notch signaling acts as a tumor suppressor in myeloid malignancies. Moreover, although T-ALL cells express Notch1 receptor, its expression is silenced in AML (Lobry *et al.*[13] and references therein). It has been previously shown that miR-125b is overexpressed in AML; thus, we investigated whether

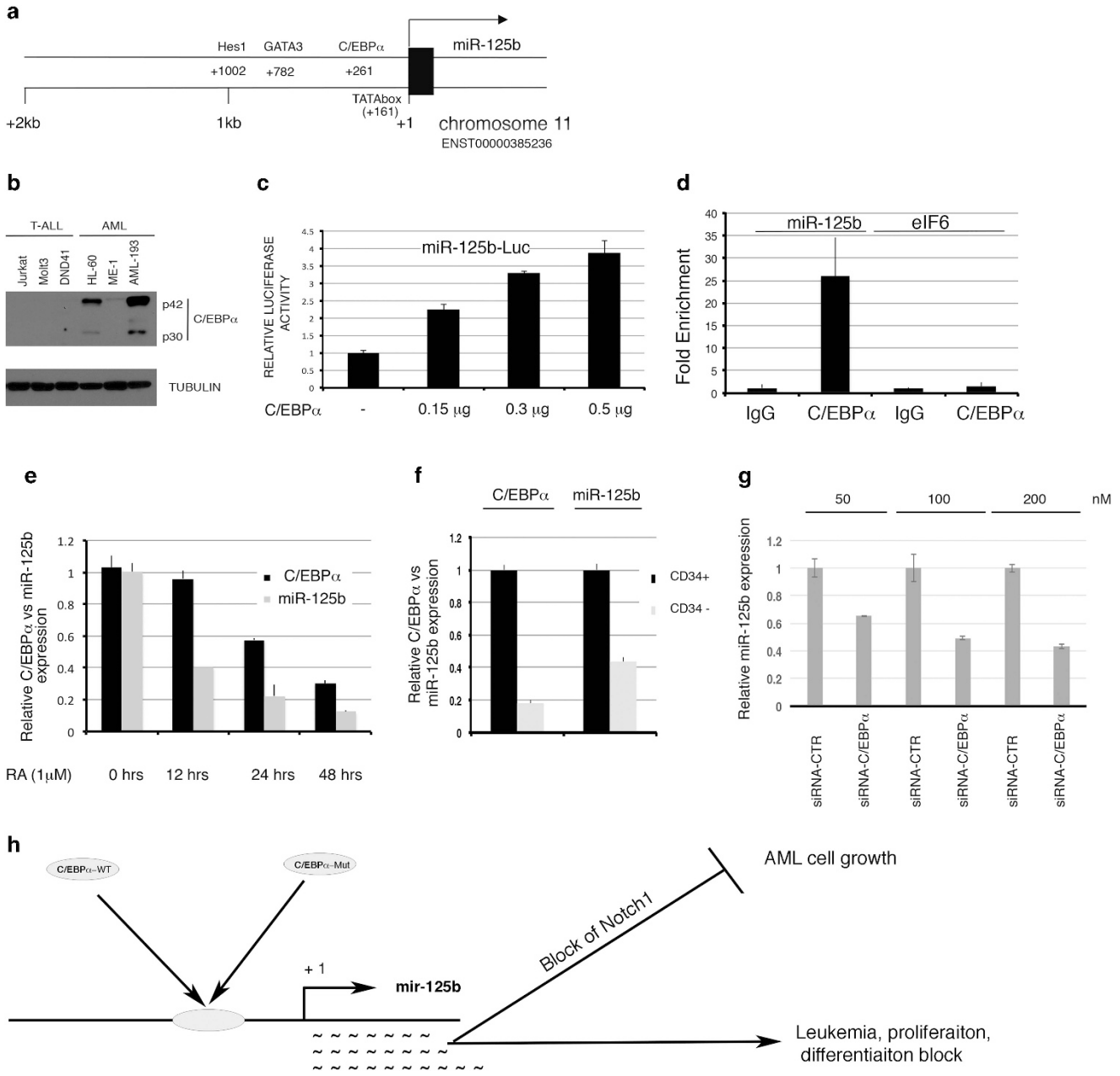

Figure 2. miR-125b is a direct target of C/EBPα. (**a**) Schematic representation of examined putative C/EBPα-binding sites in the promoter regions of miR-125b. (**b**) C/EBPα protein expression assessed by western blotting in the indicated cell lines. (**c**) HEK293T cells were transfected with miR-125b-luciferase responsive promoter construct, 0.25 μg/well in 24-well dishes, and treated with the indicated amount of C/EBPα; cells were harvested 24 h after transfection for luciferase assay. All conditions were tested in triplicate samples and s.d. is indicated. (**d**) Chromatin derived from HL-60-C/EBPα-positive cells was immunoprecipitated with anti- C/EBPα or IgG antibodies. Recovered DNA was PCR amplified with primers specific for C/EBPα-binding amplificon. Shown is the fold change in binding affinity of the anti-C/EBPα antibody normalized to IgG. Immunoprecipitation was performed three times using different chromatin samples, and the occupancy was calculated by using the ChIP-qPCR Human IGX1A Negative Control Assay (Qiagen, Milano, Italy) as a negative control. As additional control recovered DNA was PCR amplified with primers specific for Hes-1-binding amplificon in the eIF6 promoter lacking of C/EBPα-binding site.[15] (**e**) miR-125b and C/EBPα mRNA expression was analyzed at 0, 12, 24 and 48 h post RA treatment of HL-60 cells. (**f**) miR-125b and C/EBPα mRNA expression in CD34+ versus CD34 − human primary cells. (**g**) miR-125b expression of C/EBPα-silenced HL-60 cells, as assessed by qRT-PCR. (**h**) A scheme showing transcriptional regulation and function of miR-125b in AML. miR-125b is induced by both WT and mutant C/EBPα. High expression of miR-125b leads to differentiation block, proliferation and transformation. In parallel miR-125b decreases Notch1 protein level, which has also been implicated in suppression of AML cell growth.

miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML. We compared miR-125b expression pattern in both primary AML and T-ALL leukemia as well as in AML and T-ALL-derived cell lines (Figures 1a–d). Both the human primary and AML cell lines samples demonstrated significant upregulation of miR-125b expression. Conversely, both primary and T-ALL cell lines failed to show significant enrichment of this miRNA.

In order to investigate whether the deregulation of miR-125b expression occurs either at the transcriptional or processing level, primary miR-125b expression levels were analyzed in both Jurkat and HL-60 cell lines, as well as in primary AML samples (Figure 1e, Supplementary Figure S1, Supplementary Table 1 and Supplementary Figure S7). Specifically, quantitative reverse transcriptase PCR was performed to compare the levels of primary and mature miRNA. The primary miRNA levels of the miR-125b were found to parallel mature miR-125b expression in both cell lines examined (Figures 1b and e). In most primary AMLs, we found that miR-125b expression was transcriptionally upregulated (Supplementary Figure S1). Nevertheless, we observe that in some samples primary miRNAs basal transcription efficiency was associated with a low abundance of the mature miRNA (Figure 1a, AMLs 8, 9 and 10). Thus, these observations indicated that in this cellular context there is generally a high rate of primary miR-125b transcription, although an altered processing efficiency might determine the level of mature miRNAs.

We found an inverse correlation of miR-125b expression and Notch1 protein levels in both T-ALL and AML cell lines, as well as in primary AML samples (Figures 1b, d and f and Supplementary Figure S2). We observed higher level of miR-125b expression in AML when compared with T-ALL samples (Supplementary Figure S3). Importantly, the Notch1 target genes, *Hes-1* and *Deltex1*, were significantly higher in T-ALL when compared with AML (Supplementary Figure S3). Recently, we found that *NOTCH1* is a target of miR-125b;[12] thus, we analyzed the potential involvement of miR-125b in regulating the differential expression of Notch1 between T-ALL and AML cells. We analyzed Notch1 protein expression after overexpression of either miR-125b or AntagomiR-125b in T-ALL and AML cell lines, respectively.

DND41 cells, but not Jurkat and HL-60 cells, are highly transfectable. To overcome these limitations, DND41 cells were analyzed by transient transfection and both Jurkat and HL-60 cells were transduced by lentiviral infection. We found that deregulated miR-125b expression impaired Notch1 levels in DND41 (Figure 1g), and although with a lower effect also in Jurkat and HL-60 cell lines (Figure 1h). Together, these results suggest that deregulation of miR-125b expression has a critical role in the differential expression of Notch1 between T-ALL and AML. However, ME-1 cells devoid of miR-125b expression have undetectable level of Notch1 expression (Figures 1b and f). Additionally, in the T-ALL derived cell line, Molt3, miR-125b enforced expression did not affect Notch1 expression (data not shown); thus, it is likely that other mechanisms alone or synergistically with the miR-125b are involved in Notch1 downmodulation in AML[14] or alternatively an unknown mechanism antagonizes the repressive activity of miR-125b on the 3′-untranslated region of *Notch1* in a cell context-specific manner.

To explore the mechanism regulating miR-125b expression, we first characterized the miR-125b promoter region using the Genomatix MatInspector software package (Genomatix Software GmbH, Munich, Germany), focusing on those transcription factors that have been shown to have a role in either T-ALL or AML. A scan of 2 kb of genomic sequence located upstream of the predicted pre-miR-125b start site identified putative Hes-1, GATA3 and one C/EBPα consensus binding sites (Figure 2a), suggesting the involvement of those factors in the regulation of miR-125b expression. Thus, protein extracts from AML and T-ALL-derived cell lines were first analyzed for expression of those factors. Interestingly, C/EBPα expression was correlated with miR-125b expression in the cell lines examined (Figures 1b–d and 2b). We next examined the role of these transcription factors in the regulation of miR-125b expression by generating a miR-125b promoter construct and testing it in a luciferase reporter assay. As shown in the Figure 2c, we found the induction of miR-125b promoter activity by C/EBPα transfection in a dose-dependent fashion, but neither by HES-1 nor GATA3 (data not shown), indicating that C/EBPα might be a transcriptional regulator of miR-125b expression. C/EBPα is a key myeloid transcription factor, frequently mutated in AML, but none of the described mutations result in the full loss of its function.[10] Recently, it has been shown that C/EBPα-dependent activity has an important role in AML etiology.[10] Next, we investigated whether C/EBPα directly regulates miR-125b promoter. To test whether C/EBPα binds directly to the miR-125b promoter, we performed chromatin immunoprecipitation experiments in both HL-60 cells and primary AML samples. The chromatin fragments were immunoprecipitated with an anti-C/EBPα antibody. The DNA fragments were analyzed with specific primers for the indicated regions of the miR-125b regulatory region (Figure 2d and Supplementary Figure S4). We were able to observe an enrichment of DNA from the predicted C/EBPα-binding sites when compared with the immunoglobulinG control (Figure 2d). Additionally, we observed an increased recruitment of C/EBPα onto the miR-125b promoter in AML primary samples highly expressing miR-125b primary transcript (Supplementary Figures S1 and S4).

The myeloid cell lines provide an important *in vitro* model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage. Both HL-60 and NB4 pro-myelocytic leukemia cell lines have the potential to differentiate toward granulocytic lineage by exposure to retinoic acid. Thus, to explore further the role of C/EBPα in the induction of miR-125b expression, we compared C/EBPα and miR-125b expression after retinoic acid treatment (Figure 2e). Treatment with retinoic acid (1 μM) strongly decreased both C/EBPα protein and mRNA expression (Figure 2e and Supplementary Figure S5a), in parallel with induction of granulocytic differentiation (Supplementary Figure S5). Notably, in both cell lines, HL-60 and NB4, the downregulation of C/EBPα expression by retinoic acid parallels that of miR-125b (Figure 2e and Supplementary Figure S6). Interestingly a similar parallel expression was observed in CD34+ and CD34− primary cells (Figure 2f). Finally, small interfering RNA against C/EBPα in HL-60 abolished the basal level of miR-125b expression (Figure 2g), further supporting our finding that miR-125b is a direct target of C/EBPα.

In summary, several studies have made important advances in elucidating the contribution of both C/EBPα and miR-125b into the molecular mechanisms of AML development. Our study implicates the transcription factor C/EBPα as a critical determinant of miR-125b expression in AML, supporting a model whereby C/EBPα functions to enhance miR-125b expression to regulate a group of genes whose deregulation leads to acute myeloid transformation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CT designed the research, analyzed the data and wrote the paper; IS supervised the work; PVR, SC, RP and CDB performed experiments; GZ provided AML cell lines, reagents and analyzed the data; SC and DB commented on the paper. SC and RF provided AML samples. AA provided T-ALL samples. CT, PVR, SC assembled the figures.

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P Vargas Romero[1], S Cialfi[1], R Palermo[2], C De Blasio[1], S Checquolo[3], D Bellavia[1], S Chiaretti[4], R Foà[4], A Amadori[5], A Gulino[1,6,7], G Zardo[4], C Talora[1] and I Screpanti[1]

[1]*Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy;*
[2]*Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy;*
[3]*Department of Biotechnology and Medical-Surgical Sciences, Sapienza University, Latina, Italy;*
[4]*Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome, Rome, Italy;*
[5]*Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy and*
[6]*Neuromed Institute, Pozzilli, Italy*
E-mail: claudio.talora@uniroma1.it or isabella.screpanti@uniroma1.it
[7]*Dedicated to the cherished memory of Alberto Gulino.*

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A novel recurrent *EP300–ZNF384* gene fusion in B-cell precursor acute lymphoblastic leukemia

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In pediatric patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), approximately three-quarters harbor well-characterized, clinically relevant chromosomal alterations, including hyperdiploidy, hypodiploidy, t(12;21) *ETV6/RUNX1*, t(1;19) *E2A/PBX1*, t(9;22) *BCR/ABL1* and the rearrangement of *MLL* at 11q23, and they can facilitate diagnosis, risk stratification and targeted therapy.[1,2] In the remaining patients, however, major pathogenic or driver gene abnormalities and their association with the clinical outcome have yet to be fully clarified. As recent advanced genomic studies using next-generation sequencing have identified a number of novel fusion genes and stratified a high-risk subtype in BCP-ALL,[3–5] unknown genetic alterations that constitute characteristic subgroups may still exist in the remaining patients. We therefore intended to investigate unknown fusion genes in BCP-ALL by using next-generation sequencing.

As a consequence of whole transcriptome sequencing performed on complementary DNA from 55 selected samples of pediatric BCP-ALL patients without conventional genetic abnormalities (Supplementary Information), an *EP300–ZNF384* fusion gene was identified in two patients (Cases 1 and 2) as a repeatable and plausible candidate fusion gene (Figure 1a). The 372-bp fragment of the *EP300–ZNF384* fusion cDNA was amplified by RT-PCR using a pair of specific primers, and Sanger sequencing of the PCR products revealed a sequence of the products identical to that obtained by whole transcriptome sequencing (Supplementary Figure 1). The presence of *EP300–ZNF384* fusion in Case 1 was further confirmed by FISH using a combination of appropriate probes for *EP300* and *ZNF384*, respectively (Figure 1b). We screened a further 346 of pediatric ALL cases by RT-PCR, and identified 4 additional patients with *EP300–ZNF384* fusion (Cases 3–6, Supplementary Figure 1). All six patients were BCP-ALL without conventional cytogenetic abnormalities. Our RNA samples

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