Supplemental Material

BCL11A promotes myeloid leukemogenesis by repressing PU.1 target genes

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Supplemental methods and four Supplemental Figures
Supplemental Methods

Retroviral tagging
Trib1-induced AML was reported previously.\textsuperscript{14,17} Integration sites of the Trib1 retrovirus were identified by inverse PCR as previously described.\textsuperscript{14}

Plasmids
Bcl11a and Trib1 cDNAs were cloned into pMYs retroviral vectors. Lentivirus plasmids containing short hairpin RNA (shRNA) constructs of mouse Bcl11a, Kdm1a, Hdac1, Hdac2, Bcor, Smrt, Sin3a, Mta2 and Ncor, and non-target control were purchased from Sigma (Sigma-Aldrich). The shRNA sequences are listed in Supplemental Table 1.

Immunoprecipitation and immunoblotting
Immunoprecipitation was performed using cell lysates in RIPA buffer and protease inhibitor cocktail as described.\textsuperscript{48} Western blot analysis was performed using lysates of whole tumor tissues with specific antibodies listed in Supplemental Table 6.

Flow cytometry
Cells were analyzed on the FACSaria II flow cytometer (BD) and data were analyzed with FlowJo software v.10.5 (TreeStar). The list of antibodies is shown in supplemental Table 6.

Cell adhesion assay
Fibronectin was purchased from Sigma (F0895) and was immobilized 1 hour at room temperature into 24-well plates (50 μg/ml). Cell adhesion to fibronectin was evaluated as described previously with minor modifications.\textsuperscript{49} In brief, 1 X 10\textsuperscript{6} cells in cell culture medium containing 0.5% FBS were loaded to fibronectin-coated plates and were incubated for 24 h. After removal of non-adherent cells by gentle washing with PBS, adherent cells were removed by trypsinization and subjected to measurement.

OP9/leukemic cells coculture assay
Bone marrow-derived OP9 stromal cells were cultured in 6-well plates. Cobblestone formation of leukemic cells were evaluated by the method previously described.\textsuperscript{18}
Gene expression profiling

GeneChip analysis was conducted to determine gene expression profiles using murine Genome 430 PM Array (Affymetrix, Santa Clara, CA, United States) according to the procedure described. Gene Set Enrichment Analysis (GSEA) was performed using GSEA-P 2.0 software.

Quantitative RT-PCR (qRT-PCR)

Total RNA extraction, reverse transcription and RNA quantification were performed by standard methods. Conventional RT-PCR and real-time quantitative RT-PCR (Q-RT-PCR) were performed with a Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA, United States) and a 7500 Fast Real-Time PCR System (Applied Biosystems), respectively. The sequences of the oligonucleotide primers are shown in Supplemental Table 7.

Chromatin immunoprecipitation (ChIP) and ChIP-Seq

ChIP-Seq was performed using the method previous described with modifications. A total of 5 x 10^7 AML cells per immunoprecipitation were cross-linked with 1% formaldehyde for ten minutes at room temperature. Chromatin was sheared in sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris pH 8.0 to an average size of 400 to 500 bp using a Covaris S220 sonicator (Covaris, Woburn, MA, USA) for 40 min. ChIP was performed with 5 μg anti-histone H3K27ac (Active Motif, Carlsbad, CA), anti-Bcl11a (Abcam) or anti-PU.1 (Santa Cruz) antibodies. The antibody-bound protein/DNA complexes were immunoprecipitated using protein G magnetic beads. Immunoprecipitated DNA was then purified and subjected to secondary sonication to an average size 150 to 350 bp. Libraries were prepared according to instructions accompanying the ThruPLEX DNA-Seq kit (RUBICON GENOMICS, Ann Arbor, MI, USA). The ChIP DNA was end modified and adapters were ligated. DNA was PCR amplified with Illumina primers and Illumina-compatible indexes were added (San Diego, CA, USA). The library fragments of approximately 300-500 bp were band-isolated from an agarose gel. The purified DNA was sequenced on an Illumina MiSeq next-generation sequencer following the manufacturer protocols. Base calls were performed using Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). ChIP-Seq reads were aligned to the mm9 genome assembly
Peak calling was performed using MACS1.4 (http://liulab.dfci.harvard.edu/MACS). Peak distribution was calculated and neighbor genes were determined by Nucleus (https://rias.rhelixa.com). The neighbor genes on enriched genomic regions were determined using Cell Innovation Program (https://cell-innovation.nig.ac.jp). The results were visualized using IGV_2.3.80 (http://software.broadinstitute.org/software/igv). The de novo motif enrichment was performed using HOMER v 4.11.1 (http://homer.ucsd.edu/homer/motif). The ChIP-seq and microarray data are accessible through the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), with the accession number GSE147798.

**Proximity ligation assay (PLA)**
PLA was performed using the Duolink In Situ kit (Sigma). Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were then incubated with anti-myc and anti-PU.1 antibodies at 4 °C overnight. Incubation with the PLA probe, ligation, and amplification of signals were performed according to the manufacturer’s protocol. Fluorescent images were photographed with a Zeiss LSM 710 laser scanning microscope with a x60 objective (Zeiss) and LSM software ZEN 2009 (Zeiss).

**Esterase staining**
Dual esterase stains were performed using Esterase staining kit for alpha-naphthol butyrate esterase and naphthol AS-D chloroacetate esterase staining kit (Muto Pure Chemicals).

**Luciferase reporter assay**
A 1.8 kb genomic DNA fragment upstream of murine *Fcgr3* exon 1 was amplified by PCR using the forward (5’-CCTAGATAGGTCTATTGGTTGCTC-3’) and reverse (5’-GTGTATCCAAGCGTGGACTAAAGC-3’) primers. For *Clec5a*, a 1.5 kb enhancer and promoter fragments were amplified by PCR using the forward (5’-CAGTATCCACTAAACCACCCAGGC-3’) and reverse (5’-TTTACCCTACACATATGGGGTTTGGTC-3’), and the forward (5’-CAGGGCAACAGCAGTGAGAAATAGC-3’) and reverse (5’-AGTATGGGGAAGACAAATTGTGGAAGA-3’) primers, respectively. The
fragments were inserted into the pGL4.10 vector (Promega, Madison, WI, United States). Reporter plasmids and the expression plasmid of Bcl11a and/or Spi1 were transfected into U2OS cells using LipofectAmine 2000 (Life Technologies). Luciferase assays were performed by a standard method.

Chromosome conformation capture (3C) analysis
3C analysis was performed as described previously\textsuperscript{52} with minor modifications. In brief, 1 x 10\textsuperscript{7} cells were cross-linked with 1% formaldehyde for ten minutes at room temperature. Nuclei were purified using NP-40 lysis buffer containing 0.2% NP-40, 10 mM NaCl, and 10 mM Tris pH 8.0, re-suspended in restriction buffer containing 0.3% sodium dodecyl sulfate (SDS), 2% Triton-X100, and 10 mM Tris pH 8.0, and digested with 500 U of HindIII (New England Biolab) at 37 °C overnight. After inactivation of the restriction enzyme proximity ligation was performed with 800 U of T4 DNA ligase (New England Biolab) in 7mL total volume at 16 °C for 6 h. After de-crosslinking and RNase treatment, purified DNA was subjected to qPCR. Primers are listed in Supplemental Table 8. For the standard qPCR curve, purified mouse BAC DNAs containing the 5’ region of Clec5a, and the Sfi1 locus as a control.

Immunofluorescence
Tr1 and TB-13 cells were fixed with 4% paraformaldehyde for 10 min. Immunofluorescence was performed using the anti-Filamin A (supplemental Table 6) and FITC-conjugated anti-rat antibodies.

Drug treatment
GSK2879552, GSK-LSD1, pracinostat, Panobinostat, 5-Azacytidine, and decitabine were purchased from Selleck Chemicals (Houston, TX). For in vivo drug treatment assay, 1 X10\textsuperscript{7} TB-13 cells were injected intravenously after 4.0-Gy irradiation. seven weeks after the injection, GSK2879552 (1.5 mg/kg), five times per week and pracinostat (75 mg/kg), three times per week were orally administered to mice until leukemia developed. Mice were monitored carefully by checking white blood cell counts, Giemsa staining and mKO/GFP-positive fractions in peripheral blood.

Cell migration assay
The cell migration assay was performed as described previously using a cell migration chamber with 5-μm pore size membrane (Merck Millipore). 53

Supplemental References
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Supplemental Figure 1. Bcl11a promotes Trib1-induced AML development. (A) FACS showing the negative expression of CD19 and CD3 (bottom) in Tr1, TB-13, and TB-14 cells. FACS results using IgG isotype control is also shown (top). (B) Suppression of TB-14 cell proliferation (left) and self-renewal (right) by shRNA-mediated Bcl11a knockdown. Three independent shRNAs for Bcl11a are used. (C) Kaplan-Meier survival curves show inhibition of AML development by Bcl11a silencing in the recipients transplanted with TB-14 cells. ** P < 0.01, *** P < 0.001. (D) GSEA shows enrichment of the cell cycle pathway in TB-13 TB-14 cells.
**Supplemental Figure 2. Bcl11a interacts with PU.1.** (A) Co-immunoprecipitation assay shows the interaction between 3 isoforms of Bcl11a (v1, v2, and v4) and PU.1 (left). Schematic structures of each isoform are indicated in right. (B) Expression of isoforms v2 and v4 failed to show leukemia induction in vivo. Expression of isoforms are shown by western blotting using an anti-myc antibody (left).
Supplemental Figure 3. Nucleotide sequences for PU.1 binding peaks with or without Bcl11a, shown in Figure 3C and 3E. (A) ChIP-seq occupancy profiles for Bcl11a, PU.1, and H3K27ac at the Akt3 and Fam117b loci in TB-13 cells. (B) Fcgr3 promoter. (C) Clec5a promoter and enhancer. Bcl11a consensus sequences are indicated in red, and PU.1a consensus sequences in blue. (B and C) Sequences for the 5’ > 3’ DNA strand are shown in the same transcriptional orientation of each gene.
Supplemental Figure 4. Identification of Asb2 as a PU.1 target gene, repressed by Bcl11a. (A) Expression level of each gene is validated by qRT-PCR. Relative expression of individual genes in TB-13 and TB-14 cells relative to those in Tr1 is indicated as the means ± SEM. (B) CRISPR/Cas9-mediated deletion of the Bcl11a-binding motif at the
Asb2 locus. Direct sequencing show homozygous deletion of 41 bp containing Bcl11a binding sequence (TGACCA; boxed) within the Bcl11a and PU.1 binding peak. (C) Increased expression of Asb2 by deletion of the Bcl11a binding motif indicated by qRT-PCR. (D) Decreased adhesion of Bcl11a-expressing cells to fibronectin by deletion of the Bcl11a binding motif. (E) Increased migration of leukemic cells with Bcl11a expression. Overexpression of Asb2 and deletion of the Bcl11a binding motif reduced the migratory activity. (F) Knockdown efficiencies of Asb2 by shRNAs in Tr1 cells indicated by qRT-PCR. (G) Increased adhesion of Tr1 cells to fibronectin by knockdown of Asb2. (H) Increased migration of Tr1 cells by knockdown of Asb2.
Supplemental Figure 5. Inhibition of co-repressor complexes up-regulates the expression of Bcl11a and PU.1 target genes and suppress cell proliferation. (A) The knockdown efficiencies of co-repressor component genes validated by qRT-PCR. (B) Modulation of Asb2 and Clec5a expression by knockdown of co-repressor component genes. (C) Panobinostat and GSK-LSD 2HCl treatment suppresses the growth of TB-13 but not Tr1 cells. Relative growth on day 4 after treatment is shown. (D) Panobinostat upregulates Asb2 and Clec5a expression, and addition of GSK-LSD1 2HCl enhances the effect in TB-13 cells. * P < 0.05, ** P < 0.01, *** P < 0.001, NS; not significant. (E) The effects of cell growth in Tr1, TB-13 and TB-14 cells by azacytidine and decitabine treatment. No significant effects are shown.
Supplemental Figure 6. The important role of the BCL11A-PU.1 axis in human AML. (A) Kaplan-Meier survival curves for patients with normal karyotype AML among the combination of high and low expression of BCL11A and TRIB1. Hazard rate (HR) and 95% confidence intervals (CI) are indicated in right. (B) Correlative expression between BCL11A and ASB2 in the human cohort. Pearson’s correlation test was performed using the human AML cohort used in Figure 6A (GSE12417 of GEO). (C) A box plot showing ASB2 expression between TRIB1 high/BCL11A high and TRIB1 high/BCL11A low patients. (D) A box plot showing SPI1 expression between TRIB1 high/BCL11A high and TRIB1 high/BCL11A low patients. (E) Growth suppression of THP-1 cells by BCL11A
knockdown. (F) Gene expression for $ASB2$ (center) and $CLEC5A$ (right) upon $BCL11A$ knockdown in THP-1 cells is assessed by qRT-PCR. Efficiency of $BCL11A$ knockdown is shown in left. * $P < 0.05$, ** $P < 0.01$. 