BCL11A promotes myeloid leukemogenesis by repressing PU.1 target genes

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Key Points

- BCL11A promotes myeloid leukemogenesis via the repression of PU.1 target genes.

- Inhibition of corepressors abrogates the BCL11A function, inducing growth suppression and inhibition of engraftment in AML.

The transcriptional repressor BCL11A is involved in hematological malignancies, B-cell development, and fetal-to-adult hemoglobin switching. However, the molecular mechanism by which it promotes the development of myeloid leukemia remains largely unknown. We find that Bcl11a cooperates with the pseudokinase Trib1 in the development of acute myeloid leukemia (AML). Bcl11a promotes the proliferation and engraftment of Trib1-expressing AML cells in vitro and in vivo. Chromatin immunoprecipitation sequencing analysis showed that, upon DNA binding, Bcl11a is significantly associated with PU.1, an inducer of myeloid differentiation, and that Bcl11a represses several PU.1 target genes, such as *Asb2*, *Clec5a*, and *Fcgr3*. *Asb2*, as a Bcl11a target gene that modulates cytoskeleton and cell-cell interaction, plays a key role in Bcl11a-induced malignant progression. The repression of PU.1 target genes by Bcl11a is achieved by sequence-specific DNA-binding activity and recruitment of corepressors by Bcl11a. Suppression of the corepressor components HDAC and LSD1 reverses the repressive activity. Moreover, treatment of AML cells with the HDAC inhibitor pracinostat and the LSD1 inhibitor GSK2879552 resulted in growth inhibition in vitro and in vivo. High *BCL11A* expression is associated with worse prognosis in humans with AML. Blocking of *BCL11A* expression upregulates the expression of PU.1 target genes and inhibits the growth of HL-60 cells and their engraftment to the bone marrow, suggesting that BCL11A is involved in human myeloid malignancies via the suppression of PU.1 transcriptional activity.

Introduction

Hematopoiesis-associated transcription factors (TFs) play crucial roles in the malignant transformation and progression of hematopoietic neoplasms. Many TFs act as oncogenes or tumor suppressors in myeloid malignancies. They regulate transcriptional programs of their target genes, constituting specific complexes and modulating each other's functions. *Bcl11a/Evi9/Ctip1* encodes a C_2H_2-type zinc finger protein that functions as a transcriptional repressor.[1,2] *Bcl11a* was identified as a myeloid oncogene associated with a common retroviral integration in acute myeloid leukemia (AML) in BXH2 mice.[1] Subsequently, *BCL11A* was implicated in t(2;14) chromosome translocation in cases of human B-cell chronic lymphoid leukemia in which overexpression of *BCL11A* messenger RNA is achieved by juxtaposition to

the *IGH* enhancer.[3] Moreover, B-cell development is arrested at the pro-B stage in *Bcl11a* homozygous knockout mice, indicating that *Bcl11a* plays a critical role in B-cell differentiation.[4] Further studies revealed that Bcl11a is a repressor of the fetal hemoglobin gene and is essential for its switching of the fetal hemoglobin gene to the adult type.[5-8] Collectively, Bcl11a orchestrates differentiation programs in multiple lineages of hematopoiesis.

Despite several studies on the functions of BCL11A in B-cell and erythroid lineages, its role in myeloid leukemogenesis remains poorly understood. A potential association between *Bcl11a* and *Nf1* loss or *MLL-AF9* was identified using insertional mutagenesis screening.[9,10] Given that BCL11A is involved in the repression of transcription by associating with corepressor complexes,[8,11-13] it is plausible that Bcl11a promotes myeloid leukemogenesis by repressing its downstream targets. Taken together, these data strongly suggest that *Bcl11a* acts as a myeloid oncogene, although Bcl11a by itself does not possess strong transforming activity, and collaboration with other genetic aberrations is required for complete leukemogenesis.[9] In addition, Bcl11a plays a role that is distinct from those of oncogenic TFs, such as Hoxa9 and c-Myc, many of which act as transcriptional activators. In this context, Bcl11a may interact with tumor-suppressive TFs and may reverse the transcriptional programs that they regulate.

In this study, we found that *Bcl11a* cooperates with *Trib1*, a pseudokinase gene, identified as a myeloid oncogene that cooperates with *Hoxa9* and *Meis1*,[14] in the development of AML. Trib1 degrades the p42 isoform of C/EBPα, which induces myeloid differentiation and acts as a myeloid tumor suppressor.[15-17] *Bcl11a* might replace the oncogenic functions of *Hoxa9* and *Meis1* by modulating distinct transcriptional programs of downstream targets. Therefore, it is expected that Bcl11a may interact with tumor-suppressive TFs other than C/EBPα. Our study showed that *Bcl11a* promoted malignant progression of *Trib1*-expressing immortalized hematopoietic cells, whereas *Bcl11a*- and *Trib1*-coexpressing cells enabled the analysis of global DNA-binding properties of Bcl11a to address its role in aggressive phenotypes. Importantly, Bcl11a repressed the expression of the target genes of PU.1, which is a myeloid tumor suppressor.

Methods

Detailed experimental methods not presented below are described in supplemental Methods. Specific statistical tests used are described in each figure legend. All error bars represent the standard error of the mean (SEM).

Study approval

All animal experiments described in this study were performed in strict accordance with standard ethical guidelines and were approved by the animal care committee at the Japanese Foundation for Cancer Research under licenses 10-05-9 and 0604-3-13.

In vivo studies

For the bone marrow transplantations, mice were subjected to irradiation (8.5 Gy for primary bone marrow cells and 4.0 Gy for cell lines) and injected IV with 1×10^6 primary bone marrow cells infected with retrovirus or 1×10^7 Tr1, TB-13, or TB-14 cells. All mice were on the C57BL/6 background. For transplantation of HL-60 cells, NSG mice were subjected to 2.0-Gy irradiation.

Cell lines and cell culture

Bone marrow cells were prepared from 8-week-old C57BL6/J mice, 5 days after injection of 5-fluorouracil (Kyowa Hakko Kirin, Tokyo, Japan) at 150 mg/kg body weight. These cells were transduced with pMYs-Flag-Trib1-IRES-mKO, with or without pMYs-myc-Bcl11a-IRES-EGFP, according to the method described previously.[18] Human AML cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Gene knockdown and CRISPR/Cas9-mediated gene editing were performed by infecting the cells with lentiviral vectors bearing short hairpin RNAs (shRNAs; Sigma-Aldrich) and single guide RNA, respectively. The sequences of shRNAs and single-guide RNA are listed in supplemental Table 1. For pharmacological experiments, leukemia cells were treated with 100 nM pracinostat, 3 nM panobinostat, 10 μM GSK2879552, or 10 μM GSK-LSD1-2HCL for 96 hours, and expression levels of target genes or surface markers were detected by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and flow cytometry. The suppression of growth of leukemia cells was assessed following treatment with 50 nM pracinostat, 1 nM panobinostat, 1 μM GSK2879552, or 1 μM GSK-LSD1-2HCL for 96 hours.

Statistics

All in vitro experiments were performed at least in triplicate. The number of mice used per experiment is indicated in the figures. Data are expressed as means ± SEM, and statistical significance was determined using a 1-tailed Student *t* test for single comparisons and one-way analysis of variance with Dunnett multiple-comparison test for multiple comparisons on a single data set. Survival analysis was performed using the Kaplan-Meier life table method, and survival between groups was compared using the log-rank test.

Results

Bcl11a cooperates with *Trib1* in the development of AML

In a previous study, we identified 6 common integration sites in 30 murine leukemia tumor tissues from *Trib1*-expressing mouse AML.[17] As expected, *Hoxa7* and *Hoxa9* were detected at the highest frequency, which concurs with the identification of *Trib1* as a collaborator of *Hoxa9* and *Meis1*.[14] *Bcl11a* was identified as the second most frequent retroviral integration site, especially in the 175-kb region flanking *Bcl11a* (Figure 1A). Most of the integration sites were located 10 to 80 kb downstream of *Bcl11a,* which is similar to the reported distribution of common integration sites, suggesting that retroviral integrations upregulate the expression of *Bcl11a.*[9] Indeed, the expression of *Bcl11a* was found to be upregulated in integration-positive AML using qRT-PCR analysis (Figure 1A). To assess the cooperation between the expression of *Trib1* and *Bcl11a*, mouse bone marrow cells were transfected with retrovirus genes, *Trib1* and *Bcl11a*, followed by bone marrow transplantation into lethally irradiated recipients. The expression of *Bcl11a* significantly accelerated the onset of *Trib1*-induced AML (Figure 1B). Notably, the expression of *Bcl11a* alone was not associated with the development of leukemia, which is in agreement with the results of a previous study.[9] These results indicate specific cooperation between *Trib1* and *Bcl11a* in myeloid leukemogenesis. Immortalized mouse myeloid cell lines expressing *Trib1* (Tr1) or *Trib1* and *Bcl11a*
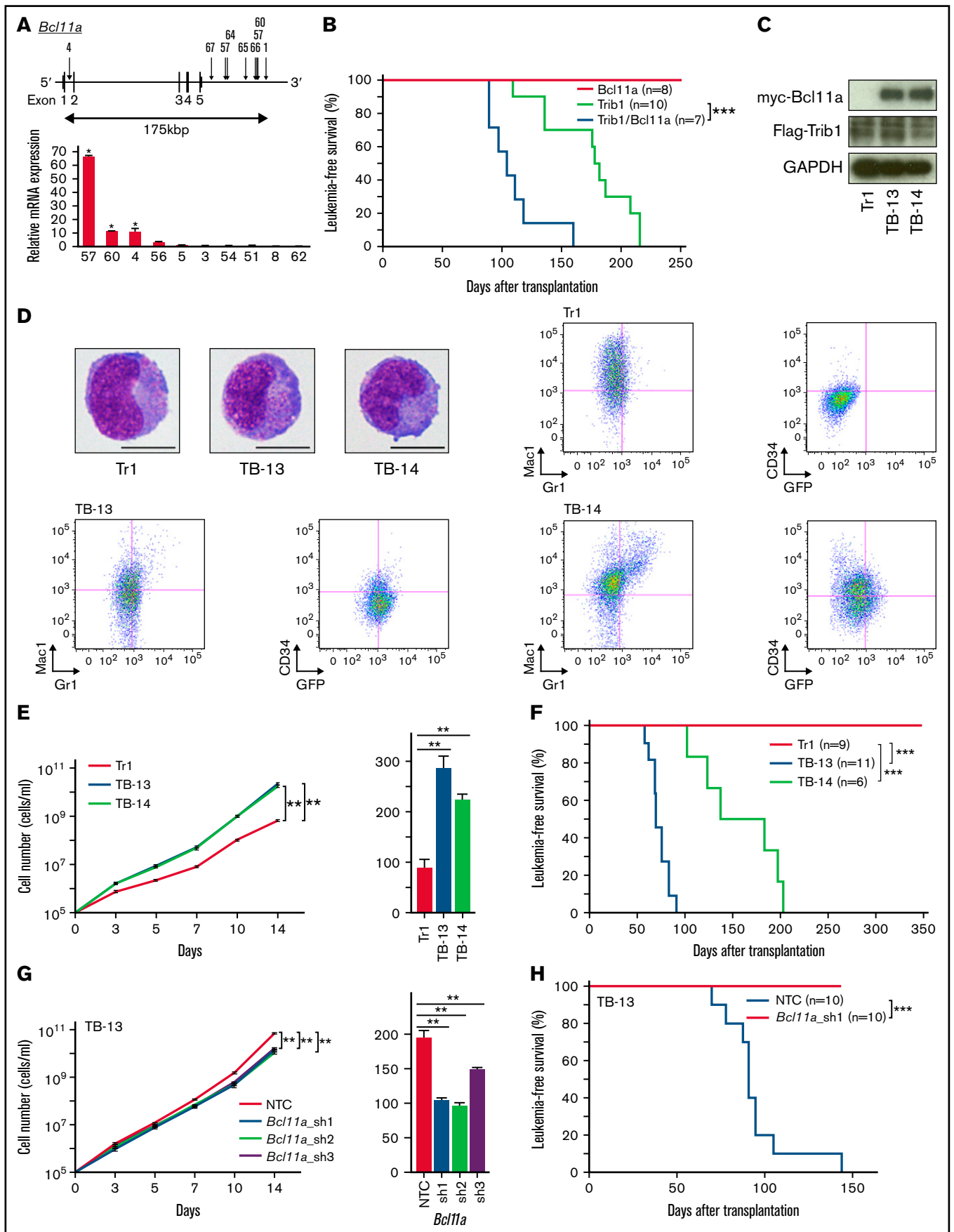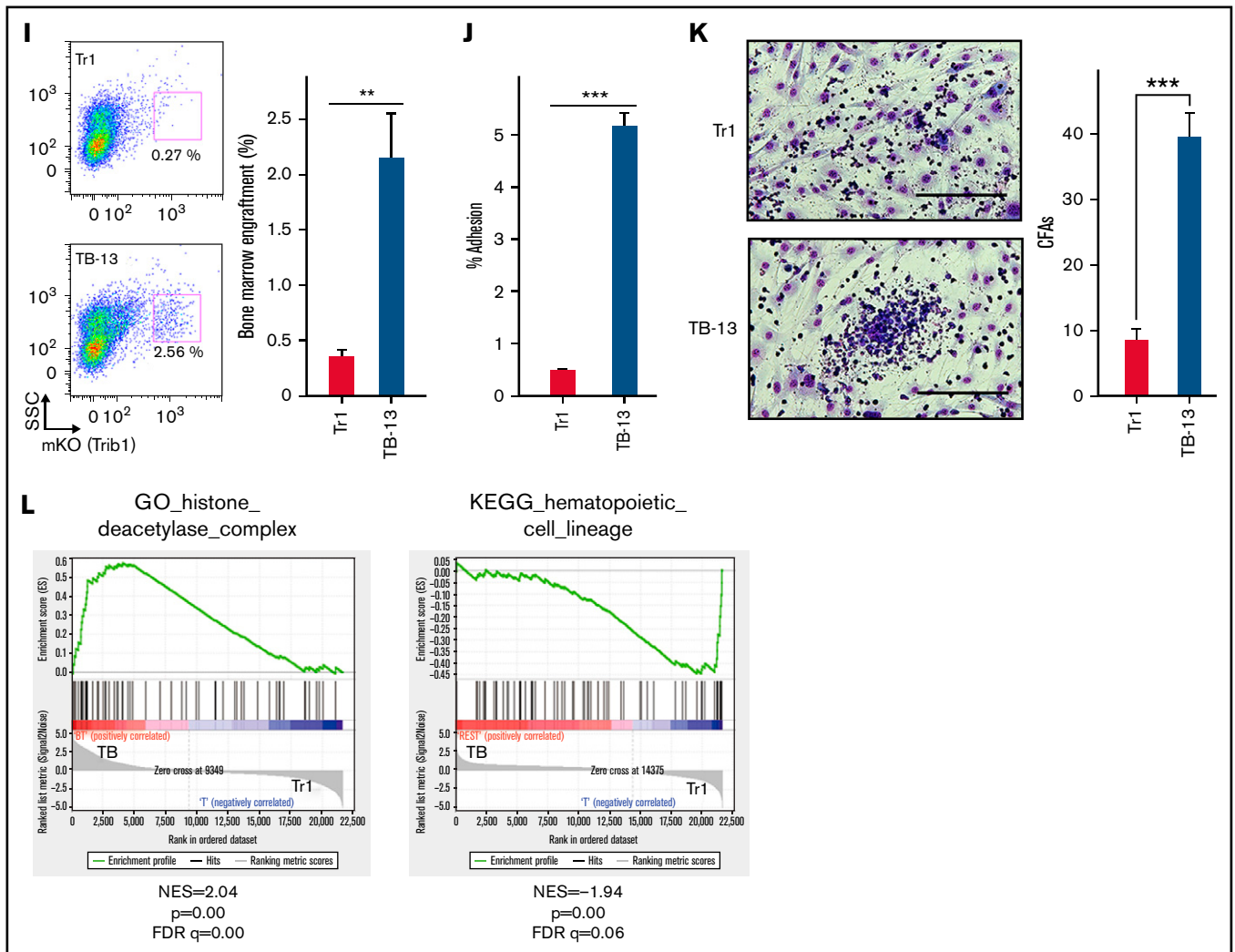
Figure 1.

Figure 1 (continued) Bcl11a promotes Trib1-induced development of AML. (A) Retroviral insertions in Trib1-induced AML. Vertical arrows indicate the locations of integrations at the *Bcl11a* locus (upper panel). Expression of *Bcl11a* in Trib1-induced AML. Asterisks indicate AML with retroviral integrations at the *Bcl11a* locus (lower panel). (B) Acceleration of the onset of AML in the *Trib1* and *Bcl11a*–coexpressing cohort in C57BL/6 mice, as shown by Kaplan-Meier survival curves. The number of animals in each cohort is indicated. (C) Western blot showing the expression of myc-tagged Bcl11a and Flag-tagged Trib1 in Tr1, TB-13, and TB-14 cells. (D) Morphology indicated by Giemsa staining (left panels) and fluorescence-activated cell sorting showing the expression of Mac1, Gr1, and CD34 in Tr1, TB-13, and TB-14 cells (right panels). Scale bars, 10 μm. (E) Increased proliferation (left panel) and colony-forming activity (right panel) of TB-13 and TB-14 cells. (F) Kaplan-Meier survival curves show the development of AML in the recipients transplanted with TB-13 or TB-14 cells, whereas no AML was induced in recipients of Tr1 cells. (G) Suppression of TB-13 cell proliferation (left panel) and self-renewal (right panel) by shRNA-mediated knockdown of *Bcl11a*. Three independent shRNAs for *Bcl11a* were used. (H) Kaplan-Meier survival curves show inhibition of the development of AML by *Bcl11a* knockdown in the recipients transplanted with TB-13 cells. (I) Tr1 and TB-13 cells in the bone marrow, 14 days after transplantation, were detected as an mKO-positive fraction using flow cytometry (left panels). A significant increase in the number of TB-13 cells was observed in the bone marrow. Frequencies of mKO-positive cells in the bone marrow are shown as mean ± SEM (right). (J) Increased adhesion of *Bcl11a*-expressing cells to fibronectin. (K) Coculture of Tr1 or TB-13 cells with OP9 cells. Cobblestone areas were significantly increased by TB-13 cells (left). The number of cobblestone areas is indicated as the means ± SEM (right). Scale bar, 200 μm. (L) GSEA shows enrichment of the histone deacetylase complex and hematopoietic cell lineage pathways by comparing AML cells expressing *Trib1*, with and without *Bcl11a*. Normalized enrichment scores (NES), nominal *P* values, and false discovery rate (FDR) q-values are provided. Trib1 and TB indicate gene expression in Tr1 and TB-13/TB-14 cells, respectively. **$P < .01$, *** $P < .001$. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mKO, monomeric Kusabira Orange; NTC, nontarget control; SSC, side scatter.

(TB-13 and TB-14) were generated, which showed immature myeloid phenotypes expressing Mac1, Gr1, and CD34 (Figure 1B-D; supplemental Figure 1A). Increased cell proliferation and colony-forming activity were observed with the expression of *Bcl11a*

(Figure 1E); more importantly, the expression of *Bcl11a* was required for the development of AML in vivo (Figure 1F). Conversely, shRNA-mediated knockdown of *Bcl11a* in TB-13 and TB-14 cells resulted in reduced proliferation in vitro and reduced leukemogenicity in vivo

(Figure 1G-H; supplemental Figure 1B-C). Furthermore, the bone marrow engraftment of leukemia cells was enhanced by the expression of *Bcl11a* (Figure 1I). The adhesion of leukemia cells to fibronectin and the interaction between leukemia cells and OP9 bone marrow stromal cells were also significantly increased by the expression of *Bcl11a* (Figure 1J-K); the latter correlated with increased bone marrow engraftment of AML.[18] Taken together, the present results indicate that *Bcl11a* cooperates with *Trib1*, which is known to suppress C/EBPα and to enhance MEK/ERK signaling.[14-16,19]

Gene expression profiles were compared between TB-13 or TB-14 and Tr1 cells. Only 216 genes were upregulated twofold, whereas 1428 genes were downregulated twofold in TB-13 cells compared with Tr1 cells (supplemental Table 2), which is consistent with the previous findings that Bcl11a functions as a transcriptional repressor.[2,5,7] Gene set enrichment analysis (GSEA) showed enrichment of histone deacetylase complex and hematopoietic cell lineage pathways (Figure 1L), suggesting that Bcl11a plays a role in myeloid cell differentiation via transcriptional repression. Enrichment of the cell cycle pathway was also observed (supplemental Figure 1D), which was consistent with increased proliferation upon expression of *Bcl11a*.

Bcl11a interacts with PU.1 and represses target gene expression

To further clarify the function of Bcl11a, chromatin immunoprecipitation sequencing (ChIP-seq) was performed to evaluate the global DNA binding of Bcl11a and H3K27Ac in TB-13 cells. We identified 10 398 DNA binding peaks of Bcl11a that were distributed most frequently in the promoter regions (44.0%), followed by intergenic regions (31.1%), and introns (23.2%) (Figure 2A; supplemental Table 3). Among 1428 genes that were downregulated twofold in Bcl11a-expressing cells, 876 genes (61.3%) were positive for Bcl11a DNA binding peaks (Figure 2B; supplemental Table 4), suggesting that several genes could be directly repressed by Bcl11a. De novo motif analysis of Bcl11a DNA binding peaks revealed that the most highly enriched motif was the consensus ETS motif of Sfpi1 (PU.1), followed by Runx and Bcl11a motifs (Figure 2C). Moreover, motif analysis in GSEA showed an inverse correlation between the expression of PU.1 target genes and *Bcl11a* (Figure 2D). These data suggest a possible association between Bcl11a and PU.1 in DNA binding. Indeed, ChIP-seq analysis for PU.1 showed cobinding between Bcl11a and PU.1, and 6109 (58.7% of the total peaks) Bcl11a binding peaks overlapped with those of PU.1 in TB-13 cells (Figure 2E-G). In contrast, Bcl11a binding peaks around the PU.1 motif were not significantly diminished by the knockdown of *Sfpi1* and vice versa (Figure 2H-J), suggesting that Bcl11a and PU.1 do not modulate the DNA binding activities and specificities of their partners. Moreover, pathway analysis using GREAT software (http://great.stanford.edu/public/html/) showed that myeloid cell pathways were significantly involved in genes with Bcl11a and PU.1 cobinding peaks, whereas genes with only Bcl11a peaks were associated with pathways related to chromatin functions (Figure 2K). Coimmunoprecipitation assay showed an interaction between exogenously expressed Bcl11a and PU.1 (Figure 2L), and interaction of endogenous proteins was also detected at the single-cell level using a proximity ligation assay (PLA) (Figure 2M). PLA is a sensitive and specific method to detect interactions of endogenous proteins using complementary oligonucleotides bound to specific antibodies.[20] Among the multiple transcript variants of Bcl11a,[1,3,13]

we found that isoforms (v1 and v2) that lacked the C-terminal zinc finger domains could interact with PU.1 (supplemental Figure 2A). However, expression of these isoforms in mouse bone marrow cells failed to show AML development in cooperation with Trib1 in vivo (supplemental Figure 2B), strongly suggesting that the DNA binding ability of Bcl11a is required for leukemogenesis. Together, these results indicate that the expression of PU.1 target genes is repressed, at least in part, by Bcl11a.

Bcl11a represses the expression of PU.1 target genes

Among the genes with overlapping peaks between Bcl11a and PU.1, 623 genes (8%) were downregulated by the expression of *Bcl11a* (supplemental Figure 3; supplemental Table 5). The downregulated expression of candidate Bcl11a target genes (Figure 3A) was validated using qRT-PCR, and their expression was found to be significantly increased upon knockdown of *Bcl11a* (Figure 3B). Consistent with the inclusion of myeloid differentiation–related genes among Bcl11a targets, granulocytic differentiation was induced by *Bcl11a* knockdown, as indicated by granulocyte-specific naphthol AS-D chloroacetate esterase staining and modulation of myeloid differentiation–associated gene expression (Figure 3C). On the contrary, PU.1 target genes without Bcl11a binding peaks, such as *Akt3* and *Fam117b*, were not downregulated by *Bcl11a* expression or upregulated by knockdown of *Bcl11a* (Figure 3A-B; supplemental Figure 3A). The high-affinity receptor for immunoglobulin G encoded by *Fcgr3*, a known PU.1 target, is required for important functions, such as phagocytosis and antimicrobial activity, in myeloid lineages.[21,22] Bcl11a and PU.1 bind to the respective consensus sequences (TGACCA for Bcl11a and AGGAAG for PU.1) in the *Fcgr3* promoter region, and the expression of *Fcgr3* was downregulated in TB-13 cells (Figure 3A,D; supplemental Figure 3B). This finding suggests that the downregulation of *Fcgr3* requires an association between the expression of Bcl11a and PU.1. The luciferase reporter assay showed that Bcl11a repressed the transactivation by PU.1 to the *Fcgr3* promoter (Figure 3E). Cobinding of Bcl11a and PU.1 was also detected at a region 10 kb upstream of *Clec5a* (−10-kb enhancer), which is a PU.1 target and is important in myeloid differentiation (Figure 3F).[23,24] In contrast to the −10-kb enhancer where Bcl11a and PU.1 cobind, the *Clec5a* promoter showed PU.1 binding without Bcl11a. The DNA binding consensus sequence of Bcl11a (TGACCA)[8] was present within the −10-kb enhancer but not within the promoter (supplemental Figure 3C), which suggests that there may be an interaction between the *Clec5a* promoter and the −10-kb enhancer. Chromosome conformation capture (3C) analysis indicated DNA looping between the *Clec5a* promoter and enhancer, regardless of the presence of Bcl11a (Figure 3G). When the −10-kb enhancer and promoter of *Clec5a* were inserted into the pGL4.0 luciferase vector, Bcl11a again repressed the transactivation by PU.1 (Figure 3H). These results suggest that, on the −10-kb enhancer, Bcl11a could access PU.1 on its own site, as well as on the promoter in the preexisting DNA loop.

Asb2 is a PU.1 target gene repressed by Bcl11a

To identify PU.1 target genes that are important for malignant progression upon the expression of *Bcl11a*, we compared gene expression profiles and ChIP-seq data for Tr1 and TB-13 cells. Among the 1428 genes that were downregulated more than
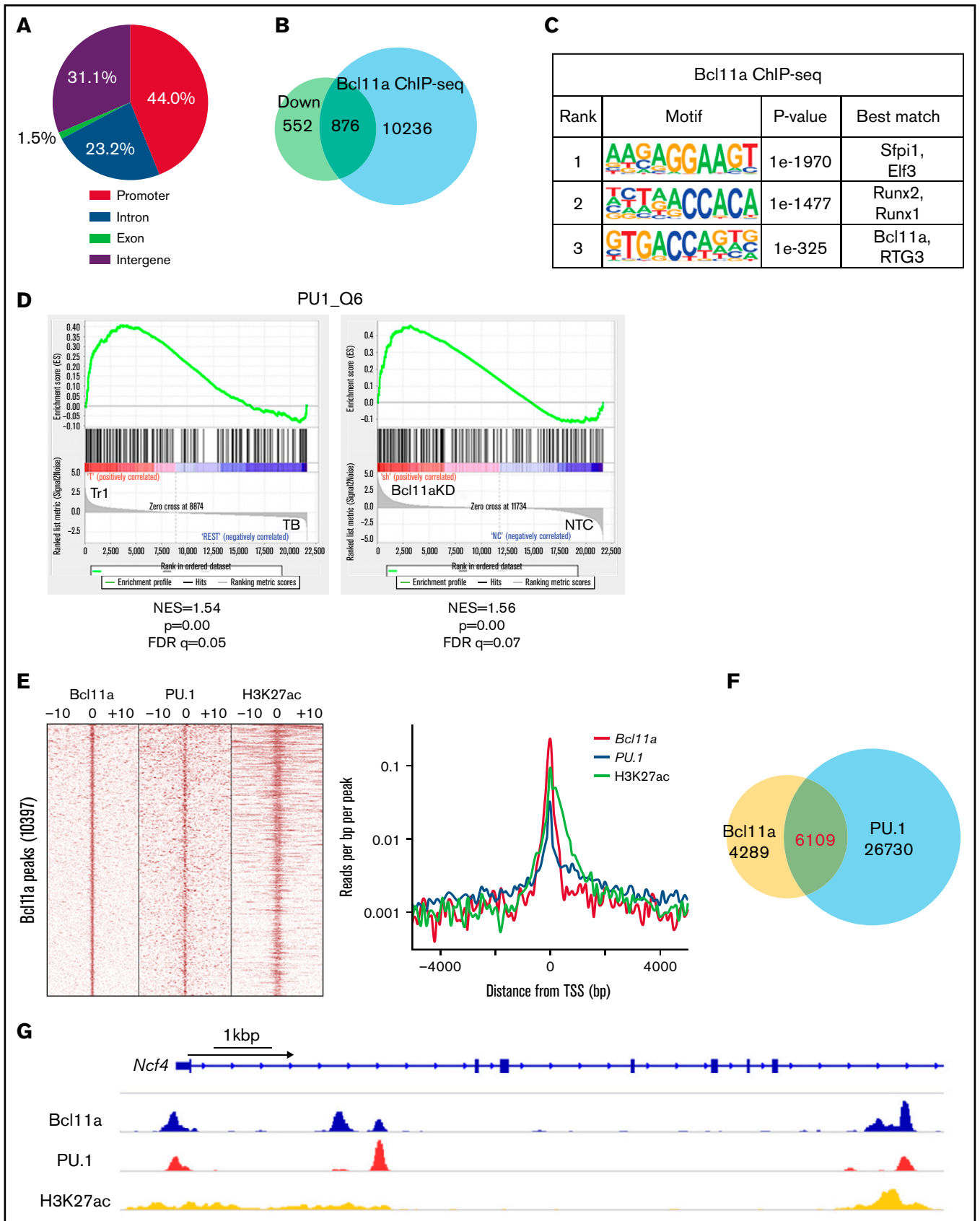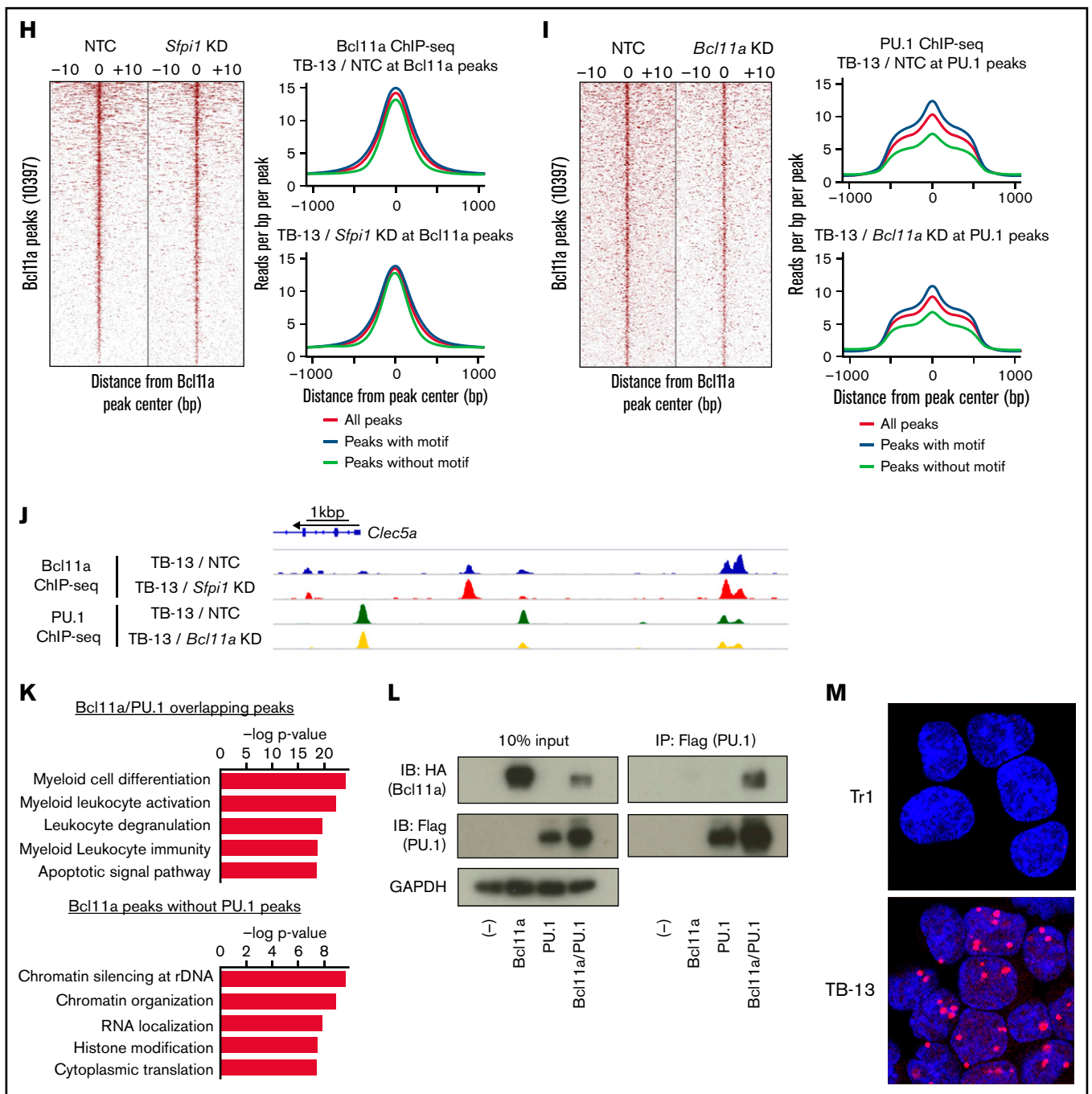
Figure 2.

H

NTC   *Sfpi1* KD
−10 0 +10   −10 0 +10

Bcl11a peaks (10397)

Distance from Bcl11a peak center (bp)

Bcl11a ChIP-seq
TB-13 / NTC at Bcl11a peaks

Reads per bp per peak

−1000   0   1000

TB-13 / *Sfpi1* KD at Bcl11a peaks

Distance from peak center (bp)

— All peaks
— Peaks with motif
— Peaks without motif

I

NTC   *Bcl11a* KD
−10 0 +10   −10 0 +10

Bcl11a peaks (10397)

Distance from Bcl11a peak center (bp)

PU.1 ChIP-seq
TB-13 / NTC at PU.1 peaks

Reads per bp per peak

−1000   0   1000

TB-13 / *Bcl11a* KD at PU.1 peaks

Distance from peak center (bp)

— All peaks
— Peaks with motif
— Peaks without motif

J

1kbp   *Clec5a*

Bcl11a ChIP-seq   TB-13 / NTC
  TB-13 / *Sfpi1* KD
PU.1 ChIP-seq   TB-13 / NTC
  TB-13 / *Bcl11a* KD

K

Bcl11a/PU.1 overlapping peaks

−log p-value
0 5 10 15 20

Myeloid cell differentiation
Myeloid leukocyte activation
Leukocyte degranulation
Myeloid Leukocyte immunity
Apoptotic signal pathway

Bcl11a peaks without PU.1 peaks

−log p-value
0 2 4 6 8

Chromatin silencing at rDNA
Chromatin organization
RNA localization
Histone modification
Cytoplasmic translation

L

10% input   IP: Flag (PU.1)

IB: HA (Bcl11a)
IB: Flag (PU.1)
GAPDH

(−)   Bcl11a   PU.1   Bcl11a/PU.1   (−)   Bcl11a   PU.1   Bcl11a/PU.1

M

Tr1

TB-13

Figure 2 (continued) Bcl11a interacts with PU.1 upon DNA binding. (A) Global distribution of 10 398 Bcl11a binding peaks. (B) Venn diagram shows that the expression of 876 of 1428 genes (61.3%) near the Bcl11a binding peaks is decreased (fold change < 2). (C) De novo motif enrichment analysis for Bcl11a binding peaks. The top 3 identified motifs are shown. (D) GSEA shows inverse correlation between genes with PU.1 binding motif and *Bcl11a* expression in Tr1 and TB-13 cells (left panel) and Bcl11a knockdown and control in TB-13 cells (right panel). (E) Heat map (left panel) and meta-profile (right panel) of Bcl11a, PU.1, and histone H3K27ac ChIP-seq signals centered on transcription start sites (TSS). (F) Venn diagram shows 6109 (58.7%) Bcl11a binding peaks overlap with those of PU.1. (G) ChIP-seq occupancy profiles for Bcl11a, PU.1, and H3K27ac in TB-13 cells at the *Ncf4* locus. (H) Bcl11a ChIP-seq signals in shNTC- or sh*Sfpi1*-treated TB-13 cells. Heat maps show 20-kb genomic regions centered on Bcl11a peaks (left panel). Composite plots of Bcl11a signals at Bcl11a sites, with or without the PU.1 motif, in shNTC-treated (upper right panel) and sh*Sfpi1*-treated (lower right panel) TB-13 cells. (I) PU.1 ChIP-seq signals in shNTC-treated or sh*Bcl11a*-treated TB-13 cells. Heat maps show 20-kb genomic regions centered on PU.1 peaks (left panel). Composite plots of PU.1 signals at PU.1 sites, with or without the PU.1 motif, in shNTC-treated (upper right panel) and sh*Bcl11a*-treated (lower right panel) TB-13 cells. (J) ChIP-seq occupancy profiles for Bcl11a and PU.1 in TB-13 cells at the *Clec5a* locus. Bcl11a and PU.1 ChIP-seq signals in shNTC-, sh*Sfpi1*-, or sh*Bcl11a*-treated TB-13 cells. (K) Pathway analysis for genes having the Bcl11a-binding peaks, with PU.1 [upper panel; 6109 genes in (F)] or without PU.1 (right panel; 4289 genes), using the GREAT algorithm. (L) Coimmunoprecipitation assay shows the interaction between Bcl11a and PU.1. (M) PLA shows a close association between endogenous Bcl11a and PU.1 in TB-13 cells (lower panel). Tr1 cells were used as a negative control (upper panel). Original magnification, ×710. FDR, false discovery rate; HA, hemagglutinin; IB, immunoblot; IP, immunoprecipitation; KD, knockdown; mKO, monomeric Kusabira Orange; NES, normalized enrichment score; NTC, nontarget control.
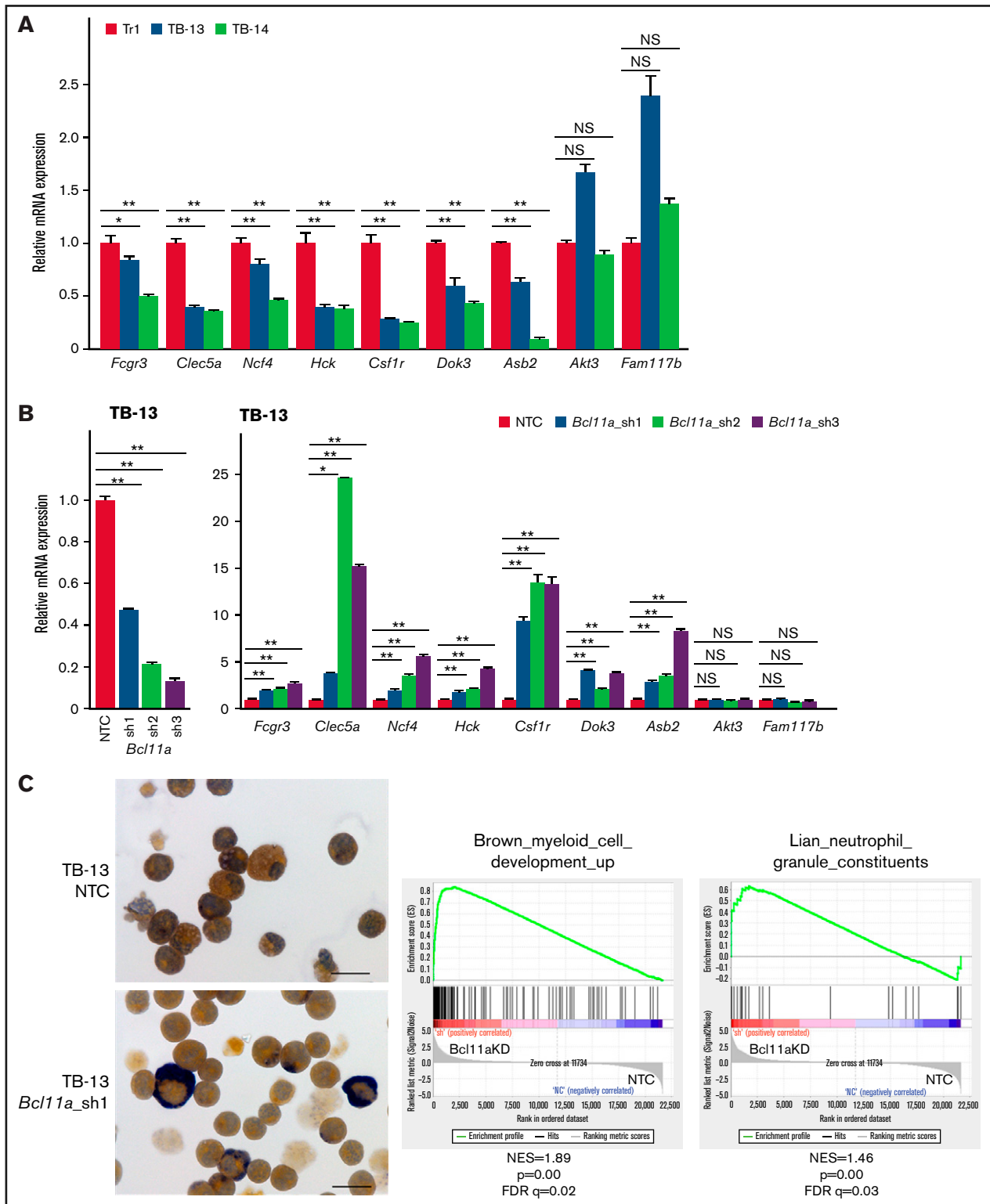
Figure 3. Repression of PU.1 target genes by Bcl11a. (A) Validation of downregulated expression of representative Bcl11a and PU.1 target genes using qRT-PCR. (B) Downregulation of gene expression was reversed by *Bcl11a* knockdown (right panel). Efficiency of Bcl11a knockdown by 3 shRNAs (left panel). (C) Naphthol AS-D chloroacetate esterase staining of TB-13 cells transfected with shNTC or shBcl11a. Esterase-positive cells are seen as dense blue granules in the cytoplasm (left panels). Scale bars, 20 μm. GSEA shows enrichment of myeloid cell development (middle panel) and neutrophil granule constituents pathways (right panel) by Bcl11a knockdown in TB-13 cells. (D) ChIP-seq occupancy profiles for Bcl11a, PU.1, and H3K27ac in TB-13 cells at the *Fcgr3* locus. (E) Luciferase reporter assays show that Bcl11a represses *Fcgr3* promoter activity in the presence of PU.1. (F) ChIP-seq occupancy profiles for Bcl11a, PU.1, and H3K27ac in TB-13 cells at the *Clec5a* locus. The positions of the
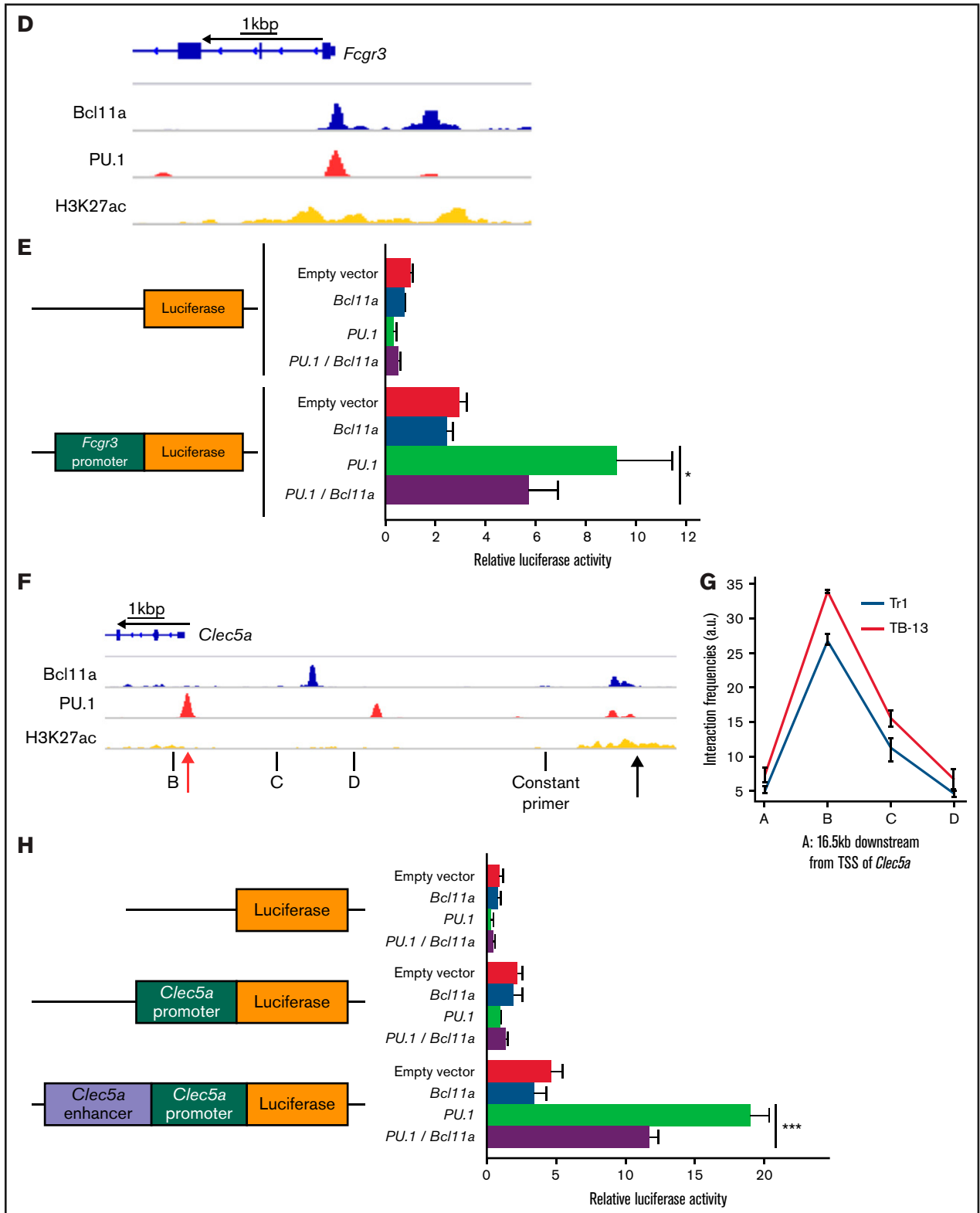
Figure 3 (continued) primers used for the 3C assay are represented by vertical lines. *Clec5a* promoter and the +10-kb enhancer are indicated by the red and black arrows, respectively. (G) The 3C assay shows DNA looping between the promoter and +10-kb enhancer of *Clec5a*. (H) Luciferase reporter assays show that Bcl11a represses *Clec5a* enhancer activity in the presence of PU.1. *$P < .05$, **$P < .01$, ***$P < .001$. a.u., arbitrary units; FDR, false discovery rate; KD, *Bcl11a* knockdown; NES, normalized enrichment score; NS, not significant; NTC, nontarget control; TSS, transcription start site.

twofold in TB-13 cells, 623 genes showed Bcl11a and PU.1 binding peaks within 30 kb from gene bodies. The expression of 46 genes was recovered upon treatment with LSD1 and HDAC inhibitors as shown in the next section. Of these, 25 genetic loci contained the Bcl11a consensus sequences; finally, 9 target candidates were validated using qRT-PCR (Figure 4A; supplemental Figure 4A). Among the 9 genes, *Asb2* was selected as a key target of PU.1 and Bcl11a interaction (Figure 4B; supplemental Figure 4B-C), because it plays an important role in the adhesion and migration of cells. *Asb2* encodes a suppressor of cytokine signaling box protein that acts as an E3 ubiquitin ligase.[25] The suppression of *ASB2* by the N^6-methyladenosine RNA demethylase FTO is considered an important genetic event in AML cells.[26] Asb2 targets and induces the ubiquitination of filamin A,[27,28] resulting in the promotion of cell mobilization. Indeed, the overexpression of *Asb2* suppressed the proliferation and self-renewal of TB-13 cells (Figure 4C-D). Furthermore, the overexpression of *Asb2* inhibited the bone marrow engraftment of TB-13 cells (Figure 4E), suggesting that Asb2 might be involved in the interaction between leukemia cells and the bone marrow stroma. As shown in Figure 1J and 1K, *Bcl11a* expression induced an increase in the adhesion to fibronectin and promoted cobblestone formation upon coculture of leukemia cells with OP9 bone marrow stromal cells. Likewise, the overexpression of *Asb2* suppressed cellular adhesion and interaction with OP9 (Figure 4F; supplemental Figure 4D). Cytoplasmic expression of filamin A was significantly increased in TB-13 cells compared with Tr1 cells and was canceled by the overexpression of *Asb2* (Figure 4G). The increase in the expression of filamin A was associated with the promotion of cell migration (supplemental Figure 4E). When the Bcl11a binding motif at the *Asb2* locus was deleted using CRISPR/Cas9-mediated genome editing (supplemental Figure 4B-C), cell adhesion, interaction with OP9 cells, and cell migration were suppressed, and filamin A staining was increased (Figure 4F-G; supplemental Figure 4D-E). Moreover, knockdown of *Asb2* in Tr1 cells promoted similar phenotypes as in TB-13 cells, such as increased interaction with OP9, increased cytoplasmic filamin A levels, and adhesion and migration of cells (Figure 4H-I; supplemental Figure 4F-H). Taken together, *Asb2* is a critical downstream target gene of PU.1 that is repressed by Bcl11a, potentiating the malignant characteristics of AML.

Bcl11a suppresses the function of PU.1 via corepressor complexes

Given that Bcl11a interacts with corepressor complexes to repress the expression of its target genes,[11,12] we tested whether knockdown of specific components of the complexes would recover the expression of Bcl11a target genes. The knockdown of *Kdm1a* (encoding LSD1), *Hdac1*, *Hdac2*, *Ncor1*, or *Ncor2* consistently induced the upregulation of *Clec5a* and *Asb2*, whereas the knockdown of *Sin3a* was not effective for the expression of these genes (Figure 5A; supplemental Figure 5A-B). The suppression of leukemia cell growth by pracinostat and GSK2879552, or by panobinostat and GSK-LSD1, was significantly associated with the expression of *Bcl11a* (Figure 5B-C; supplemental Figure 5C). Furthermore, pracinostat treatment, with or without GSK2879552, significantly suppressed AML cells coexpressing *Trib1* and *Bcl11a* in vivo (Figure 5D). Pracinostat and GSK2879552 treatment significantly modified the gene expression profiles. GSEA showed that genes downregulated by Bcl11a were inversely correlated with those upregulated upon treatment with LSD1 and HDAC inhibitors, and genes upregulated by LSD1 and HDAC inhibitors were significantly enriched in the upregulated genes without the expression of *Bcl11a* (Figure 5E). Treatment of AML cells with the HDAC inhibitors pracinostat and panobinostat induced the upregulation of *Clec5a* and *Asb2*; the addition of the LSD1 inhibitors GSK2879552 and GSK-LSD1 enhanced these effects (Figure 5F; supplemental Figure 5D). DNA hypomethylating agents, 5-azacytidine and decitabine, did not significantly suppress the growth of Tr1, TB-13, and TB-14 cells (supplemental Figure 5E). Together, these data indicate that Bcl11a represses the expression of PU.1 target genes via the recruitment of corepressor complexes, suggesting that the corepressor complex might be a promising drug target for AML with upregulation of *BCL11A*.

Modulation of PU.1 target gene expression by BCL11A in human AML

We assessed whether the expression of *BCL11A* and *TRIB1* correlated with prognosis in human normal-karyotype AML using the PrognoScan online platform (http://www.prognoscan.org/).[29,30] We selected a cohort of 163 patients with AML[29]; 156 patients (95.7%) were enrolled in the multicenter AMLCG-1999 trial of the German AML Cooperative Group between 1999 and 2003,[31] and all received 1 or 2 courses of high-dose cytarabine plus mitoxantrone therapy, followed by autologous stem cell transplantation or maintenance chemotherapy. Increased expression of *BCL11A*, with or without *TRIB1*, correlated significantly with worse prognosis (Figure 6A; supplemental Figure 6A), suggesting that the overexpression of *BCL11A* might contribute to malignant progression of human AML. In addition, an inverse correlation between the expression of *BCL11A* and *ASB2* was observed in this cohort (supplemental Figure 6B-C). The expression of *SPI1* encoding PU.1 was not significantly different between *BCL11A*-high and *BCL11A*-low groups (supplemental Figure 6D). Next, to investigate the possible involvement of BCL11A in human AML, the expression of *BCL11A*, as well as of *SPI1*, encoding PU.1, was examined using AML cell lines in vitro. Among 8 cell lines tested, HL-60, EOL1, and THP-1 cells showed coexpression of *BCL11A* and *SPI1* (Figure 6B). The knockdown of *BCL11A* suppressed the proliferation of HL-60 and THP-1 cells (Figure 6C; supplemental Figure 6E). *BCL11A* knockdown and treatment with HDAC and LSD1 inhibitors induced an upregulation of *CLEC5A* and *ASB2* in HL-60 and THP-1 cells (Figure 6D-E; supplemental Figure 6F), suggesting that repression of PU.1 target genes by BCL11A might be achieved in human AML by similar mechanisms as in mouse AML. Despite moderate suppression of the growth of HL-60 cells by knockdown of *BCL11A*, the in vivo engraftment of HL-60 cells in NSG mice was significantly inhibited (Figure 6F). Knockdown of *BCL11A* suppressed cellular adhesion of HL-60 to fibronectin (Figure 6G). Collectively, these data strongly suggest that BCL11A functions primarily as a repressor of target genes that are driven by PU.1.

Discussion

Bcl11a encodes a C_2H_2-type zinc finger TF with 6 zinc fingers in the largest isoform.[3,13] Multiple splicing variants of Bcl11a have been reported,[13] but only the largest isoform contains the 3 C-terminal zinc fingers that possess the major DNA binding activity.[8] In the present study, the DNA binding activity was required for the oncogenic function in AML. BCL11A interacts with multiple partner proteins, such as
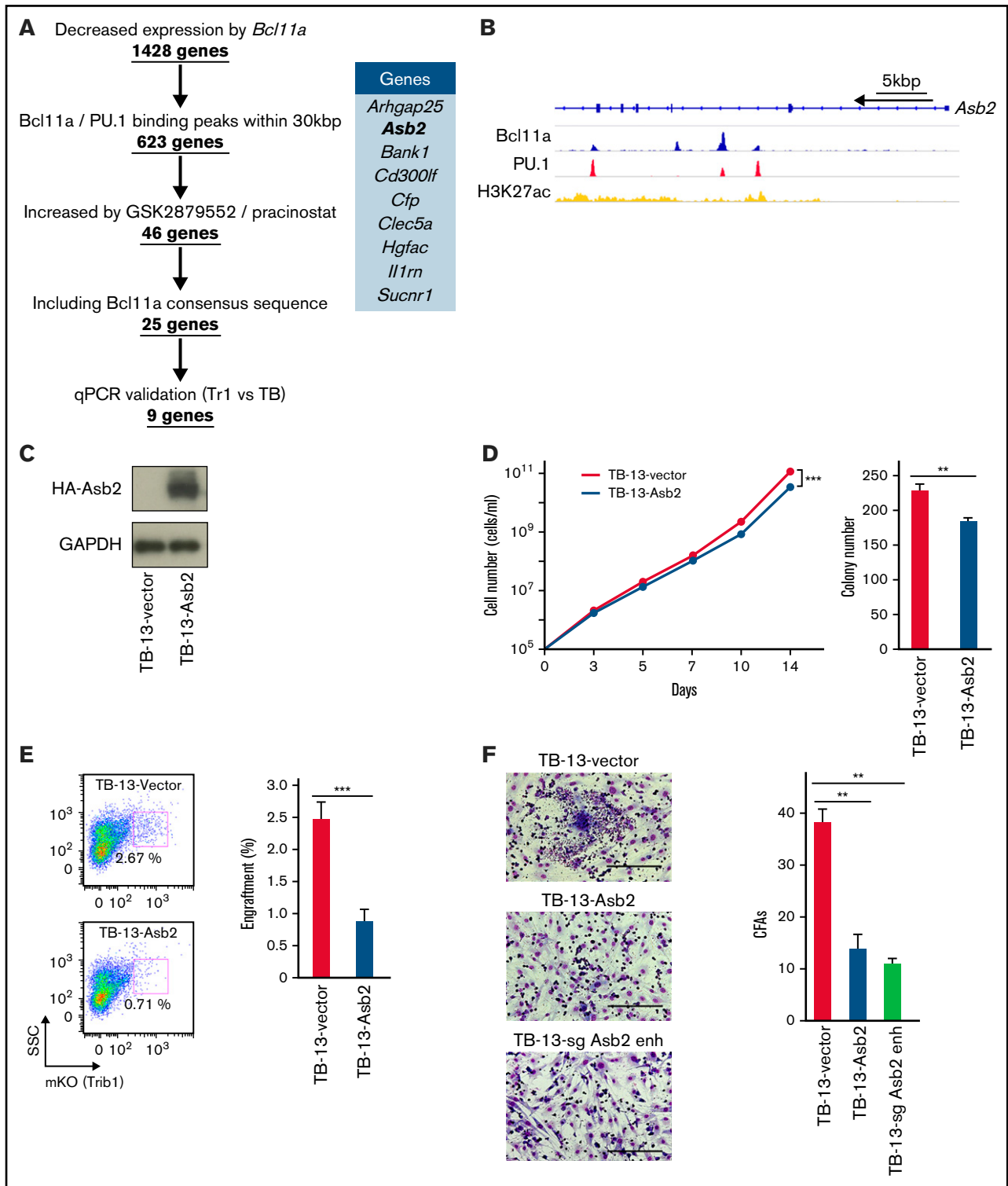
Figure 4. Identification of *Asb2* as a PU.1 target gene, repressed by Bcl11a. (A) A schematic diagram for the identification of target genes. Nine candidate targets are shown in the box on the right. (B) ChIP-seq occupancy profiles for Bcl11a, PU.1, and H3K27ac in TB-13 cells at the *Asb2* locus. (C) Western blot showing the expression of hemagglutinin (HA)-tagged Asb2 in TB-13 cells. (D) Suppression of TB-13 cell proliferation (left panel) and self-renewal (right panel) by overexpression of *Asb2*. (E) Decreased engraftment of TB-13 cells by overexpression of *Asb2* in the bone marrow, 14 days after transplantation (left panels). A significant decrease in the number of TB-13 cells in the bone marrow was observed. Frequencies of mKO⁺ cells in the bone marrow are shown as means ± SEM (right). (F) Interaction between TB-13 and OP9 cells is inhibited by overexpression of *Asb2*. Cobblestone areas are significantly decreased by overexpression of *Asb2* and homozygous deletion of Bcl11a binding sequence at the *Asb2* enhancer (enh; left panels). Scale bars, 200 μm. The number of cobblestone areas is shown as the mean ± SEM (right panel).
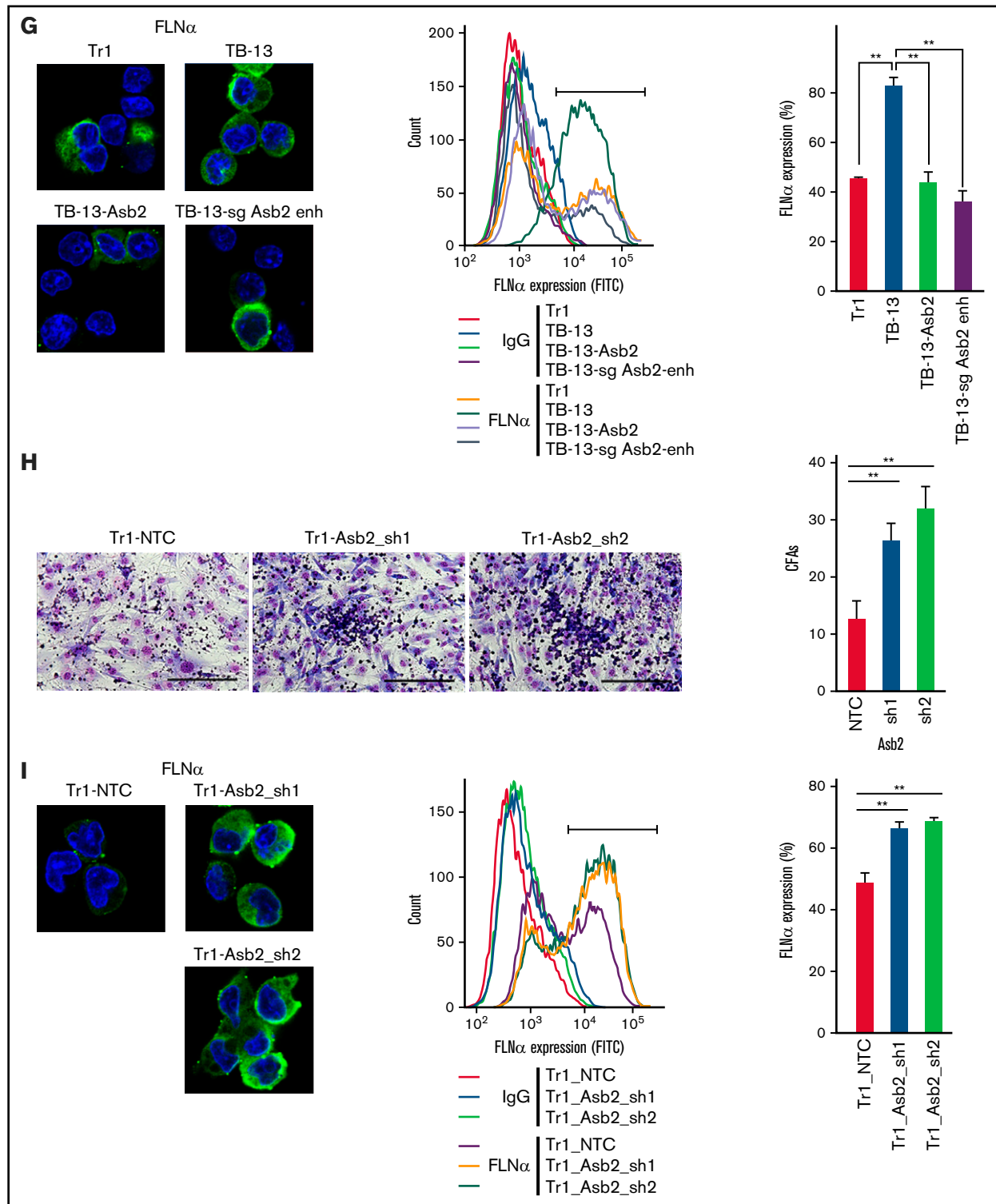
Figure 4 (continued) (G) Cytoplasmic filamin A (FLNa) is reduced by the upregulated status of Asb2, as in (F). Filamin A expression is visualized by FITC fluorescence (middle panel; flow cytometric analysis for the expression of filamin A). The isotype control–stained cells are indicated. FLNa[+] fractions are quantitated in the bar graph (right panel). (H) Interaction between Tr1 and OP9 cells is promoted by the knockdown of *Asb2*. Cobblestone areas are significantly increased by the knockdown of *Asb2* (left panels). Scale bars, 200 μm. The number of cobblestone areas is shown as means ± SEM (right panel). (I) The level of cytoplasmic FLNa is increased by the knockdown of *Asb2* in Tr1 cells (left panels, original magnification, ×490). FLNa expression is visualized by FITC fluorescence (middle panel; flow cytometric analysis for the expression of FLNa). The isotype control–stained cells are indicated. FLNa[+] fractions are quantitated in the bar graph (right panel). **P < .01, ***P < .001. CFA, cobblestone forming area; mKO, monomeric Kusabira Orange; NTC, nontarget control; qPCR, quantitative polymerase chain reaction.
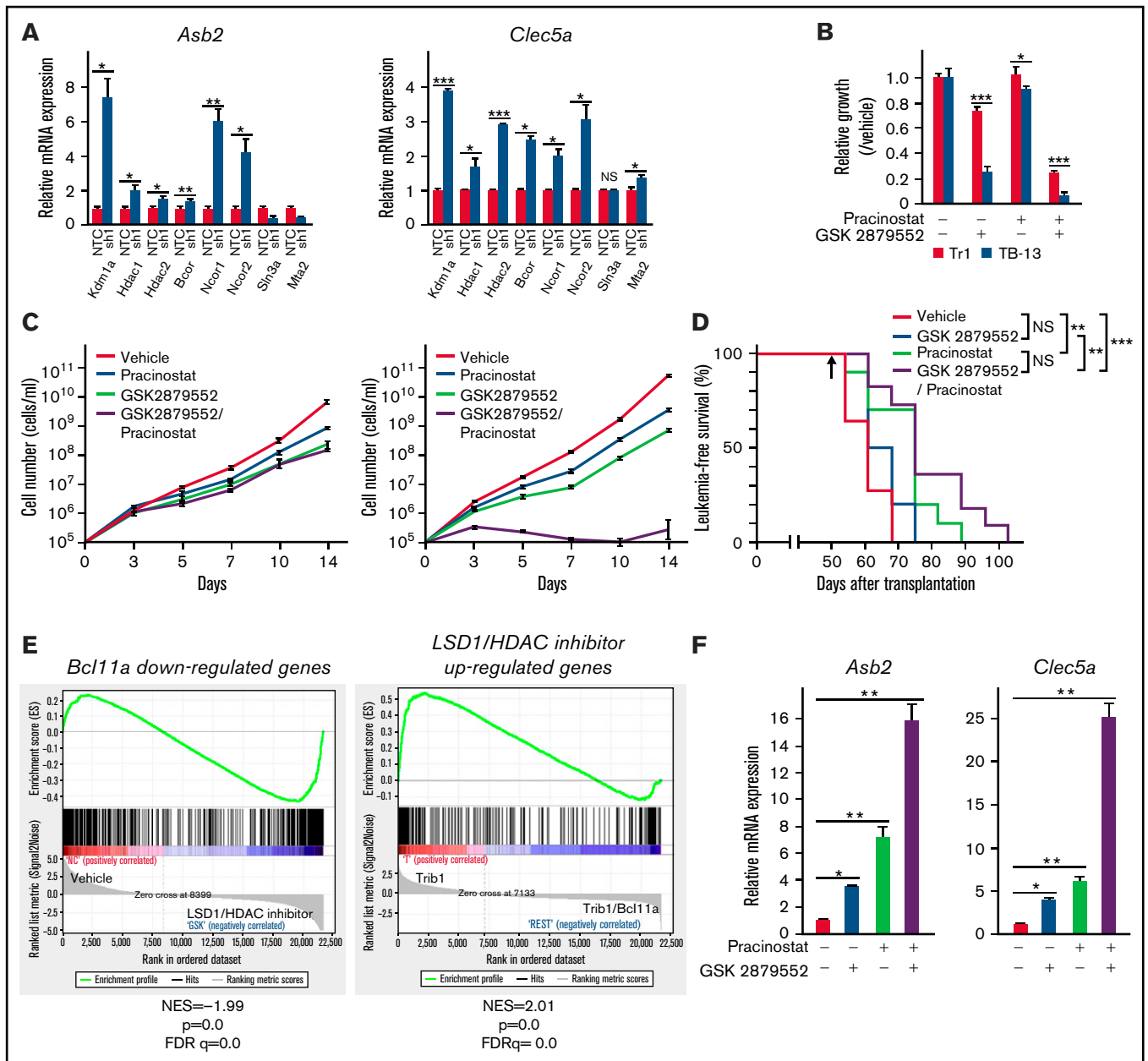
Figure 5. Bcl11a suppresses the function of PU.1 via the corepressor complexes. (A) Modulation of the expression of *Asb2* and *Clec5a* by knockdown of corepressor component genes. (B) Pracinostat and GSK2879552 treatment suppresses the growth of TB-13 cells but not of Tr1 cells. Relative growth on day 4 after treatment is shown. (C) Effects of pracinostat and GSK2879552 on cell proliferation during 14 days of treatment in Tr1 (left) and TB-13 (right) cells. (D) Kaplan-Meier survival curves showing improvement in survival with pracinostat and GSK2879552 treatment. The arrow shows the beginning of treatment. (E) GSEA shows enrichment of the Bcl11a-downregulated gene set for the gene expression profile upon treatment with the LSD1/HDAC inhibitor (left panel) and upregulated gene set upon treatment with the LSD1/HDAC1 inhibitor for the gene expression profile upon expression of *Bcl11a* (right panel). (F) The HDAC inhibitor pracinostat upregulates the expression of *Asb2* and *Clec5a*, and addition of an LSD1 inhibitor, GSK2879552, enhances the effect in TB-13 cells. *$P < .05$, **$P < .01$, ***$P < .001$. FDR, false discovery rate; mRNA, messenger RNA; NES, normalized enrichment score; NS, not significant.

BCL6, COUP-TF, RBBP4/7, and SOX2, as well as a broad spectrum of corepressor components, including NuRD, CoREST, and NCoR complexes.[1,2,12,32] In this study, we unraveled a novel mechanism by which Bcl11a represses the expression of PU.1 target genes in association with the corepressor complex in myeloid leukemogenesis. The repression of PU.1 target genes by Bcl11a is frequently associated with the cobinding of PU.1 and Bcl11a to DNA, and interaction of these TFs was shown by coimmunoprecipitation assay and PLA; the latter is a sensitive and specific method to detect the interaction of endogenous proteins. Previous retroviral tagging studies indicated that Bcl11a cooperates with *Nf1* loss and *MLL-AF9* expression, and downregulation of *Cdkn1a* by Bcl11a was reported
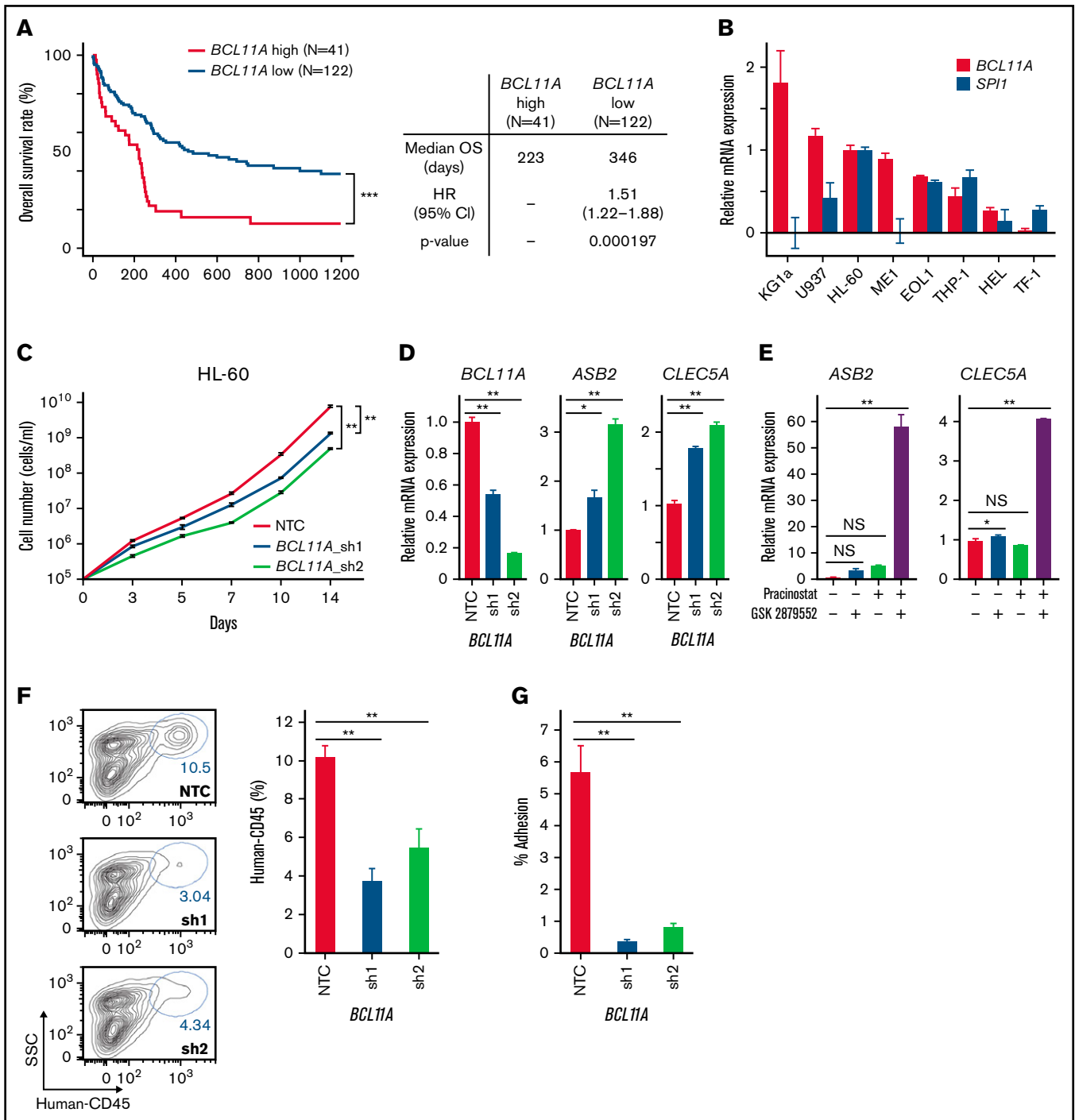
Figure 6. Important role of the BCL11A-PU.1 axis in human AML. (A) Kaplan-Meier survival curves for patients with normal-karyotype AML[29] having high or low expression of *BCL11A* (left panel). Hazard ratio (HR) and 95% confidence intervals (CI) are shown (right panel). (B) qRT-PCR for *BCL11A* and *SPI1* in human AML cells. Relative expression values normalized to the expression in HL-60 cells are shown. (C) The suppression of the growth of HL-60 cells by *BCL11A* knockdown. (D) Gene expression of *ASB2* (middle panel) and *CLEC5A* (right panel) upon knockdown of *BCL11A* in HL-60 cells was assessed using qRT-PCR. Efficiency of *BCL11A* knockdown (left panel). (E) The HDAC inhibitor pracinostat upregulates the expression of *ASB2* and *CLEC5A*, and addition of an LSD1 inhibitor, GSK2879552, enhances the effect in HL-60 cells. (F) Fluorescence-activated cell sorting (FACS) analysis shows engraftment of human CD45$^+$ HL-60 cells, with or without the knockdown of *BCL11A*, into NSG mice 1 week after transplantation. Representative results of FACS (left panel) and frequencies of human CD45$^+$ cells (right panel). (G) Decreased adhesion of *BCL11A*-silenced HL-60 cells to fibronectin. *$P < .05$, **$P < .01$, ***$P < .001$. mRNA, messenger RNA; NS; not significant; NTC, nontarget control; OS, overall survival.

in the cooperation between *Bcl11a* expression and *Nf1* loss.[9,10] Although the expression of *Cdkn1a* was not altered by the expression of *Bcl11a* in our model, the suppression of PU.1 function by Bcl11a might also be important in these models.

PU.1 regulates the differentiation of myeloid and lymphoid cell lineages, and it is essential for normal myelopoiesis.[33,34] Mice bearing hypomorphic *Sfpi1* alleles developed AML,[35] and p53 loss promotes malignant progression in homozygous deletion of the upstream regulatory element of *Sfpi1*.[36] These data suggest that the inhibition of PU.1 function disrupts signaling pathways that are important for myeloid differentiation. In support of this, many genes involved in myeloid differentiation, such as *Clec5a*, *Fcgr3*, *Csf1r*, and *Ncf4*, are identified as targets for PU.1, which were downregulated by Bcl11a in the present study. *Asb2* has been identified as a novel Bcl11a target gene that is downregulated by interaction with Bcl11a. *Asb2* is a known target of N^6-methyladenosine, promotes myeloid differentiation, and encodes a component of the ECAS E3 ubiquitin ligase complex.[26,37] Filamin A is a target of ECAS/Asb2-mediated protein degradation. We found the disappearance of filamin A when *Asb2* was upregulated in AML cells. Asb2/filamin A is involved in the remodeling of the actin cytoskeleton[28] and is associated with cell adhesion, interaction with bone marrow stromal cells, and engraftment of leukemia cells. In addition, the migratory activity of leukemic cells was increased by the Bcl11a-induced expression of filamin A. Previous studies showed that filamin A promoted the migration of Jurkat and melanoma cells[38,39] but inhibited the migration of dendritic cells,[40] suggesting that the function of the Asb2/filamin A axis in cell motility might be cell context dependent. It remains to be clarified how the downregulation of Asb2 and the increase in filamin A induce interaction between leukemia cells and bone marrow stromal cells; however, our study highlights the possible role of Asb2 in the engraftment of leukemia cells. Asb2 was also found to induce the degradation of MLL,[41] and this mechanism might also be associated with the suppression of the growth of *Bcl11a*-expressing AML. In both mechanisms, Asb2 plays an important tumor-suppressive role in the progression of leukemia downstream of the PU.1/Bcl11a axis.

Cooperation between TRIB1 and BCL11A functions as a powerful driving force in leukemogenesis by abrogating the 2 major myeloid suppressors: PU.1 and C/EBPα. Trib1 pseudokinase degrades C/EBPα by COP1-mediated ubiquitination.[15] In the cooperation between Trib1 and Hoxa9, the C/EBPα p42 isoform is selectively degraded, resulting in the modulation of the Hoxa9 transcriptional program and of the superenhancer at the *Erg* locus.[42] Recruitment of the corepressor complex to PU.1 by BCL11A promotes leukemogenic activity independent of, but cooperating with, the Trib1-induced degradation of C/EBPα. In a previous study, it was reported that deficiency of Runx1 in AML led to the recruitment of PU.1 to the corepressor complex.[43] Moreover, inhibition of LSD1 induced the differentiation of AML cells by interfering with the GFI1-mediated repression of PU.1 target genes.[44] Thus, the rescue of PU.1 target gene expression by inhibiting the corepressor components is a promising therapeutic tool for *BCL11A*-expressing AML. In contrast, the decrease in the expression of PU.1 in MLL-AF9–expressing AML induced resistance to LSD1 inhibition.[45] However, it has been reported that LSD1 inhibition sensitizes glioma cells to HDAC inhibition,[46] and combination therapy consisting of LSD1 and HDAC inhibitors against AML has been proposed.[47] HDAC inhibition might rescue the effect of LSD1 inhibitors on the diminished PU.1 activity. Therefore, it is expected that the expression level of *BCL11A* will be a reliable biomarker indicating response to therapy with HDAC and LSD1 inhibitors. A previous study indicated that LSD1 cooperates with Bcl11a in silencing the expression of globin,[11] suggesting the specific interaction between Bcl11a and LSD1 in the hematopoietic system.

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Authorship

Contribution: Y.S. and T.N. designed the study; Y.S., T.Y., S.Y., T.T., and Y.Y. performed research and analyzed data; H.H. provided critical reagents; and T.N. supervised the study and wrote the manuscript.

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