Received: 23 June 2021 | Revised: 30 September 2021 | Accepted: 11 October 2021

DOI: 10.1111/cas.15164

ORIGINAL ARTICLE

Cancer Science WILEY

C/EBPβ induces B-cell acute lymphoblastic leukemia and cooperates with *BLNK* mutations

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Funding information
Ministry of Education, Culture, Sports, Science and Technology, Grant/Award Number: 17013086; Japan Society for the Promotion of Science, Grant/Award Number: 23240125 and 23790434

Abstract

BLNK (*BASH/SLP-65*) encodes an adaptor protein that plays an important role in B-cell receptor (BCR) signaling. Loss-of-function mutations in this gene are observed in human pre-B acute lymphoblastic leukemia (ALL), and a subset of *Blnk* knock-out (KO) mice develop pre-B-ALL. To understand the molecular mechanism of the *Blnk* mutation-associated pre-B-ALL development, retroviral tagging was applied to KO mice using the Moloney murine leukemia virus (MoMLV). The *Blnk* mutation that significantly accelerated the onset of MoMLV-induced leukemia and increased the incidence of pre-B-ALL *Cebpb* was identified as a frequent site of retroviral integration, suggesting that its upregulation cooperates with *Blnk* mutations. Transgenic expression of the liver-enriched activator protein (*LAP*) isoform of *Cebpb* reduced the number of mature B-lymphocytes in the bone marrow and inhibited differentiation at the pre-BI stage. Furthermore, *LAP* expression significantly accelerated leukemogenesis in *Blnk* KO mice and alone acted as a B-cell oncogene. Furthermore, an inverse relationship between *BLNK* and *C/EBPβ* expression was also noted in human pre-B-ALL cases, and the high level of *CEBPB* expression was associated with short survival periods in patients with *BLNK*-downregulated pre-B-ALL. These results indicate the association between the C/EBPβ transcriptional network and BCR signaling in pre-B-ALL development and leukemogenesis. This study gives insight into ALL progression and suggests that the BCR/C/EBPβ pathway can be a therapeutic target.

KEYWORDS
acute lymphoblastic leukemia, B-cell receptor, BLNK, C/EBPβ, retroviral tagging

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy among children. Although the 5-y overall survival is approximately 90% in children with optimal diagnosis and treatments, it remains the most frequent cause of death in patients younger than 20 y of age.[1,2] The majority of childhood ALL originates from B-cell precursors such as pre-B-cells and pro-B-cells in which the B-cell receptor (BCR) signaling pathway plays an important role in cellular differentiation and survival.[3] BCR signaling is frequently suppressed in ALL and its recovery induces cell death, indicating a tumor suppressor role for this pathway.[4-6]

BLNK/SLP65/BASH is an adaptor protein involved in BCR signaling, and it is required for B-cell maturation.[7] Recurrent loss-of-function mutations in *BLNK* have been reported in childhood pre-B-ALL,[8,9] and a subset of *BLNK* homozygous knock-out (KO) mice develop pre-B-cell leukemia with a relatively long latency.[10,11] These data indicate that *BLNK* mutations represent important molecular aberrations for the pre-B component, but are not sufficient for complete leukemogenesis. Therefore, additional mutations might be required as cooperative genetic events for the genesis of pre-B-ALL. In addition, a specific combination of oncogenic signaling is required to promote pre-B leukemogenesis. For example, *Stat5b* oncogenic signaling is enhanced by the deletion of *Blnk* in B-cell transformation.[12] Although genetic mutations that affect BCR signaling such as *PAX5*, *EBF1*, or *IKZF1* deletions have been identified,[13] it is important to identify additional genes that cooperate with *BLNK* mutations and/or aberrations of BCR signaling for understanding the leukemogenic mechanisms and to identify novel target candidates for therapies.

In this study, we identified cooperative genes for *Blnk* deletion using Moloney murine leukemia virus (MoMLV)-based retroviral tagging. Retroviral tagging is a powerful tool to identify important genetic events that are responsible for conferring a growth advantage to leukemic cells and facilitating disease progression.[14] The technique has been efficiently utilized to identify unknown genetic interactions for the certain mutations. Using this technique, we identified a common insertion site (CIS) in *Cebpb*, which encodes a CCAAT/enhancer-binding protein β that acts as a sequence-specific transcription factor. There are 2 major isoforms of the encoded protein, namely liver activating protein (LAP) and liver inhibitory protein (LIP).[15] We identified that the *Blnk* KO and *Cebpb LAP* overexpression significantly cooperated to accelerate leukemogenesis. Therefore, this study demonstrates the important role of the C/EBPβ LAP isoform in the development of B-cell malignancy and the early stages of B-cell differentiation.

2 | MATERIALS AND METHODS

2.1 | Mice

Blnk KO mice have been described previously.[16] To generate *Cebpb* transgenic mice, *Cebpb* cDNA, covering the entire coding regions of either *LAP* or *LIP*, were amplified using PCR and subcloned into the p3xFLAG-CMV-10 vector (Sigma). After sequence verification, FLAG-tagged LAP or LIP were inserted into the Lck proximal promoter and the intronic enhancer of the Ig heavy chain (*Lck/EμH*).[17] Transgenic mice were generated using microinjecting constructs as previously described.[18] Three independent transgenic lines for each construct were generated, and each 1 line showed protein expression (Figure S1). These animals were mated with *Blnk* KO mice. Transgenic founders were identified using Southern blot hybridization using an hGH sequence as the probe. Genotyping was performed using PCR. The sequences of all PCR primers are listed in Table S1.

Mice were monitored daily for evidence of disease, and all of the diseased animals were subjected to necropsy. Tissues were analyzed morphologically and by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). All experiments involving mice were approved by the Institutional Review Board of the Cancer Institute, Japanese Foundation for Cancer Research.

2.2 | Retroviral infection and Isolation of retroviral integration sites (RIS)

MoMLV was produced by infecting murine SC1 cells with retroviral stock. The virus-containing medium was harvested and a high titer ($>1 \times 10^4$ pfu/mL) was confirmed by performing an XC cell assay.[19] Newborn *Blnk* homozygous KO, heterozygous KO or wild type mice were inoculated ip with 100 μL of viral medium.

Southern blot analysis was carried out to assess the clonal insertion and copy numbers of the retrovirus. Genomic DNA was digested with appropriate restriction enzymes, subjected to agarose gel electrophoresis and transferred to a Hybond-N nylon filter (GE Healthcare). The filter was hybridized with the *env* sequence of MoMLV as a probe.

RISs were identified using the inverse polymerase chain reaction (IPCR) approach as described previously.[18] Briefly, genomic DNA was digested with *Eco*RI, *Bam*HI, *Bgl*II, *Nco*I, *Hin*dIII, or *Sac*I, self-ligated, and subjected to nested PCR. The PCR primers for each restriction digestion are available on request. The PCR products were analyzed using agarose gel electrophoresis, subcloned into the pGEM T-easy plasmid (Promega), and subjected to sequence analysis.

2.3 | Flow cytometry

Single-cell suspensions of 1×10^6 bone marrow cells were incubated with specific antibodies, as indicated in Table S2 and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Pre-BI and pre-BII cells were defined as $B220^+CD24^{low}CD43^+$ and $B220^+CD24^{high}CD43^+$ fractions, respectively.

2.4 | Cell separation

CD3-positive ($CD3^+$) T cells and $CD19^+$ B-cells were purified from whole spleen by magnetic separation over columns using the

MACS Multisort kit in accordance with manufacturer's instructions (Miltenyi Biotec Inc).

2.5 | Immunoblot analysis

Western blot analysis was performed using total cell lysates as described previously.[20] Primary antibodies used were anti-C/EBPβ (Santa Cruz Biotech), anti-BLNK (Santa Cruz Biotech), anti-FLAG (Sigma-Aldrich), anti-GAPDH (Santa Cruz Biotech), anti-phospho-Stat5 (Cell Signaling Technologies), and anti-Stat5 (Cell Signaling Technologies).

2.6 | Preparation of RNA and real-time quantitative PCR

RNA was extracted from fresh-frozen lymph node or spleen samples using an RNeasy Mini Kit (Qiagen). Reverse transcription and RNA quantification were performed in accordance with methods described previously.[21]

2.7 | Patient samples

Blood samples were taken from 73 B-ALL patients at the University of Tokyo Hospital and Gunma Children's Medical Center. Diagnoses were made in accordance with World Health Organization criteria. Peripheral blood samples from healthy volunteers were taken at Tokyo Medical and Dental University with Informed consent obtained from all individuals. This study was approved by the Ethics Committees of the Cancer Institute, Japanese Foundation for Cancer Research, University of Tokyo, Gunma Children's Medical Center and Tokyo Medical and Dental University, and all procedures were performed in accordance with the ethical standards established by these committees.

2.8 | Microarray and database analysis

The Mouse Genome 430 2.0 Array and HT MG-430 PM Array (Affymetrix) were hybridized with cRNA probes generated from B220-positive bone marrow B-cells and leukemia samples from *Blnk* KO/LAP tg mice, and BKO418 cells in accordance with methods described previously.[22,23] The data were analyzed using GeneSpring v.12.6 software (Agilent Technologies). Pathway analyses were performed using gene set enrichment analysis (GSEA) software.[24] The microarray data are accessible through the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo) with the accession number GSE110739. Datasets for Pediatric Acute Lymphoid Leukemia—Phase II (TARGET, 2018) samples were used in cBioPortal (https://www.cbioportal.org) using TCGA data. [25,26]

2.9 | Statistical analysis

Significant differences were demonstrated for the quantitative analysis of mRNA expression using SPSS statistical software. The *P*-values were calculated using the Student *t* test, chi-square test, Scheffé method, Mann-Whitney *U*-test, and log-rank test. *P*-values $< .05$ were considered significant.

3 | RESULTS

3.1 | *Blnk* mutation accelerates the induction of MoMLV-induced pre-B-ALL

To identify the genes that cooperated with *BLNK* mutations during B-lymphoid leukemogenesis, MoMLV was injected intraperitoneally (ip) into wild type (N = 24), *Blnk* heterozygous KO (N = 48), and *Blnk* homozygous KO (N = 25) newborn mice. After MoMLV infection, the induction of leukemia in *Blnk* homozygous KO mice was associated with an earlier disease onset, compared with that in heterozygous and wild type mice, with a mean survival time of 97 d (Figure 1A). Most cases developed pre-BCR-positive B-cell leukemia and they involved lymph nodes and livers (Figure 1B,C). More importantly, 16 of 25 *Blnk* homozygous KO mice developed B220- and pre-BCR-positive pre-B-ALL (Figure 1D), whereas the majority of leukemias were of T-cell origin in heterozygous and wild type mice, indicating that the *Blnk* mutation accelerates MoMLV-induced leukemogenesis and shifts leukemogenic activity in a B-tropic direction. In addition, 3 of 5 pre-B-ALL cases in *Blnk* heterozygous KO mice were associated with loss of heterozygosity (LOH) at the *Blnk* locus (Figure 1E), further suggesting the oncogenic role of the *Blnk* homologous deletion in pre-B-ALL development. A reduced signal of the KO allele was observe in one of the heterozygous leukemias (tumor #27), suggesting the loss of the KO allele, although the exact mechanism remains unclear.

3.2 | Identification of *Cebpb* as a common MoMLV retroviral integration site

Southern blot analysis of MoMLV-induced leukemia using an *env* probe showed clonal integrations of the retrovirus in ALL with an average copy number of 7.6 (Figure 2A). RISs were identified using inverse PCR followed by sequencing and sequence mapping to the mouse genome as previously described.[20] In total, 97 RISs were identified in 16 pre-B-ALL and 1 null-type ALL samples developed from *Blnk* homozygous KO mice (Table S3). *Cebpb*, *Ahi1*, *Gfi1b*, *Myb*, *Myc*, *Sos1*, *Sfpi1*, and *Bmi1* were identified as CIS (Table 1). Most CIS had been already identified as CIS in other systems (http://variation.osu.edu/rtcgd/index.html)[27]; however, *Cebpb* and *Sos1* had not been identified as CIS in B-cell malignancies. Moreover, the *Cebpb* locus was identified at one of the highest frequencies among 8 CIS, and most integrations at the locus were found at the 3′ end of the *Cebpb* gene (Figure 2B). Although distant locations of integration sites at 3′

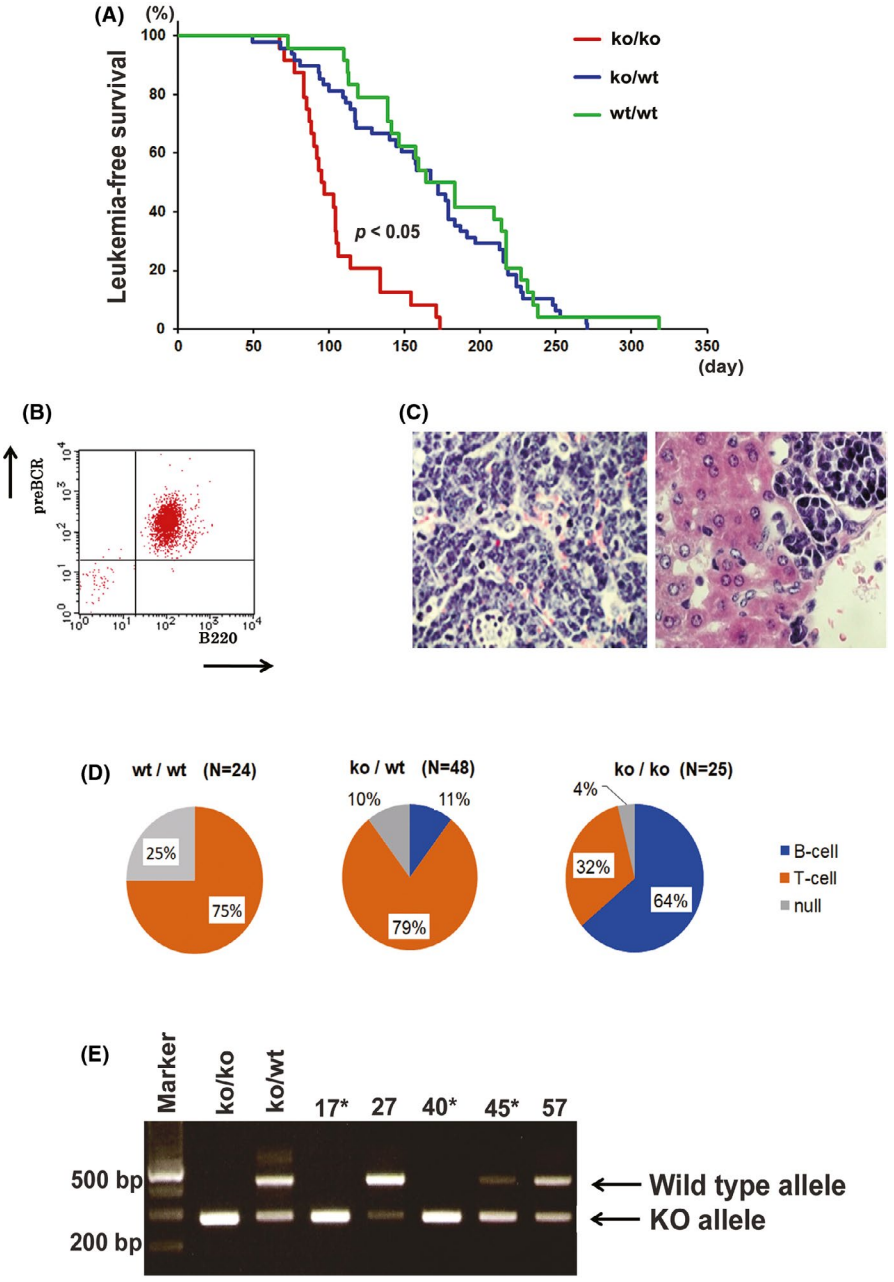

FIGURE 1 *Blnk* deficiency promotes Moloney murine leukemia virus (MoMLV)-induced B-cell leukemogenesis. A, Kaplan-Meier survival curve for *Blnk* homozygous (ko/ko), heterozygous KO (ko/wt) and wild type (wt/wt) mice infected with MoMLV. Statistical significance values between homozygous KO and the other groups were calculated by performing a log-rank test. B, Representative phenotypes of pre-B-acute lymphoblastic leukemia (ALL) that developed in *Blnk* homozygous KO mice. Flow cytometric analysis of pre-BCR and B220 expression. C, Histological feature of leukemic tissue of the lymph node (left panel) and liver with leukemic infiltration (right). D, Distribution of lineage of retroviral inducted lymphoid leukemia. E, *Blnk* loss of heterozygosity in pre-B-ALL developed in heterozygotes. The asterisks indicate tumors showing loss of the wild type allele

regions are not very common, previous studies have reported a similar integration tendency at 3′ sites.[28,29] Real-time quantitative RT-PCR showed that *Cebpb* mRNA was highly expressed in all pre-B-ALL samples compared with that in CD19-positive bone marrow cells derived from wild type and *Blnk* homozygous KO mice regardless of retroviral integration (Figure 2C). High expression of the C/EBPβ protein was also confirmed by immunoblotting, and both LAP and LIP isoforms were expressed in MoMLV-induced pre-B-ALL (Figure 2D).

3.3 | Lymphoid-specific expression of the LAP isoform of C/EBPβ cooperates with the *Blnk* mutation in pre-B-ALL development

To confirm the cooperative activity between the *Blnk* mutation and candidate genes identified as CIS, *Cebpb* transgenic mice were generated. *Cebpb* was identified as one of the most frequent CIS and a unique integration site during B-cell malignancy based on retroviral tagging experiments. The cDNA sequences encoding either C/EBPβ LAP or LIP isoforms were expressed under the control of a *Lck* promoter and *Igh* enhancer (Figure S1A). Expression of LAP or LIP in transgenic spleen cells was confirmed by immunoblot analysis using a FLAG antibody (Figure S1B), and the mice were crossed with *Blnk* KO animals to obtain *Cebpb* transgene-expressing *Blnk* homozygous KO mice.

Transgenic expression of the *LAP* isoform significantly accelerated leukemogenesis in *Blnk* KO mice. All *LAP* transgenic/*Blnk* KO developed pre-B-ALL with a median survival time of 173 days (Figure 3A). Expression of the *LIP* isoform did not have such a cooperative effect, and *LIP* expression by itself did not induce ALL, whereas 73% and 37% of *Blnk* homozygous KO and *LAP* transgenic mice, respectively, developed ALL within 600 days (Figure 3A).

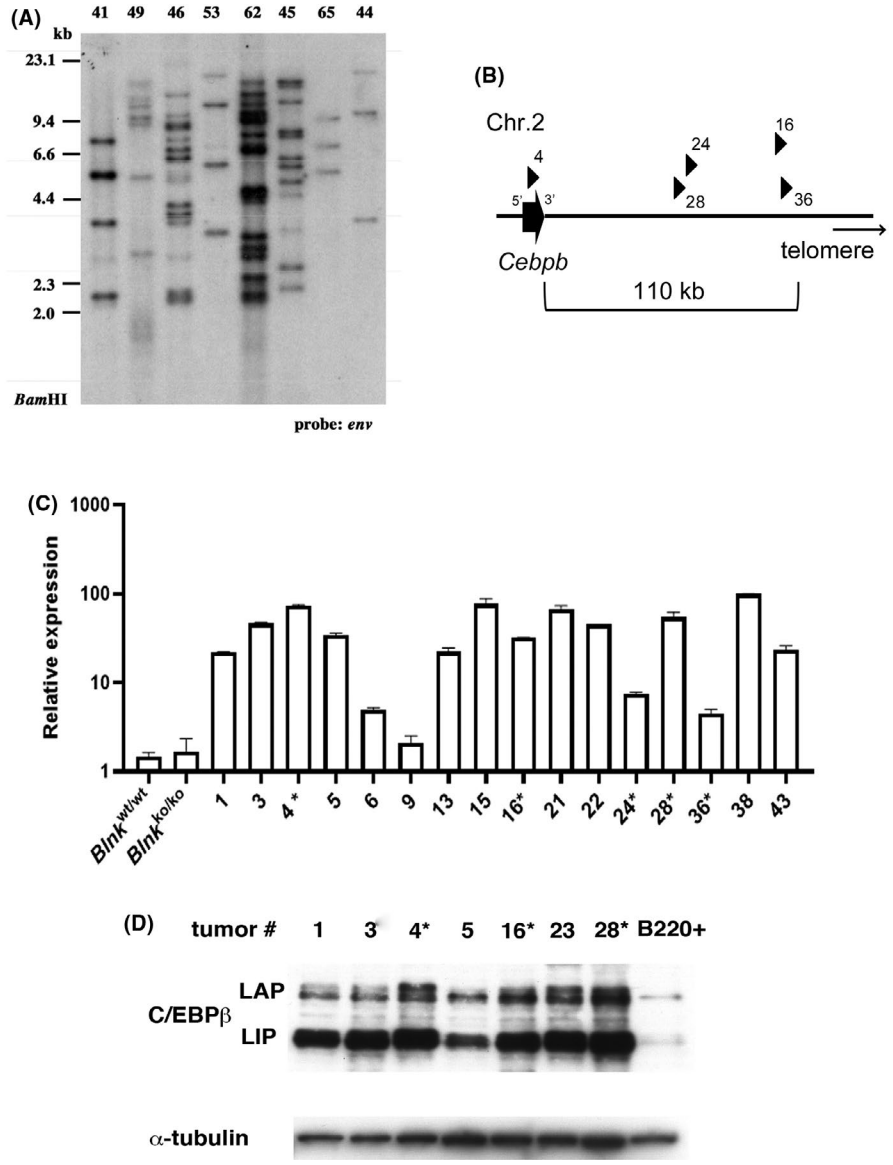

FIGURE 2 Moloney murine leukemia virus (MoMLV) retroviral integrations at the *Cebpb* locus. A, Southern blot analysis of viral integration in each tumor. A probe for MoMLV *env* was applied to detect viral integrations. B, A physical map of retroviral integration sites at the *Cebpb* locus in pre-B acute lymphoblastic leukemia (ALL) of *Blnk* homozygous KO mice. C, Expression of *Cebpb* in pre-B-ALL in *Blnk* homozygous KO mice. Higher expression of *Cebpb* mRNA was observed in all pre-B-ALL samples, compared with that in pre-BCR-positive bone marrow B-cells regardless of viral integration. Asterisks indicate tumors with retroviral integration at the *Cebpb* locus. Error bars represent the mean ± SD of 3 independent experiments. D, Immunoblotting shows upregulated expression of both liver activating protein (LAP) and liver inhibitory protein (LIP) isoforms of the C/EBPβ protein regardless of viral integration. Asterisks indicate tumors with retroviral integration at the *Cebpb* locus

Typically, the leukemias that developed in both *Blnk* KO and *LAP/Blnk* KO mice were pre-B ALL[30] and these were positive for pre-BCR, CD19, B220, IL-7Rα, CD24, and CD43 (denoted as type 1 pre-B-ALL). In contrast, 2 subgroups of leukemia developed in the *LAP* transgenic mice including type 1 and type 2 pre-B-ALL, the latter was positive for CD43 but negative for CD19, CD24, B220, pre-BCR, and IL-7Rα (Figure 3B,C). In total, 54 of 55 (98%) leukemias that developed in the *LAP/Blnk* homozygous KO mice were type 1 pre-B-ALL, whereas 13 of 37 (35%) developed in *LAP* transgenic mice (Figure 3D). The data indicate that *LAP* acts as a strong driving force to enhance pre-B-ALL development in the *Blnk* KO condition.

Gene expression profiling of type 1 pre-B-ALL that developed in *LAP* transgenic, *Blnk* homozygous KO and *LAP/Blnk* homozygous KO mice, and of type 2 B-ALL in *LAP* transgenic mice, was performed. Principal component analysis (PCA) showed that ALLs from each genetic background were independently clustered (Figure 3E). GSEA was conducted to compare gene expression profiles among *LAP* transgenic, *Blnk* KO, and *LAP/Blnk* KO leukemia. Gene expression profiles between *Blnk* KO and *LAP/Blnk* KO were not significantly different, and the AKT pathway was only the significantly pathway that was enriched in *LAP*-expressing *Blnk* KO leukemia compared with *Blnk* KO pre-B-ALL (Figure 3F).

3.4 | Lymphoid-specific expression of the LAP isoform of C/EBPβ enhances differentiation block of pre-B-cell in *BLNK* homozygous KO mice

To understand the significance of *LAP* overexpression in *Blnk* KO-associated leukemogenesis, its effect on B-cell differentiation was examined. The number of mature B-cells in the bone marrow was significantly decreased in both *LAP* transgenic and *Blnk* KO mice (Figures 4A and S2). Moreover, an accumulation of IL-7Rα/

TABLE 1 Retroviral integration sites of pre-B-ALL derived from BLNK$^{ko/ko}$ mice

Candidate gene	Product	Locus (Chr.)	Incidence (n = 16)	Tumor #
C/ebpb	CCAAT/enhancer-binding protein beta	**2:**167,688,915 −167,690,432	5	4, 16, 24, 28, 36
Ahi1	Jouberin	**10:**20,952,547 −21,080,429	5	3, 4, 21, 23, 43
Gfi1b	Growth factor independent 1B	**2:**28,609,450 −28,621,982	3	3, 6, 28
c-*myb*	Myeloblastosis proto-oncogene product	**10:**21,124,930 −21,160,984	3	4, 6, 28
c-*myc*	*myc* proto-oncogene product	**15:**61,985,341 −61,990,361	3	1, 9, 38
Sos1	Son of sevenless homolog 1	**17:**80,393,752 −80,480,453	2	3, 16
Sfpi1	PU.1	**2:**91,096,678 −91,115,759	2	38, 43
Bmi1	Bmi1 polycomb ring finger protein	**2:**18,677,018 −18,686,629	2	3, 24

B220 double-positive cells was observed in *Blnk* homozygous KO mice, and LAP expression significantly enhanced this effect (Figure 4B,C), indicating that *LAP* expression enhanced the *Blnk* mutation-associated block in differentiation at the pre-BI stage, defined as B220^{high}CD43^{high}CD24^{intermediate} (Figure 4D). STAT5, a downstream molecule of IL-7R signaling, was significantly phosphorylated in *Blnk* homozygous KO B-cells and *LAP* expression (Figure 4E).

Gene expression profiles of non-neoplastic B220-positive cells were compared among wild type, *LAP* transgenic, *Blnk* KO, and *LAP/Blnk* KO bone marrow samples. PCA showed a distinct expression pattern in each group, however there was close similarity between *Blnk* KO and *LAP/Blnk* KO bone marrow (Figure 4F). GSEA showed that gene sets associated with immature lineages such as pre-BI and hematopoietic stem cells were significantly enriched by *LAP* expression, consistent with its effect on pre-BI accumulation (Figure 4G).

3.5 | Involvement of *CEBPB* and *BLNK* in human B-ALL

Expression of *CEBPB* in human B-ALL cells was examined using real-time quantitative RT-PCR analysis and bone marrow samples from 73 cases of human childhood B-ALL including pro-B and pre-B-ALL, as well as non-neoplastic B-cells from 6 healthy individuals. Significantly higher *CEBPB* expression was observed in B-ALL compared with that in non-neoplastic B-cells obtained from healthy donors (Figure S3A). When our B-ALL cases were divided into 2 groups in accordance with the *BLNK* expression level, the *BLNK*-low group showed significantly high expression of *CEBPB* (Figure 5A). Furthermore, an inverse correlation between *BLNK* and *CEBPB* expression was observed in 203 cases of human pediatric ALL obtained from the data sets of Pediatric Acute Lymphoid Leukemia—Phase II (TARGET, 2018) (Figure 5B). The *BLNK*-low and *CEBPB*-high (BLCH) ALL patients showed significantly shorter survival times compared with that in the other groups (Figure 5D) and that in the *BLNK*-low/*CEBPB*-low (BLCL) group (Figure S3B). Although gene expression analysis at the DNA and RNA levels did not distinguish expression of LAP and LIP proteins, increase in the LAP protein requires upregulation of *CEBPB* expression. In addition, the analysis of copy number changes using 682 cases of pediatric pre-B-ALL showed deep deletions of *BLNK* in 9 cases (1.3%) (Figure S3C). *BLNK* deletions and other mutations related to BCR signaling, such as deletions of *PAX5*, *EBF1*, and *IKZF1*, were mutually exclusive except for a single case with *BLNK* and *PAX5* deletions (Figure S3C). These findings are consistent with the results of our mouse experiments.

4 | DISCUSSION

In this study, we identified the *LAP* isoform of C/EBPβ as a B-cell oncoprotein and a cooperative factor for a *Blnk* mutation that accelerates pre-B leukemogenesis (Figure 5D). In MoMLV-induced B-ALL on a *Blnk* KO background, retroviral integrations were observed downstream of the *Cebpb* locus, suggesting that enhancer activation of *Cebpb* was induced by the retroviral sequence, which acts as a *cis*-element. Most *Blnk* KO B-ALL examples without retroviral integrations at the *Cebpb* locus also showed upregulation of the gene. The exact mechanism of this *Cebpb* upregulation remains unclear; however changes in the epigenetic environment around the *Cebpb* locus may contribute to the upregulation. Human and murine C/EBPβ consists of 2 isoforms, LAP and LIP, which are produced using alternative start codons.[31] LAP and LIP

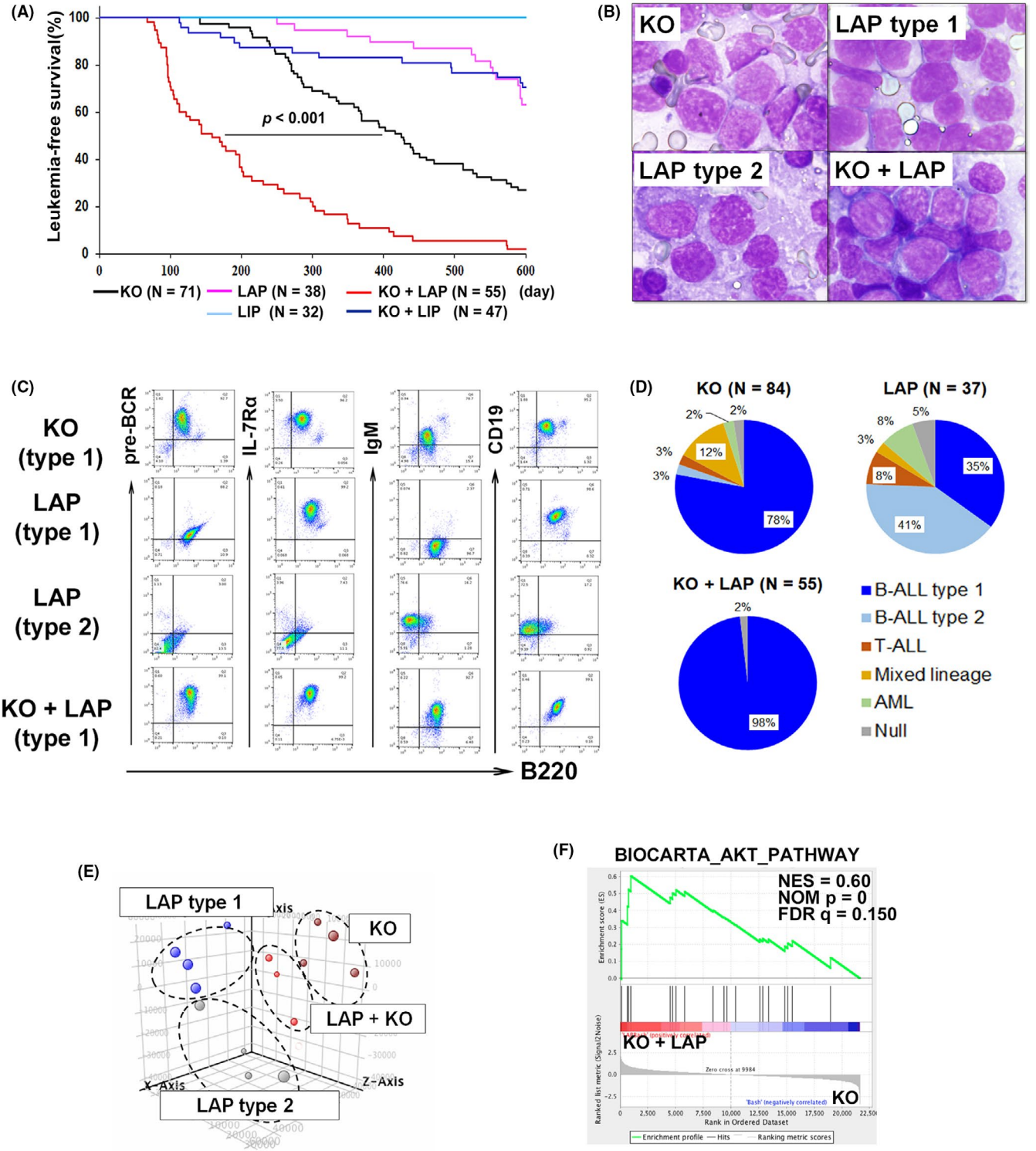

FIGURE 3 Transgenic expression of the Cebpb LAP isoform accelerated pre-B-ALL development in *Blnk* homozygous KO mice. A, Kaplan-Meier survival curve for mice of indicated genotypes. Giemsa staining. B, Representative morphologies of pre-B-ALL developed in *LAP* transgenic, *Blnk* homozygous KO and *LAP/Blnk* homozygous KO mice, and B lymphoblastic leukemia in *LAP* transgenic mice. C, Surface marker expression in ALL of indicated genotypes. D, Distribution of disease types developed in *LAP* transgenic, *Blnk* homozygous KO, and *LAP/Blnk* homozygous KO mice. E, Principal component analysis for gene expression profiles in leukemia of each genotype. F, GSEA of *LAP/Blnk* homozygous KO vs *LAP* transgenic leukemia shows enrichment of gene sets involved in the AKT pathway

share an identical DNA binding domain, however LIP lacks the transactivation domain and its function is antagonistic to LAP.[32] A previous report showed that LIP can induce cell proliferation and collaborates with *Evi1* in acute myeloid leukemia.[28] In addition, LIP can induce *Myc* expression in hematopoietic progenitor cells.[33] An appropriate cellular context and epigenetic status might be

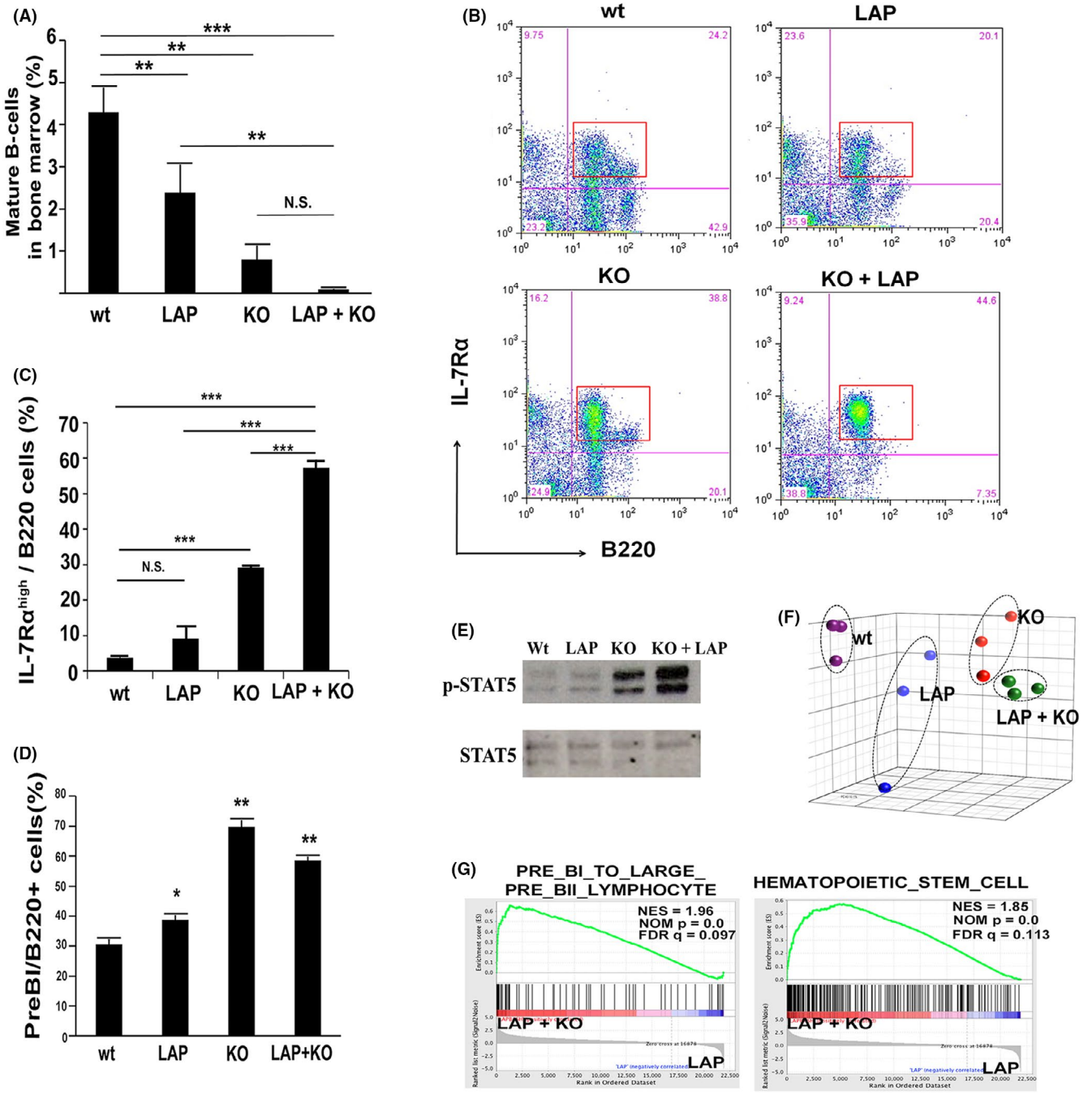

FIGURE 4 Transgenic expression of the Cebpb LAP isoform enhances differentiation block in *Blnk* homozygous KO mice. A, The number of B220-positive and IgM-high mature B-cells in bone marrow was measured in mice of 6 wk of age for the indicated genotype. Statistical significance was assessed using Scheffé test (**$P < .01$, ***$P < .001$). B, Representative results of flow cytometric analysis. Frequencies of B220- and IL-7Rα-positive B-cells are indicated. C, The number of B220- and IL-7Rα-positive B-cells in the bone marrow was measured in mice of 6 wk of age for the indicated genotype. Statistical significance was assessed using Scheffé test (***$P < .001$). D, Differential arrest at the pre-BI stage ($B220^{high}CD43^{high}CD24^{intermediate}$) of KO and LAP + KO mice. E, Immunoblotting shows increased phosphorylation of STAT5 in bone marrow B-cells. F, PCA of all groups of bone marrow cells. G, GSEA of bone marrow B-cells from *LAP/Blnk* homozygous KO shows enrichment of gene pathways involved in pre-BI lymphocyte differentiation (left) and hematopoietic stem cells (right)

required for the transforming activity of the LAP isoform. Notably, a previous study has demonstrated that *Cebpb* deficiency results in a significant decrease in bone marrow B-cells and the proliferative responsiveness of B-cells to IL-7.[34] In addition, *CEBPB* upregulation was found to be involved in human B-cell ALL,[35,36] and CEBPB is required for ALK-mediated transformation in anaplastic large cell lymphoma.[37] Our data clearly indicated that deregulated expression of the *LAP* isoform is important for defective pre-B-cell differentiation and the development of B-cell malignancies in collaboration with impaired BCR signaling.

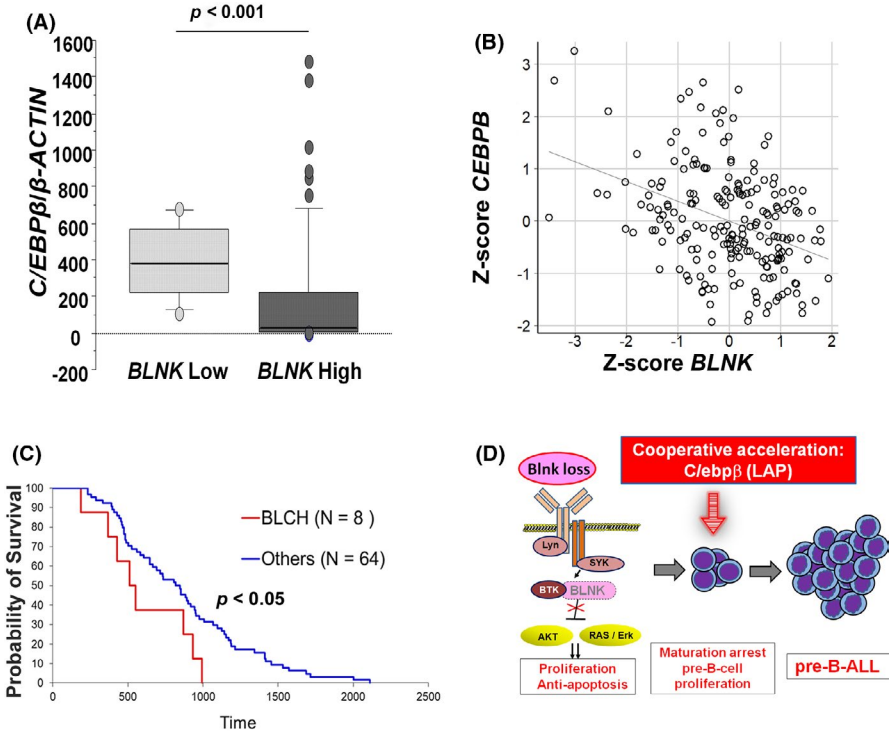

FIGURE 5 Correlation between *BLNK* and *CEBPB* expression in human pre-B-ALL. A, Gene expression of *CEBPB* in human patients with B-ALL (n = 73). The patients were divided into *BLNK*-low (n = 8) and *BLNK*-high (n = 65) expression groups. B, Inverse correlation between *BLNK* and *CEBPB* expression in human pediatric pre-B-ALL in TCGA database by cBioportal. N = 203 cases, $r = -0.35$, $P < .01$ using Spearman's rank correlation coefficient. C, High expression of *CEBPB* and low expression of *BLNK* (BLCH) is associated with poor prognosis among all types of pediatric pre-B-ALL. In total, 72 patients in the TARGET Phase II cohort for whom survival information was available from the cBioPortal were divided into 8 BLCH and 64 others. Hazard ratio, 2.75, using log-rank test. D, Schematic representation of *BLNK* and *CEBPB* changes in leukemogenesis

Activation of BCR signaling is essential for pre-B-cell differentiation, and impaired signaling blocks the differentiation at the pre-B stage.[38,39] The *BLNK* mutation causes a reduction in mature B-cells, resulting in immunodeficiency with hypogammaglobulinemia.[40] The malignant transformation of pre-B-cells involves several differential impairments and activation of oncogenic signals. Pre-BCR activation suppresses IL-7Rα signaling through an interaction between BLNK and JAK/STAT5. [41] Conversely, IL-7Rα signaling can induce Foxo1, a coactivator of Blnk, via the PI3K-Akt pathway, and BLNK expression also antagonized Akt activation.[42] Although genetic mutations of *BLNK* have not been frequently observed using the recent analysis of 1988 pre-B-ALL cases,[43] the impact of expression of *BLNK* might not be fully understood and cross-talk with other signaling molecules should be addressed. Indeed, Imoto and colleagues reported that BLNK is a target of repression by PAX5-PML important for ALL development.[44] In our mouse model, the abnormal accumulation of IL-7Rα-positive pre-B-cells in the bone marrow of *Blnk* KO mice was further enhanced by *LAP* expression, suggesting that C/EBPβ might also function in IL-7R α-JAK/STAT and/or AKT signaling.

Our retroviral tagging experiment also identified several candidate genes that cooperate with the *Blnk* mutation, including *Myc*, *Myb*, and *Ahi1*, most of which have been already identified as important genes for leukemia.[45] *Sos1*, which encodes a guanine nucleotide exchange factor for small GTPases RAS and RAC,[46] was identified as a unique CIS in Blnk KO ALL. Transgenic expression of *Sos1* using the same *Lck/IgH* enhancer that was used to generate *Cebpb* transgenic mice failed to accelerate leukemogenesis in *Blnk* KO mice (data not shown). In this case, the use of a more appropriate enhancer for efficient expression of *Sos1* might be investigated in a future experiment, given the rather low expression of the transgene. Nevertheless, upregulation of *Sos1* might contribute to the malignant progression of pre-B-ALL, as a recent study indicated that phosphorylation of SOS1 promotes BCR-ABL-associated leukemogenesis.[47]

The exact mechanisms of the C/EBPβ LAP isoform-mediated transcriptional modulation in pre-B-cells with *Blnk* deficiency remains unclear. Difficulty in maintaining pre-B-ALL cells that express *LAP* hinders the analysis of global LAP binding in leukemic cells. Nevertheless, our present study underscores the important role of the C/EBPβ LAP isoform in the malignant progression of pre-B-ALL, and suggests that the BCR/C/EBPβ pathway could represent a potential therapeutic target.

ACKNOWLEDGMENTS

We thank Masato Kubo for the *Lck/IgH* cassette, Hidetoshi Ikeda for MoMLV, and Yohei Kanno, Ayako Hiyama, Aya Nakamura, and Mizuki Homme for technical assistance. The results shown here are in whole or part based upon data generated by TCGA Research Network: https://www.cancer.gov/tcga. This work was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (17013086 to TN), and by a Grant-in-Aid from Japan Society for the Promotion of Science (23240125 to TN and 23790434 to MK).

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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How to cite this article: Kurata M, Onishi I, Takahara T, et al. C/EBPβ induces B-cell acute lymphoblastic leukemia and cooperates with *BLNK* mutations. *Cancer Sci*. 2021;112:4920–4930. https://doi.org/10.1111/cas.15164