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

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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-B1 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. 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. BCR signaling is frequently suppressed in ALL and its recovery induces cell death, indicating a tumor suppressor role for this pathway.

BLNK/SLP65/BASH is an adaptor protein involved in BCR signaling, and it is required for B-cell maturation. Recurrent loss-of-function mutations in BLNK have been reported in childhood pre-B-ALL, and a subset of BLNK homozygous knock-out (KO) mice develop pre-B-cell leukemia with a relatively long latency. 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 leukemia. For example, Stat5b oncogenic signaling is enhanced by the deletion of Blnk in B-cell transformation. Although genetic mutations that affect BCR signaling such as PAX5, EBF1, or IKZF1 deletions have been identified, 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. 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 Cebp, 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). We identified that the Blnk KO and Cebp 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. To generate Cebp transgenic mice, Cebp 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). Transgenic mice were generated using microinjecting constructs as previously described. 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 FACS Calibur 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 × 10^5 pfu/mL) was confirmed by performing an XC cell assay. 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. Briefly, genomic DNA was digested with EcoRI, BamHI, BglII, Ncol, HindIII, or SacI, 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 FACS Calibur flow cytometer (Becton Dickinson). Pre-B1 and pre-BII cells were defined as B220*CD24lowCD43+ and B220*CD24highCD43+ 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
2.5 | Immunoblot analysis

Western blot analysis was performed using total cell lysates as described previously. 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.

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. The data were analyzed using GeneSpring v.12.6 software (Agilent Technologies). Pathway analyses were performed using gene set enrichment analysis (GSEA) software. 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.

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 observed 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. 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, Spi1, 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), 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’
regions are not very common, previous studies have reported a similar integration tendency at 3′ sites. 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 Igβ enhancer (Figure S1A). Expression of 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 LAP 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).
Typically, the leukemias that developed in both Blnk KO and LAP/Blnk KO mice were pre-B ALL 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 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/
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-B1 stage, defined as B220<sup>high</sup>CD43<sup>high</sup>CD24<sup>intermediate</sup> (Figure 4D). STATS, 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 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 S3B). These findings are consistent with the results of our mouse experiments.

### 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 Cebpβ locus, suggesting that enhancer activation of Cebpβ was induced by the retroviral sequence, which acts as a cis-element. Most Blnk KO B-ALL examples without retroviral integrations at the Cebpβ locus also showed upregulation of the gene. The exact mechanism of this Cebpβ upregulation remains unclear; however changes in the epigenetic environment around the Cebpβ 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. LAP and LIP

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**TABLE 1** Retroviral integration sites of pre-B-ALL derived from BLNK<sup>ko/ko</sup> mice

| Candidate gene | Product | Locus (Chr.) | Incidence (n = 16) | Tumor # |
|---------------|---------|-------------|------------------|--------|
| C/ebpβ       | CCAAT/enhancer-binding protein beta | 2167688915-167690432 | 5 | 4, 16, 24, 28, 36 |
| Ah1           | Jouberin | 1020952547-21080429 | 5 | 3, 4, 21, 23, 43 |
| Gfi1b         | Growth factor independent 1B | 1028609450-28621982 | 3 | 3, 6, 28 |
| c-myb         | Myeloblastosis proto-oncogene product | 1021124930-21160984 | 3 | 4, 6, 28 |
| c-myc         | myc proto-oncogene product | 1561985341-61990361 | 3 | 1, 9, 38 |
| Sos1          | Son of sevenless homolog 1 | 1780393752-80480453 | 2 | 3, 16 |
| Spi1          | PU.1 | 2191096678-91115759 | 2 | 38, 43 |
| Bmi1          | Bmi1 polycomb ring finger protein | 218677018-18686629 | 2 | 3, 24 |
share an identical DNA binding domain, however LIP lacks the transactivation domain and its function is antagonistic to LAP. A previous report showed that LIP can induce cell proliferation and collaborates with Evi1 in acute myeloid leukemia. In addition, LIP can induce Myc expression in hematopoietic progenitor cells. An appropriate cellular context and epigenetic status might be...
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 up-regulation 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 4** Transgenic expression of the Cebpb LAP isoform enhances differentiation block in Blink 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-7Ra-positive B-cells are indicated. C, The number of B220- and IL-7Ra-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\(^{\text{high}}\)CD43\(^{\text{high}}\)CD24\(^{\text{intermediate}}\)) 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/Blink homozygous KO shows enrichment of gene pathways involved in pre-BI lymphocyte differentiation (left) and hematopoietic stem cells (right).
Activation of BCR signaling is essential for pre-B-cell differentiation, and impaired signaling blocks the differentiation at the pre-B stage. The BLNK mutation causes a reduction in mature B-cells, resulting in immunodeficiency with hypogammaglobulinemia. 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. Conversely, IL-7Rα signaling can induce Foxo1, a coactivator of Blnk, via the PI3K-Akt pathway, and BLNK expression also antagonized Akt activation. Although genetic mutations of BLNK have not been frequently observed using the recent analysis of 1988 pre-B-ALL cases, 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. Sos1, which encodes a guanine nucleotide exchange factor for small GTPases RAS and RAC, 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 Cebpβ 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.

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.

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DISCLOSURE
The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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