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Dual retrovirus integration tagging: identification of new signaling molecules *Fiz1* and *Hipk2* that are involved in the IL-7 signaling pathway in B lymphoblastic lymphomas

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RECEIVED NOVEMBER 9, 2009; REVISED FEBRUARY 11, 2010; ACCEPTED MARCH 5, 2010. DOI: 10.1189/jlb.1109748

ABSTRACT

IL-7R, FLT3, and CD43 are surface antigens expressed during the transition from pro-B to pre-B cells in BM. To understand interactions between their signaling pathways, we analyzed spontaneous mouse B-LBLs with dual MLV integration into *Stat5a* and *Fiz1* or *Stat5a* and *Hipk2*. MLV integration resulted in up-regulation of these genes in lymphoma cells compared with normal pro-B cells from the BM. In lymphomas with both integrations into *Stat5a* and *Fiz1*, increases in phosphorylated STAT5A and expression of c-Myc, a target gene of STAT5A, were observed following stimulation of the FLT3. Clones with the dual integrations grew faster in IL-7 and FLT3L-supplemented medium than clones with *Stat5a* integration alone. On the other hand, in lymphomas with integrations into *Stat5a* and *Hipk2*, increases in phosphorylated STAT5A and expression of c-Myc were observed following cross-linking of CD43. In conclusion, FLT3 and CD43 signaling pathways involve STAT5A via *Fiz1* and *Hipk2* in B-LBLs. Identification of the dual MLV integration sites in B-LBLs, therefore, will provide an excellent tool for identification of the signaling pathways in B-LBLs. *J. Leukoc. Biol.* 88: 107–116; 2010.

Introduction

Retroviral insertional mutagenesis in several inbred strains of mice induces a high incidence of myeloid leukemia and B and T cell lymphomas, as well as other types of tumors [1]. The retroviral integration sites in these tumors thus provide powerful tools for the identification of novel oncogenes and tumor-related genes [2]. The inbred strain of mice SL/Kh develops lymphoma spontaneously as a result of genetically acquired MLV integration [3, 4]. More than 90% of these mice develop $B220^+$ $BP\text{-}1^+$ $CD43^+$ $CD24^+$ $sIgM^-$ B-LBLs spontaneously by 6 months of age [5]. As development of spontaneous B-LBLs is rare in most other mice, the SL/Kh strain is an important model for studying the pathogenesis of B-LBLs. By analyzing (SL/Kh×NFS/N) × NFS/N F2, we have mapped a highly significant quantitative trait locus, named Bomb1, on the distal segment of mouse chromosome 3, which is responsible for pre-B expansion [6]. NFS/N is an inbred mouse strain without an endogenous, ecotropic MLV genome and with low spontaneous lymphoma incidence [7]. We generated a congenic strain, NFS/N.SL/Kh-Bomb1 mice, in which the Bomb1 locus is replaced by the identical locus in the SL/Kh mice genome. We observed that the congenic mice showed pre-B cell expansion after 4 months of the birth and demonstrated that the Bomb1 locus is crucial for the pre-B expansion [8]. Such spontaneous lymphoma strains have been used as a tool to search for novel oncogenes by identification of common integration sites of MLV [1, 2]. Reintegration of endogenous MLV genomes into the cancer-related genes *Stat5a* [9] and *c-myc* [10] induced these lymphomas. In this study, we show that these mice are also a useful model for investigation of oncogene pathway interactions by analysis of lymphomas with dually integrated, clonal MLVs. Wu et al. [11] reported that MLV integration favors transcriptionally active genes. Therefore, when lymphoma cells have more than one clonally integrated MLV ge-

Abbreviations: B-LBL=B-lymphoblastic lymphoma, BM=bone marrow, Bomb1=bone marrow pre-B-1, D_H-J_H=Ig heavy chain diversity-joining, *Emv11*=endogenous ecotropic MuLV11, *Fiz1*= fms-like tyrosine kinase 3-interacting zinc finger protein 1, FLT3L=fms-like tyrosine kinase 3 ligand, *Hipk2*=CD43-interacting homeodomain-interacting protein kinase 2, *IgH*=Ig heavy chain, *IgL*=Ig light chain, ITD=internal tandem duplication, MLV=murine leukemia retrovirus, sIgM=surface IgM

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nome, the host genes are likely to be commonly active, and their interaction can be observed in the host cells. We reported previously that one of the common integration sites of MLV in SL/Kh B-LBLs is *Stat5a*, whose constitutive activation is responsible for pre-B cell lymphomagenesis in vivo and in vitro [9]. Activated STAT5A forms a dimer and influences transcriptional activity of other genes by binding to the γ-activated site element in the promoter of target antiapoptotic genes, such as *c-myc*, *pim-1*, *bcl-xL*, and *cyclin D1* [12–15]. STAT5A is recruited in the IL-7R pathway that operates selectively during the precursor stage of the pro- to pre-B cell transition in BM, as well as in T cells [15, 16].

In the current study, we show two novel types of signaling pathways that were activated by MLV integration. We also identify a novel pathway involved in cellular signaling transmission by analysis of the signaling pathways. We examined the *c-myc* expression via the STAT5A phosphorylation [9]. STAT5 is essential for early B cell development but not for B cell maturation and function [17]. This IL-7R pathway is key in pro-B cell development and is known to regulate IgH recombination and B cell precursor expansion [18, 19]. STAT5 and IL-7 signaling control cell survival and the developmental ordering of Ig gene rearrangements by suppressing premature Igκ recombination in pro-B cells [20]. Thus, the occurrence of B-LBLs in SL/Kh mice caused by *Stat5a* integration is a promising model for investigation of one of the signaling pathways in early B lymphocytes [5]. The development stage of B cells was according to Hardy's standard classification of murine B cell lineage [21]. In the current study, we identified novel integration sites in SL/Kh B-LBLs including *Fiz1* and *Hipk2*. *Fiz1* encodes a novel zinc finger protein with C_2H_2-type zinc fingers that interacts with the receptor tyrosine kinase FLT3 [22], a surface antigen of pro- to pre-B cells. On the other hand, *Hipk2* encodes a member of a novel family of serine/threonine kinases [23] that interacts with the cytoplasmic domain of CD43, which is expressed on most hematopoietic cells, including pro- to pre-B cells. Although the host genes activated by MLV integration were different, the type of lymphoma was consistently B-LBLs. This suggests that cross-talk may be involved in the intracellular pathways activated by the MLV integration. As several clones of cells from SL/Kh B-LBLs had dual *Stat5a* and *Fiz1* or *Stat5a* and *Hipk2* integrations, we studied the cross-talk among signaling molecules in the IL7-R pathway. These data provide novel insights into the pathogenesis of B-LBLs.

MATERIALS AND METHODS

Animals

The origin, endogenous MLVs, and host genetic factors in lymphomagenesis in SL/Kh mice have been reported previously [3–6]. All animal experiments were carried out with approval from the Ethical Committee for Animal Experiments, Kyoto University Graduate School of Medicine (Japan).

Inverse PCR for identification of MLV integration sites

The virus-host junctions of the MLV integration site were amplified with inverse PCR [9]. In brief, genomic DNA (100 ng) from each lymphoma was digested with *Sad*I for 2 h and self-ligated with T4 ligase (Takara Bio, Otsu, Japan) at 14°C overnight. PCR amplification was carried out in three steps in a thermal cycler (Takara Bio) under the following conditions: initial denaturation (1 min at 95°C); then 10 cycles (30 s at 94°C, 40 s at 62°C, and 4 min at 68°C; step 1); then 20 cycles (30 s at 94°C, 40 s at 62°C, and 4 min plus extension of 20 s in one cycle at 68°C; step 2); and finally, elongation (10 min at 72°C). In some cases, nested PCR was added to the above reaction. The primers for inverse PCR were located within the MLV genomes. Their

TABLE 1 *Phenotypic Features of Lymphoma*

			IgH								
ID	Sex	Age	D_HJ_H	V_HD_H	IgL	CD43	IL-7R	CD24	V_{preB}	λ5	Integration site
1	f	6	c	n.d.	g	+	+	+	+	–	*Stat5a*
2	m	7	c	n.d.	g	+	+	+	+	–	*Stat5a*
3	f	4	c	n.d.	g	+	+	+	+	–	*Stat5a*
4	m	6	c	c	g	+	+	+	+	–	*Stat5a*
5	f	7	c	c	g	+	+	+	+	–	*Stat5a*
6	f	6	c	c	g	+	+	+	+	–	*Stat5a*
7	m	7	c	n.d.	g	+	+	+	+	–	*Fiz1* + *Stat5a*
8	f	6	c	n.d.	g	+	+	+	+	–	*Fiz1* + *Stat5a*
9	f	10	c	n.d.	g	+	+	+	+	–	*Fiz1* + *Stat5a*
10	m	5	c	n.d.	g	+	+	+	+	–	*Fiz1* + *Stat5a*
11	m	4	c	c	g	+	+	+	+	–	*Fiz1* + *Stat5a*
12	f	9	c	c	g	+	+	+	+	+	*Fiz1*
13	m	6	c	c	g	+	+	+	+	+	*Fiz1*
14	f	7	c	c	g	+	+	+	+	+	*Fiz1*
15	f	5	c	c	g	+	+	+	+	+	*Hipk2*
16	m	8	c	n.d.	g	+	+	+	+	–	*Hipk2*
17	f	4	c	n.d.	g	+	+	+	+	–	*Hipk2* + *Stat5a*
18	m	5	c	c	g	+	+	+	+	–	*Hipk2* + *Stat5a*
19	f	6	c	c	g	+	+	+	+	–	*Hipk2* + *Stat5a*
20	m	7	c	c	g	+	+	+	+	–	*Hipk2* + *Stat5a*
21	m	3	c	c	g	+	+	+	+	–	*Hipk2* + *Stat5a*

f, Female; m, male; c, completed (D_HJ_H); n.d., not detected (V_HD_H); g, germ line (IgL); V_HD_H, variable of IgH.

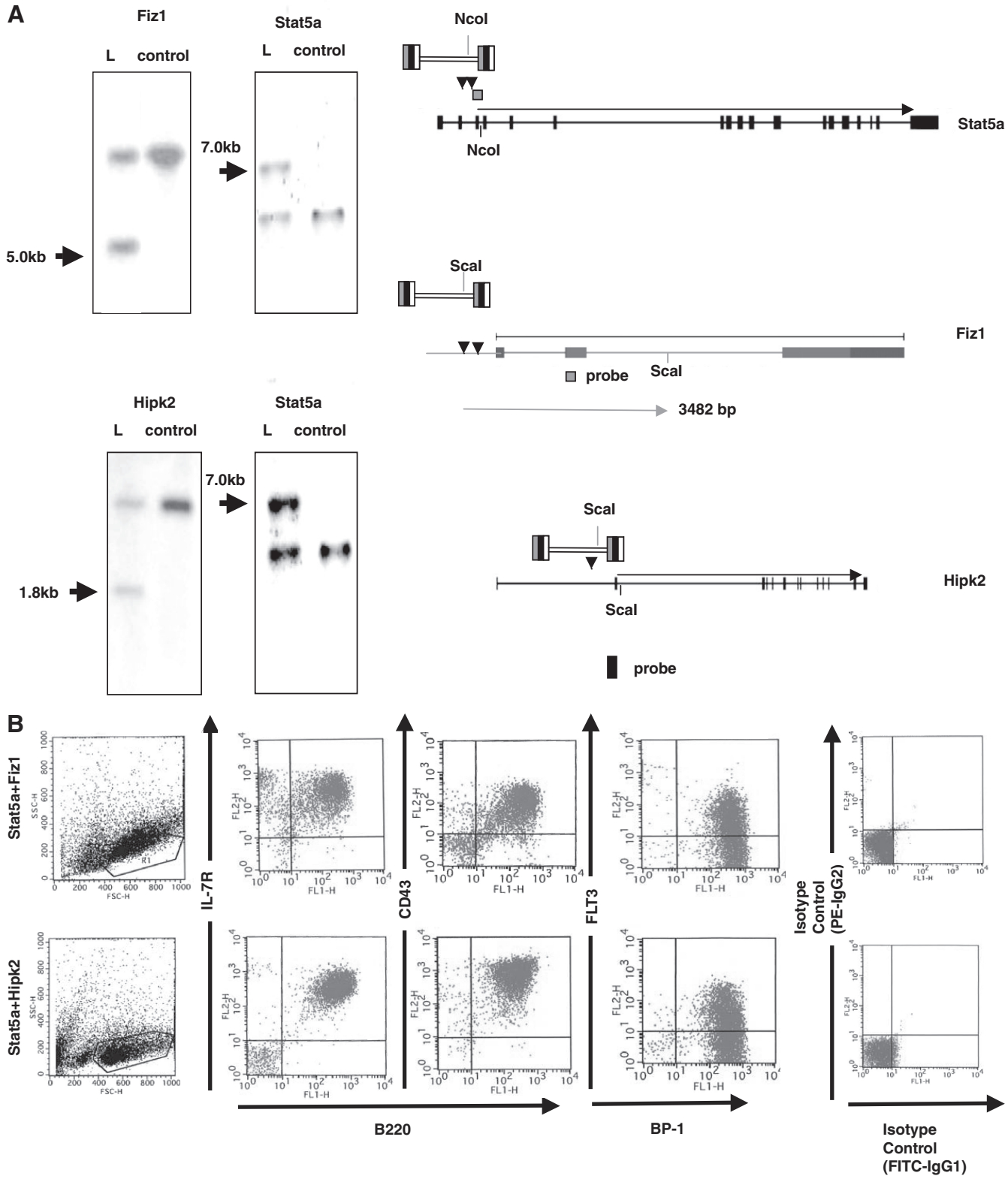

Figure 1. Clonality of B-LBL cells with dual integration. (A) Southern hybridization of LBL cells with *Stat5a* and *Fiz1* or *Stat5a* and *Hipk2* integration. The right scheme shows *Nco*I and *Sca*I sites within the MLV genome and immediately near the third exon of the *Stat5a* gene. The fragments of MLV-mouse genome DNA were visualized on the blot (indicated by arrows), and in these blots, the intensity of the fragments from provirus-integrated *Fiz1* and *Hipk2* was nearly identical to that of fragments from the nonintegrated allele. (B) Flow cytometry analysis of B-LBL cells with dual integration into *Stat5a* and *Fiz1* or *Stat5a* and *Hipk2*. FL2-H, Fluorescence 2-height. *(continued on next page)*

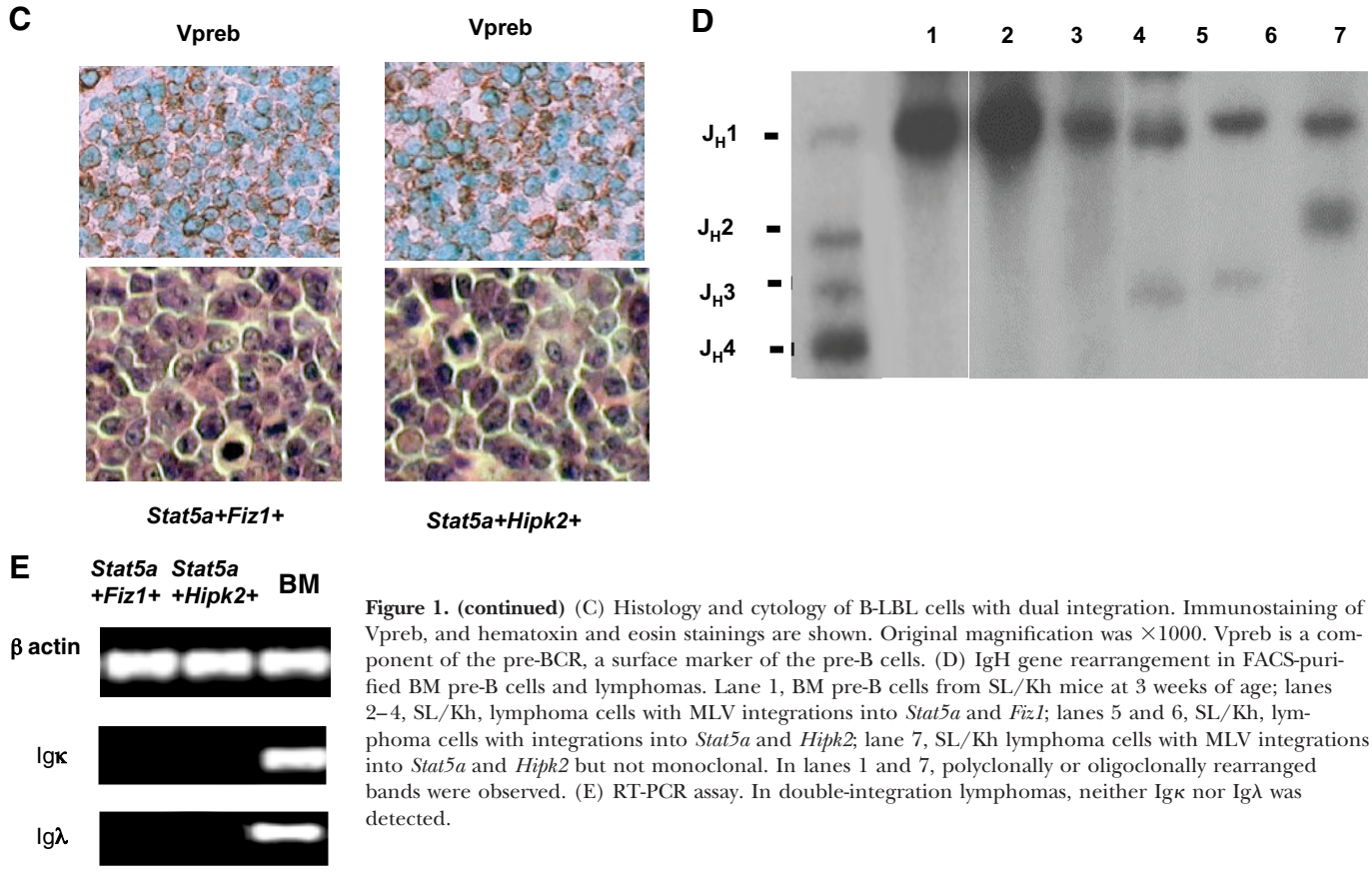

Figure 1. (continued) (C) Histology and cytology of B-LBL cells with dual integration. Immunostaining of Vpreb, and hematoxin and eosin stainings are shown. Original magnification was ×1000. Vpreb is a component of the pre-BCR, a surface marker of the pre-B cells. (D) IgH gene rearrangement in FACS-purified BM pre-B cells and lymphomas. Lane 1, BM pre-B cells from SL/Kh mice at 3 weeks of age; lanes 2–4, SL/Kh, lymphoma cells with MLV integrations into *Stat5a* and *Fiz1*; lanes 5 and 6, SL/Kh, lymphoma cells with integrations into *Stat5a* and *Hipk2*; lane 7, SL/Kh lymphoma cells with MLV integrations into *Stat5a* and *Hipk2* but not monoclonal. In lanes 1 and 7, polyclonally or oligoclonally rearranged bands were observed. (E) RT-PCR assay. In double-integration lymphomas, neither Igκ nor Igλ was detected.

sequences were: 5B4, 5′-GAGGGCTTGGACCTCTCGTCTCCTA-AAAAACCACG-3′, and 5F1, 5′-GTCTCTCCCAAACTCTCCCCCTCTC-CAACC-3′, in the first step and 5F2, 5′-CCTCCTCTGACGGAGATGGC-GACAGAGAAGAGG-3′, and 5B1, 5′-GAGGGCTTGGACCTCTCGTCTC-CTAAAAGAACCACG-3′, in the second step of cycles for nested PCR. Sequence analysis of the amplification products was performed with a Thermosequenase kit (GE Healthcare, Buckinghamshire, UK).

Southern blot of lymphoma DNA

The detailed protocol for detection of provirus integration into the host gene has been reported previously [9]. In brief, lymphoma DNA was digested with *Nco*I and *Sca*I (New England Biolabs, Ipswich, MA, USA). Probes for *Fiz1* and *Hipk2* were synthesized by PCR from the genomic DNA of SL/Kh mice and labeled with the Megaprime DNA-labeling system (GE Healthcare). The sequences were: 5′-CTTCCGTCATGGAAGACTCC-3′ and 5′-AGCAGGCCAGTG-GAGTCTCG-3′ for *Fiz 1* (exon 2) and 5′-TACCTTACGAGCAGACCATC-3′ and 5′-CAAAGACTAAGCACGTGTGG-3′ for *Hipk2* (exon 2). When the integration was completely clonal, the density ratio of the digested fragment, including MLV and the host gene DNA from the integrated allele, to the native *Stat5a* gene from the intact allele was nearly 1.0.

Colony growth assay

Prior to stimulation, lymphoma cells were grown for 6 h in RPMI. Lymphoma cells were washed once with PBS. After washing, they were suspended in 10 ml of a semi-solid medium.

Methocult (Stemcell Technologies, Vancouver, Canada), containing IL-7, was used as a basic semisolid medium in the colony assay. To detect early growth before 48 h, we defined the colony as a cluster of greater than five cells but after 48 h, only those of >20 cells. The cells were incubated in the medium with 10% FCS, 50 mM β-ME, and 100 mg/ml penicillin-streptomycin. Cultures were established in a humidified atmosphere at 37°C with 5% CO_2.

Ig gene recombination in the genome of lymphoma tissue

We confirmed the clonality by detection of a single IgH D_HJ_H recombination [8]. In brief, the lymphoma genomic DNA was subjected to PCR with primers designed to amplify four possible junctions between the D-Q52 and J_H regions. The primers for the first round were: 5′-CACAGAGAATTCTCCATAGTTGAT-AGCTCAG-3′ (D_HQ52-1; sense) and 5′-AGGCTCTGAGATCCCTAGACAG-3′ (J_H4-1; antisense). PCR conditions were as follows: denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2.5 min at 72°C (28 cycles). Two microliters of each reaction was subjected to a second round of PCR using a pair of internal primers: 5′-GCCTCAGAATTCCTGTGGTCTCT-GACTGGT-3′ (D_HQ52-2; sense) and 5′-GGGTCTAGACTCTCAGCCGGCTC-CCTCAGGG-3′ (J_H4-2; antisense). PCR was repeated as above. The PCR products were electrophoresed in an agarose gel, blotted onto a nylon membrane (GE Healthcare), and probed with a J_H4 probe for confirmation of the clonality of the cells by detection of IgH rearrangement [20]. RT-PCR assay for IgL will be described below. IgH J_H probe was the *Eco*RI-*Hin*dIII fragment encoding J_H4 [5, 24].

Flow cytometry

We used a protocol that has been described previously [8]. In brief, a single-cell suspension was prepared from femur BM plugs and axillary lymphoma tissue. The cell density was adjusted to 106 cells/ml, and the cells were analyzed in a FACScan apparatus (Becton Dickinson, Mountain View, CA, USA). The following antibodies were purchased commercially: FITC-labeled anti-BP-1 (clone 6C3), PE-labeled anti-B220 (clone RA3-6B2), anti-IL-7R (clone A7R34), and anti-CD24 (clone 30-F1;

eBioscience, San Diego, CA, USA) and anti-CD43 (clone S7), FITC-anti mouse IgG1, and PE-anti mouse IgG2 (BD Biosciences, San Diego, CA, USA). We stained BM early B cells and their fate in SL/Kh, NFS.SL/Kh-Bomb1, and NFS. It was contained with 1:10,000-diluted mAb for 15 min.

Western blots and immunohistochemistry

The extract of sorted BP-1$^+$ B220$^+$ pre-B cells from the SL/Kh BM were used as a control. Antibodies to Fiz1 (sc-101955), Flt3 (sc-1496-1), Hipk2 (sc-10294), and Vpreb (sc-33128) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Western blotting protocol has been described previously [5]. Phospho-specific antibodies against STAT5A (Tyr694) were obtained from Cell Signaling Technology (Beverly, MA, USA). Total cell lysate was precleared using protein L-Sepharose (Pierce Biotechnology, Rockford, IL, USA) or protein G-Sepharose (Sigma-Aldrich, St. Louis, MO, USA) at 4°C with 1 h of agitation. For each immunoprecipitation reaction, 400 microliters of the cleared lysate was incubated with 50 microliters MEM59 at 4°C overnight, and then 50 microliters protein L-Sepharose or protein G-Sepharose was added and incubated for an additional 1 h. Immunocomplexes were precipitated by centrifugation, washed three times with a radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet P-40, a protease inhibitor mixture (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany)], and finally, resuspended in the sample buffer and boiled for 5 min. The released proteins were examined by Western blotting. Antibody against mouse Ig polycolonal antibody was used for control of the blot of the immunoprecipitate (Dako, Gostrup, Denmark). The intensity of the blot was measured by using LumiVision (Aishin, Nagoya, Japan).

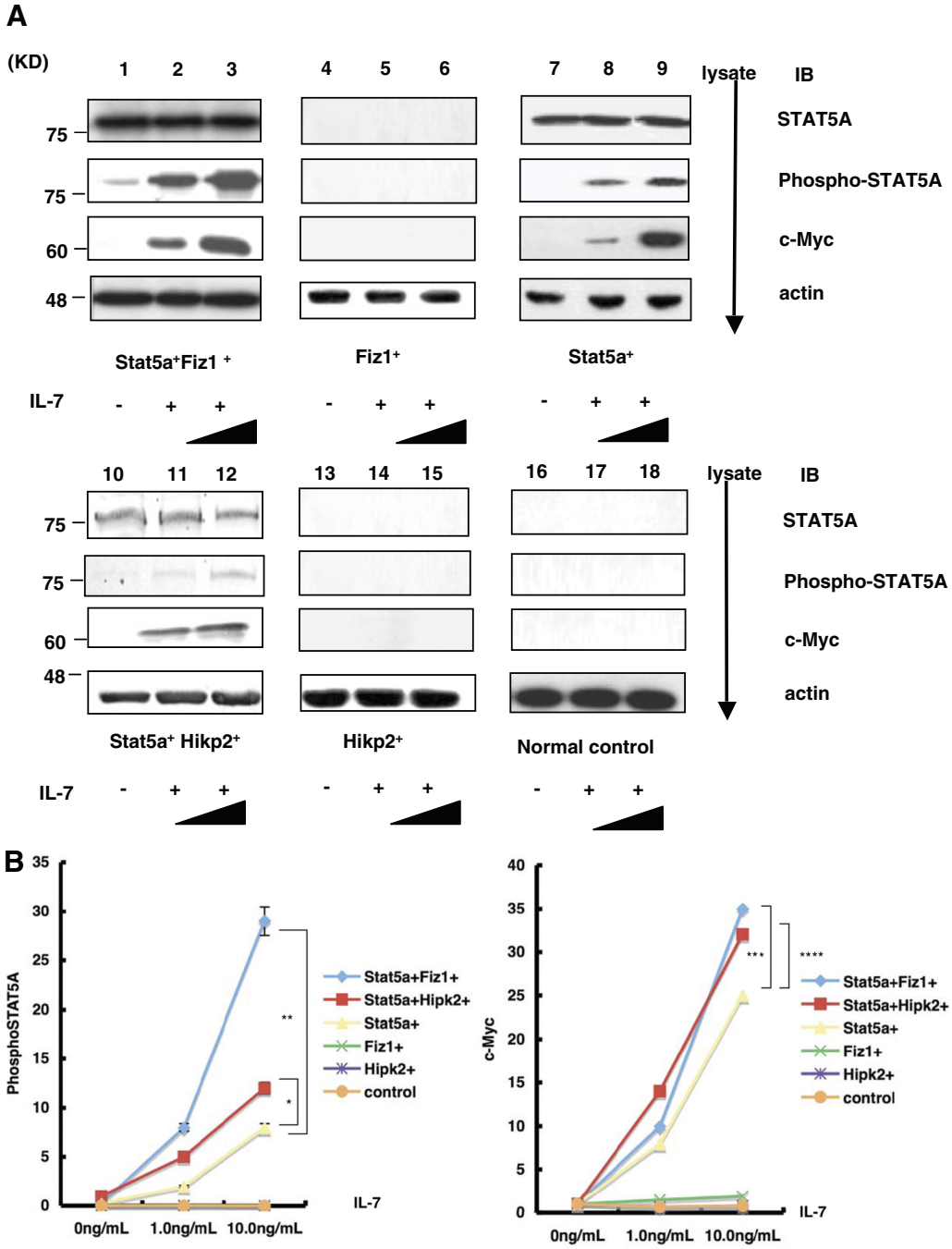

Figure 2. IL-7 induced phosphorylation of STAT5A and expression of c-Myc. (A) A representative blot of LBL cells with dual integration. Lanes 1, 4, 7, 10, 13, and 16, No IL-7 in the medium; lanes 2, 5, 8, 11, 14, and 17, 1.0 ng/mL IL-7; lanes 3, 6, 9, 12, 15, and 18, 10 ng/mL IL-7. IB, Immunoblot; Phospho, phosphorylated. (B) Intensity of phosphorylated STAT5A and c-Myc. The measurement was performed independently three times following IL-7R stimulation of the clone (the mean±SD; n=5).

RT-PCR

Total RNA was extracted from tissues using the ISOGEN reagent (Nippon Gene, Toyama, Japan). First-strand cDNA synthesis was performed using a Ready-To-Go T-primed first-strand kit (Amersham, Piscataway, NJ, USA). RT-PCR was performed as described previously [5]. The primers for β-actin were also prepared as reported previously. The primers for other genes were as follows: Igκ, 5′-GGCTGCAGSTCCAGTGGCAGTGGRTCWGGRA-3′ and 5′-CATTCCTGTTGAAGCTCTTGACAATGGGTG-3′; Igλ, 5′-GCCTTTCTACACTGCAGTGGGTATGCAACAAT-3′ and 5′-AGCCACTYACCTAGGACAGTSASYTTGGTTCC-3′ [25, 26]. A control β-actin probe was amplified by using RT-PCRmer™ β-actin (Gene Link, Hawthorne, NY, USA). For real-time RT-PCR, total RNA was reverse-transcribed using the Omniscript RT kit (Qiagen, Valencia, CA, USA). For relative quantification with RT-PCR, 20 ng each cDNA was analyzed using a FastStart DNA Master SYBR Green kit (Roche Diagnostics) with software, Version 3.5. For each primer pair, a standard curve was developed.

Statistical analysis

Analysis was done with the unpaired *t*-test. A P value <0.05 was considered to be statistically significant.

RESULTS

Identification of *Emv11* that is crucial for lymphomagenesis in SL/Kh mouse genome

In the current study, we found that studied SL/Kh mice share the *Emv11* that is mapped to 15.14cM on chromosome 7. Provirus *Emv11* was identified previously in strain AKR/J [27]. To study the susceptibility to pro-B cell lymphomagenesis by this proviral integration, we observed (SL/Kh×NFS/N)F1, backcrossed them to NFS/N, which did not possess pathogenic

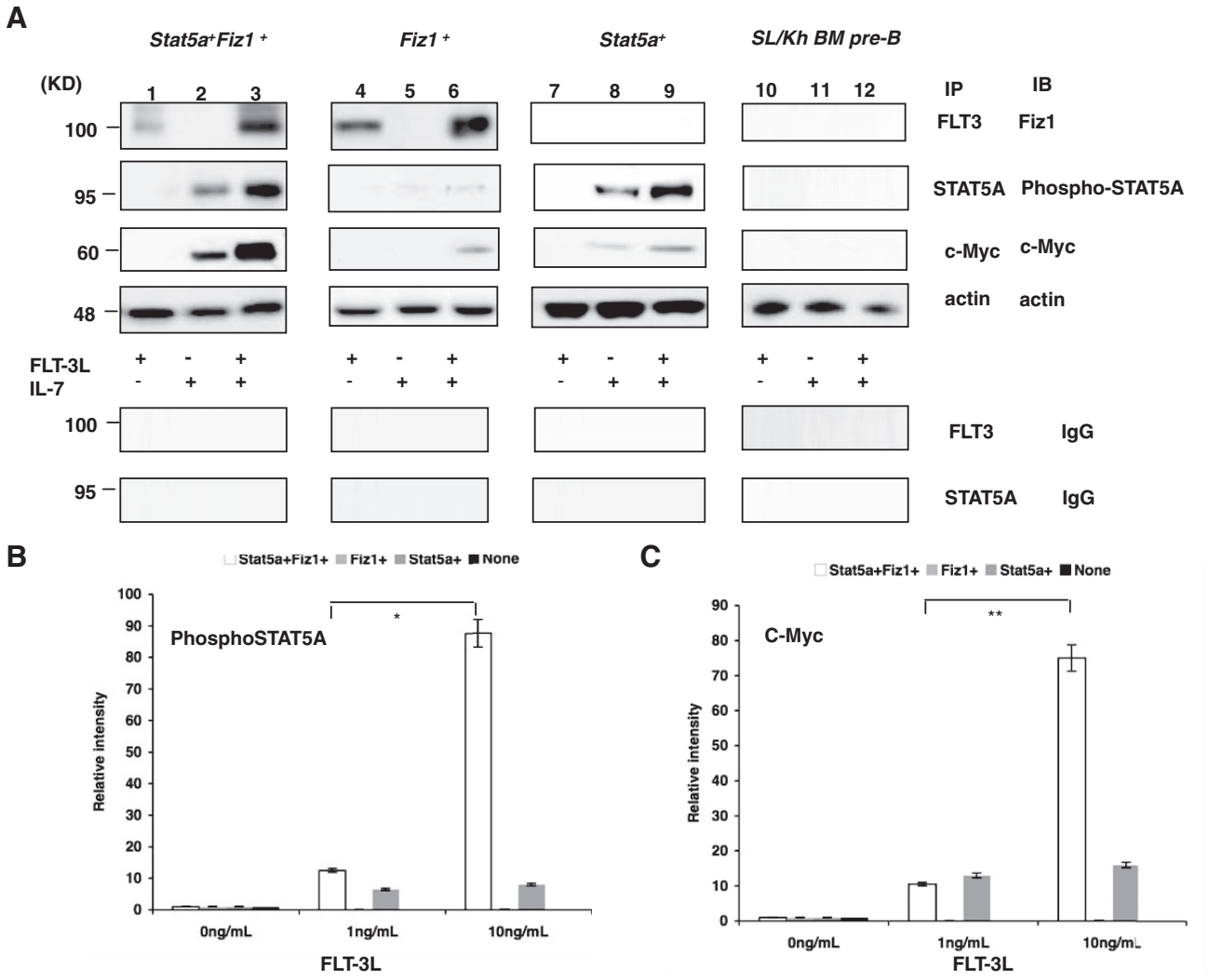

Figure 3. FLT3L induces phosphorylation of STAT5A and expression of c-Myc. (A) Blots of lymphoma cells with dual integration (*Stat5a*$^+$*Fiz1*$^+$) and single integration (*Stat5a*$^+$). Lanes 1, 4, 7, and 10, 1.0 ng/mL FLT3L and no IL-7; lanes 2, 5, 8, and 11, no FLT3L and 10 ng/mL IL-7; lanes 3, 6, 9, and 12, 1.0 ng/mL FLT3L and 10 ng/mL IL-7. IP, Immunoprecipitation. Normal control (no MLV integration) was BP-1$^+$B-220$^+$ BM pro-B cells from SL/Kh. (B) Relative intensity of phospho-STAT5A in immunoprecipitates in using an antibody recognizing STAT5A (the mean±SD; n=5). (C) Relative intensity of c-Myc in immunoprecipitates in using an antibody recognizing c-Myc (the mean±SD; n=5).

MLV, and analyzed spontaneous tumors. In F1, 32 of 62 (51%) individual mice developed pro-B cell lymphomas. In the backcross of the F1 to NFS/N, 38 of 102 (37.2%) mice developed lymphomas. Significantly, none of the backcross mice without the *Emv11* developed them. This evident cosegregation of lymphoma development with the *Emv11* plays a pivotal role in lymphomagenesis.

B-LBL clones with dual MLV integration

From primary B-LBL lymphomas of SL/Kh mice, we isolated several B-LBL clones with the *Emv11* integration(s) (**Table 1**). Southern blot analysis of *Nco*I- or *Sca*I-digested DNA with probes hybridizing to *Stat5a*, *Fiz1*, or *Hipk2* (**Fig. 1A**) revealed clonal rearrangements of these genes induced by the *Emv11* integration. FACScan analysis revealed that these clones were composed homogenously of BP-1^+ $CD43^+$ sIgM-$B220^+$ cells, indicating that the lymphoma cells were pro- to early pre-B cells (Fig. 1B). Histology of the lymphomas showed positive for Vpreb, one of the surrogate light chain components, and lymphoblastic morphology (Fig. 1C). PCR-Southern blot analysis of IgH rearrangement demonstrated monoclonal DQ52-J_H1 or -J_H2 rearrangement, indicating that the lymphoma cells used in this study were monoclonal (Fig. 1D, lanes 2–6). In addition, neither Igκ nor Igλ was detected in the lymphomas by the RT-PCR assay (Fig. 1E). The lymphoma clones used in this study are listed in Table 1.

Stimulation with IL-7R of B-LBL clones with double integration

Using these B-LBL clones, we studied the effect of IL-7R stimulation on STAT5A phosphorylation and induction of c-Myc, one of the downstream targets of STAT5A. Sorted BM pro-B cells were used as the control. It was found that the *Stat5a* target genes were commonly up-regulated in concomitance with the *Emv11* integration (**Fig. 2A**). After incubation in IL-7-free medium for 6 h, the B-LBL cells were added with 1.0 or 10.0 ng/ml IL-7 for 1 h. In B-LBL cells, with clonal *Stat5a* integration, the phosphorylation of STAT5A and the expression of c-Myc increased in a dose-dependent manner (Fig. 2A, lanes 2, 3, 8, 9, 11, and 12). In the absence of IL-7, phosphorylation of STAT5A and expression of c-Myc were nearly undetectable. Therefore, IL-7R stimulation was followed by STAT5a phosphorylation in B-LBL cells, with constitutive expression of STAT5A driven by the *Emv11* integration. Individual visualized blots of phosphorylated STAT5A and c-Myc were measured quantitatively (Fig. 2B). The results revealed that increases in STAT5A phosphorylation and c-Myc expression in the clones with the integration into the *Stat5a* and *Fiz1* or *Hipk2* were more than in the clones with the single integration into the *Stat5a* (*, P=0.003; **, P=0.004; ***, P=0.003; ****, P=0.012; Fig. 2B).

STAT5A phosphorylation was induced by stimulation of FLT3 with FLT3L in *Fiz1*$^+$ *Stat5a*$^+$ B-LBL cells

Next, we examined the effect of FLT3 stimulation with FLT3L on STAT5A phosphorylation. After 6 h in IL-7-free culture medium, B-LBL cells with the *Stat5a* and *Fiz1* integrations (*Stat5a*$^+$*Fiz1*$^+$) or with only *Stat5a* integration (*Stat5a*$^+$) were stimulated with 1.0 ng/mL FLT3L (**Fig. 3A**). IL-7 (1.0 ng/mL) was supplied again or not. The result revealed that in the *Stat5a*$^+$*Fiz1* and *Fiz1*$^+$ B-LBL cells, *Fiz1* was found to be associated with FLT3 when stimulated by FLT3L (Fig. 3A, lanes 1, 3, 4, and 6), and more phosphorylated STAT5A was observed following the supply of FLT3L (Fig. 3A, lanes 2 vs. 3 and 8 vs. 9). Moreover, we changed concentration of FLT3L from 1.0 ng/mL to 10 ng/mL and investigated the effects on phosphorylation of the STAT5A and c-Myc expression in the presence of 10 ng/mL IL-7. The result revealed that phosphorylation of STAT5A was increased in the *Stat5a*$^+$*Fiz1*$^+$ B-LBL cells following stimulation with 10 ng/mL FLT3L (Fig. 3B, lanes 2 vs. 3; *, P=0.008) but not in the *Stat5a*$^+$ clone (Fig. 3B, P=0.07). c-Myc expression was also up-regulated in the *Stat5a*$^+$*Fiz1*$^+$ clone following stimulation with 10 ng/mL FLT3L (Fig. 3B, *, P=0.012), but this up-regulation was not seen in the *Stat5a*$^+$ clone (Fig. 3B, P=0.051).

Colony growth of B-LBL cells on a semisolid medium

Next, we examined the effect of increased expression of these genes in the *Emv11* integration sites by culturing the cells on Methocult medium, semisolid methylcellulose medium containing 1.0 ng/mL IL-7, which was optimized for the culture of pre-B cells. We added 10 ng/mL FLT3L to the medium and observed the growth of *Stat5a*$^+$*Fiz1*$^+$ and *Stat5a*$^+$ clones, which began to grow after 48 h, forming col-

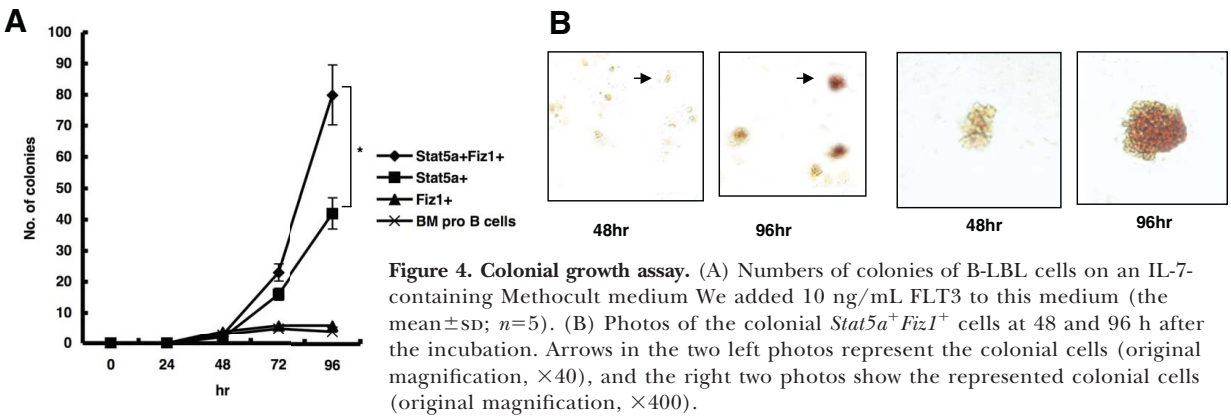

Figure 4. Colonial growth assay. (A) Numbers of colonies of B-LBL cells on an IL-7-containing Methocult medium We added 10 ng/mL FLT3 to this medium (the mean±SD; n=5). (B) Photos of the colonial *Stat5a*$^+$*Fiz1*$^+$ cells at 48 and 96 h after the incubation. Arrows in the two left photos represent the colonial cells (original magnification, ×40), and the right two photos show the represented colonial cells (original magnification, ×400).

onies in the semisolid medium. Colonies were grown on an average of six dishes that each contained 1.3×10^6 cells. Following the initial incubation, *Stat5a*$^+$*Fiz1*$^+$ clones grew faster than *Stat5a*$^+$ clones (**Fig. 4, A** and **B**). This result indicates that *Fiz1* up-regulation in expression promotes the growth of B-LBL cells with a supplement of FLT3L and IL-7 (*, P=0.003; Fig. 4A).

STAT5A phosphorylation was induced by stimulation with anti-CD43 in *Stat5a*$^+$*Hipk2*$^+$ B-LBL cells

Next, we examined the effect of CD43 stimulation by cross-linking in B-LBLs. After 6 h in IL-7-free medium, CD43 was cross-linked using an anti-CD43 antibody. The result revealed that *Hipk2* was found to be associated with CD43 following the cross-linking (**Fig. 5A**, lanes 1, 2, 7, and 8) in the *Stat5a*$^+$*Hipk2*$^+$ and *Hipk2*$^+$ clones. In the *Stat5a*$^+$*Hipk2*$^+$ clone, phosphorylated STAT5A was increased significantly by CD43 cross-linking in the *Stat5a*$^+$*Hipk2*$^+$ B-LBL cells (Fig. 5A, lanes 2 vs. 3; *, P=0.008; Fig. 5B) but not significantly in the *Stat5a* single integration clone (Fig. 5A, lanes 5 vs. 6; P=0.07; Fig. 5B). In close correlation with this, the significant increase in c-Myc expression in the *Stat5a*$^+$*Hipk2*$^+$ clones was observed following the cross-link (Fig. 5A; lanes 2 vs. 3; **, P=0.006; Fig. 5C), but it was not observed in the *Stat5a*$^+$ clones (Fig. 5A, lanes 5 vs. 6; P=0.09; Fig. 5C).

DISCUSSION

Constitutive activation of *Stat5a* by retrovirus integration contributes to lymphomagenesis via activation of the IL-7R signaling pathway [9]. In this study, we identified *Fiz1* and *Hipk2* as novel targets for integration. FLT3 is one of the markers and

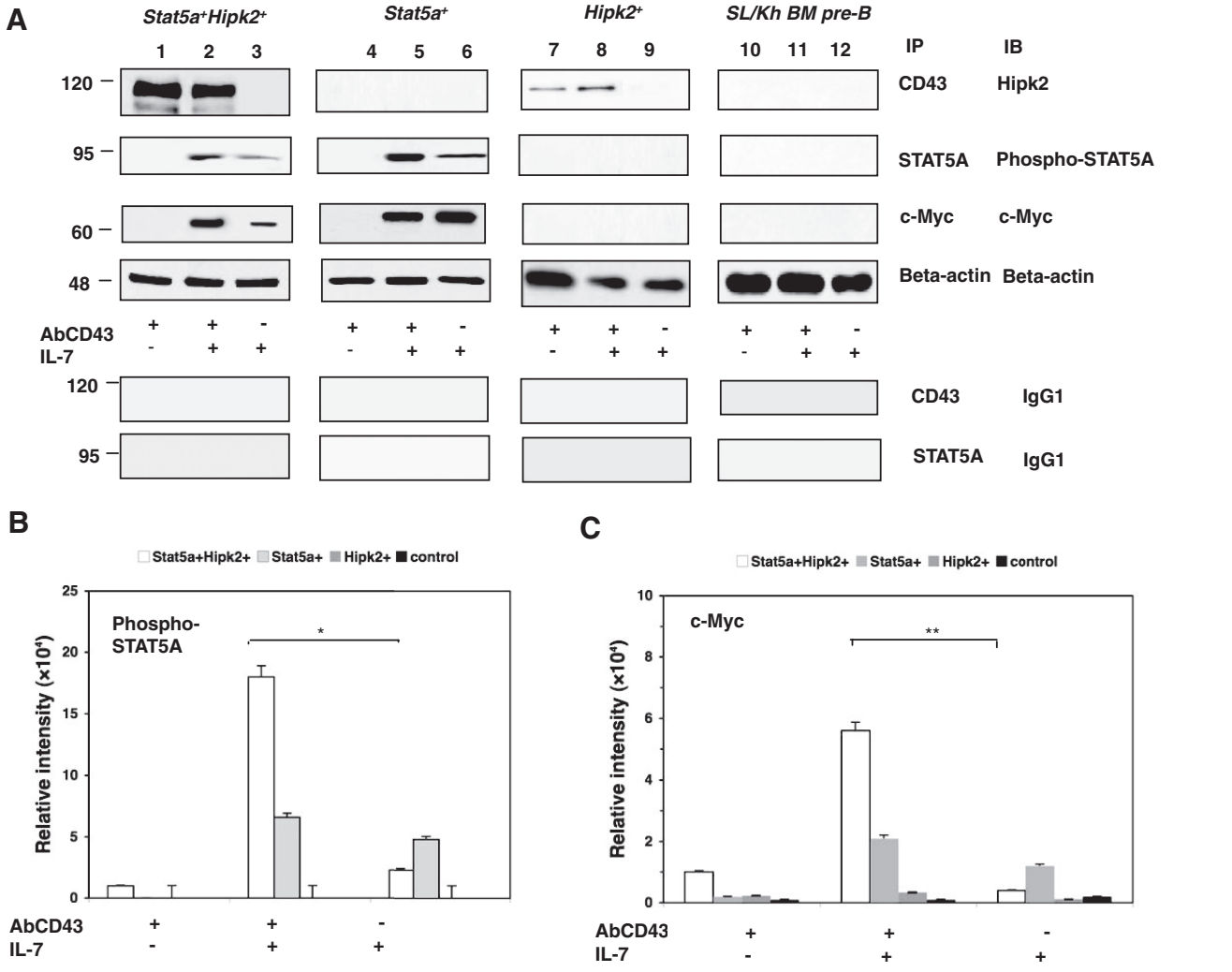

Figure 5. Cross-linking of CD43 induces phosphorylation of STAT5A and expression of c-Myc. (A) Lanes 1, 4, 7, and 10, No IL-7 with cross-linking; lanes 2, 5, 8, and 11, 1.0 ng/mL IL-7 with cross-linking; lanes 3, 6, 9, and 12, 1.0 ng/mL IL-7 without cross-linking. Anti-CD43 (AbCD43) was used at 20 ng/mL (lanes 1, 2, 4, 5, 7, 8, 10, and 11). (B) Relative intensity of phospho-STAT5A in blots of immunoprecipitates using an antibody recognizing phosphorylated STAT5A (the mean±SD; n=5). (C) Relative intensity of c-Myc in immunoblots of immunoprecipitates using an antibody recognizing c-Myc (the mean±SD; n=5).

functional molecules expressed in early B lymphoid progenitor cells [28–32]. As these pathways act selectively in the pro-B cell stage of B cell development, constitutive activation of these molecules may stabilize the immature host cellular phenotype and prevent further differentiation. Choudhary et al. [30] reported that STAT5A is involved in the FLT3 pathway. Mutations in the receptor tyrosine kinase FLT3 are a commonly occurring genetic lesion in acute myeloid leukemia [31]. ITD mutations clustered in the juxta-membrane domain are the most frequent and best-characterized type of mutation found in FLT3. FLT3-ITD-induced STAT5 activation is independent of Src, JAKs, and FLT3. The juxta-membrane domain of FLT3 is critical for ligand-dependent activation of FLT3 and for the transforming potential of oncogenic FLT3 mutants. In addition, phosphorylated STAT5 in the nucleus was detectable in 20% of B-lymphoblastic leukemia cells in the reported cases, and FLT3 is involved in B-acute lymphocytic leukemia with karyotype t (13; 13)(q12; q22) [32]. Thus, the FLT3 pathway can contribute to lymphomagenesis via STAT5A activation. We showed that the activation of the FLT3 pathway by increasing *Fiz1* expression may also contribute to STAT5 activation and B-LBL pathogenesis. As aforementioned, this pathway is enhanced by the supply of IL-7, suggesting the interaction between the IL-7R pathway and the FLT3 pathway.

In addition, we demonstrated a possibility that the CD43 pathway involved Hipk2 and STAT5A. In T cell development, TCR-dependent cell response is modulated by CD43 engagement [33]. Lee et al. [34] demonstrated that *Hipk2* promotes Wnt/Wg signaling by stabilizing β-catenin/Armadillo and stimulating target gene expression. This group also showed that *Hipk2* interacts with lymphoid-enhancing factor 1, which acts as a transcriptional factor, promoting c-Myc and cyclin D1 expression. In the erythroleukemic cell line TF-1, STAT5A is not associated with CD43-mediated cell proliferation [35]. Recently, it was found that CD43 functions as an E-selectin counter-receptor in human pre-B cell leukemia NALL-1 cell line [36]. Thus, CD43 signaling pathway is one of the candidate pathways that actively operates in the B-LBLs. In our study, CD43 cross-linking resulted in an increase in STAT5A phosphorylation and c-Myc expression in *Stat5a*$^{+}$*Hipk2*$^{+}$ B-LBL cells when IL-7 was supplied. These data suggest that CD43 enhances the IL-7R signal pathway, which normally enhances c-Myc expression via STAT5A phosphorylation. Taken together, we propose a scheme of interactions among the IL-7, CD43, and FLT3 signaling pathways (**Fig. 6**). Thus, we hypothesize that these three pathways form an interacting network and affect B-LBL development. SL/Kh mice serve as one of the appropriate models for studying the signaling pathways leading to lymphomagenesis, using MLV integration as a tool. The data we report here will provide new insights into the aberrant signaling pathways leading to lymphomagenesis.

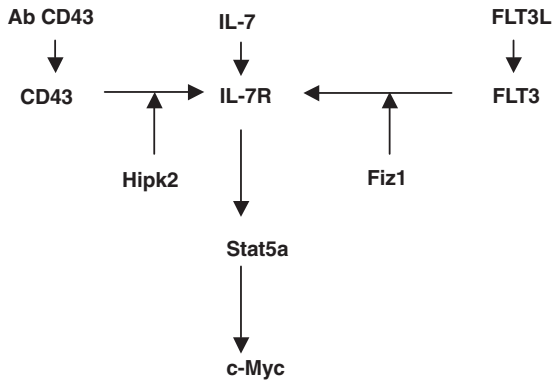

Figure 6. Plausible signaling pathways in B-LBL cells.

AUTHORSHIP

Tatsuaki Tsuruyama planned all of the experiments. Tatsuaki Tsuruyama, Yukiko Imai, Haruya Takeuchi, Takuya Hiratsuka, Yasuhiro Maruyama, Kazuya Kanaya, Richard Kaszynski, and Guang Jin conducted all of the experiments. Tatsuaki Tsuruyama, Tomoko Okuno, Munetaka Ozeki, Takuro Nakamura, Tetsuya Takakuwa, Toshiaki Manabe, and Hiroshi Hiai critically advised on the signaling pathway and morphology of the lymphoma tissue. Tatsuaki Tsuruyama and Keiji Tamaki analyzed the data. Tatsuaki Tsuruyama and Richard Kaszynski wrote the paper.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for cancer research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and a grant for strategic research on cancer from the Ministry of Health, Labor, and Welfare, Japan. We are grateful for the generous financial support from the Kyoto Shimizu Immunology Foundation.

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KEY WORDS:
murine leukemia · Stat5a · B-LBL · Flt3 · CD43