PIM1 gene cooperates with human *BCL6* gene to promote the development of lymphomas

Beverly W. Baron[a,1], John Anastasi[a], Elizabeth M. Hyjek[a], Juraj Bies[b], Poluru L. Reddy[a], Jingfang Dong[c], Loren Joseph[a], Michael J. Thirman[c], Kristen Wroblewski[d], Linda Wolff[b], and Joseph M. Baron[c]

[a]Department of Pathology, University of Chicago, Chicago, IL 60637; [b]Leukemogenesis Section, Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and Departments of [c]Medicine and [d]Health Studies, University of Chicago, Chicago, IL 60637

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Diffuse large B-cell lymphomas in humans are associated with chromosomal rearrangements (~40%) and/or mutations disrupting autoregulation (~16%) involving the *BCL6* gene. Studies of lymphoma development in humans and mouse models have indicated that lymphomagenesis evolves through the accumulation of multiple genetic alterations. Based on our prior studies, which indicated that carcinogen-induced DNA mutations enhance the incidence of lymphomas in our mouse model expressing a human *BCL6* transgene, we hypothesized that mutated genes are likely to play an important cooperative role in *BCL6*-associated lymphoma development. We used retroviral insertional mutagenesis in an effort to identify which genes cooperate with *BCL6* in lymphomagenesis in our *BCL6* transgenic mice. We identified *PIM1* as the most frequently recurring cooperating gene in our murine BCL6-associated lymphomas (T- and B-cell types), and we observed elevated levels of PIM1 mRNA and protein expression in these neoplasms. Further, immunohistochemical staining, which was performed in 20 randomly selected BCL6-positive human B- and T-cell lymphomas, revealed concurrent expression of BCL6 and PIM1 in these neoplasms. As *PIM1* encodes a serine/threonine kinase, PIM1 kinase inhibition may be a promising therapy for BCL6/PIM1-positive human lymphomas.

Diffuse large B-cell lymphomas in humans are associated with a high mortality rate, and the majority of these contain mutations or chromosomal rearrangements that involve the *BCL6* gene (1). Prior studies of deregulated BCL6 expression (2, 3) in mouse models have suggested that lymphoma development in these animals is hastened through the accumulation of multiple oncogenic hits. In an effort to define more targeted therapies for BCL6-associated lymphomas, we sought to identify which mutated genes cooperate with *BCL6* in lymphomagenesis. Here, through the use of retroviral insertional mutagenesis in our mouse model of the human *BCL6* transgene (2), we detected multiple retroviral insertion sites in the lymphomas (T- and B-cell types) in our animals, a phenomenon that resembles the mutational load noted in human B-cell lymphomas (4). We identified the gene called proviral integration site for Moloney murine leukemia virus 1 (*PIM1*) as the most frequently recurring cooperating gene in our murine BCL6-associated lymphomas. Immunohistochemical staining, which was performed in 20 randomly selected BCL6-positive human B- and T-cell lymphomas, revealed concurrent expression of BCL6 and PIM1. Because *PIM1* encodes a serine/threonine kinase, our findings suggest that PIM1 specific kinase inhibitors may be promising therapies for BCL6/PIM1-associated lymphomas.

Results

MOL4070LTR Infection Accelerates Lymphomagenesis in *BCL6* Transgenic Mice. Our *BCL6* transgenic mice are doubly transgenic animals, containing two transgenes: (*i*) human *BCL6* cDNA under control of the tetracycline-responsive minimal promoter (tet-*o*-BCL6) and (*ii*) the tetracycline-transactivating protein (tTA) under control of the Ig heavy-chain enhancer and the SRα promoter (EμSR-tTa) (2, 5). As previously described (2), we generated two transgenic mouse lines, one containing four copies of the human *BCL6* transgene, the other 20 copies. We used the retrovirus MOL4070LTR (6) for retroviral insertional mutagenesis in our *BCL6* transgenic and control animals (nontransgenic mice of the same background). All animals were followed until natural death or necessity for euthanasia. It was possible to analyze the tissues from 92 of 99 transgenic mice injected with retrovirus and from 32 of 33 nontransgenic injected controls. Two animals in the transgenic group died on weaning, and tissues from all other excluded animals were too autolyzed to be analyzed.

Of 53 animals in the four-copy transgenic line, 24 (45.3%) developed lymphomas (18 T-cell, six B-cell; representative histology and flow data shown in Fig. 1). Of 39 mice in the 20-copy line, 10 (25.6%) developed lymphomas (three T-cell, seven B-cell). Lymphomas (BCL6-negative) also were anticipated in the control group, as the MOL4070LTR retrovirus is known to cause lymphomas and leukemias in mice (23% of FVB mice develop T-cell lymphomas by 270 d and approximately 50% of mice develop myeloid leukemias) (6). In our study, of 32 injected control (i.e., nontransgenic) animals, 11 (34.4%) developed lymphomas (five T-cell, six B-cell), with an unexpectedly high incidence of B-cell tumors compared with what is reported in the literature (6). Of 124 evaluable animals, 20 did not develop hematopoietic neoplasms (lymphoid or other, e.g., myeloid leukemias). Of these, three were in the control (i.e., nontransgenic) group and lived an average of 155 d (range 53–246 d), five were in the four-copy line and survived an average of 312 d (range, 254–392 d), and 12 were in the 20-copy line and lived an average of 249 d (range, 180–338 d).

We hypothesized that genes cooperating with *BCL6* in lymphomagenesis would be likely to promote faster lymphoma development in transgenic animals compared with control animals. Thus, for statistical purposes, we primarily focused on the first 180 d. Our four-copy *BCL6* transgenic line comprised 58% (53 of 92) of the analyzable mice in the study group, and the majority of the early lymphomas developed in this line (14 T-cell, two B-cell). Fig. 2 shows that the study (i.e., transgenic) group in this four-copy line had worse survival (hazard ratio, 3.8; 95% confidence interval, 1.1–13.0; $P = 0.03$, Cox regression) and, specifically, had a significantly worse 180-d lymphoma mortality rate (30.2%) than the control (i.e., nontransgenic) group (9.4%; $P = 0.03$, Fisher exact test).

The lymphomas usually were aggressive neoplasms. Most of the B-cell lymphomas were mature B-cell (7), $CD19^+$, $B220^+$, with associated splenomegaly (Fig. 1*A*) and often lymphadenopathy. The majority of T-cell tumors (Fig. 1*B*) could be

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[1]To whom correspondence should be addressed. E-mail: beverly.baron@uchospitals.edu.

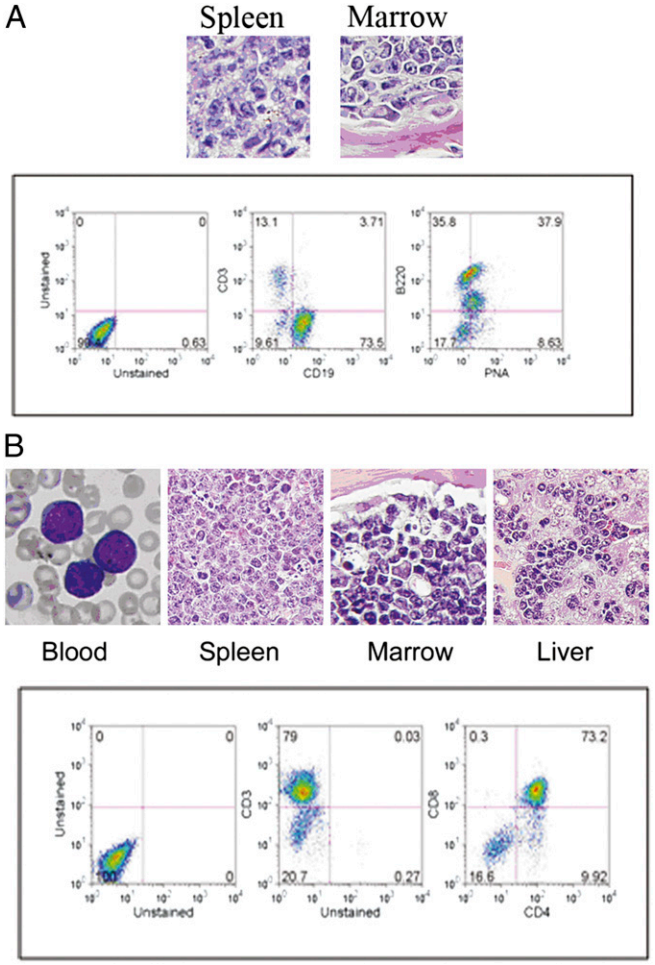

Fig. 1. Representative lymphomas in *BCL6* transgenic mice injected with MOL4070LTR retrovirus. (*A*) Mature B-cell aggressive lymphoma. *Upper*: Representative histologic sections. The spleen and bone marrow are infiltrated with large malignant lymphoid cells. *Lower*: The tumor is a B-cell lymphoma that is $CD19^+$ (*Center*) and $B220^+$ (*Right*); the peanut agglutinin positivity (*Right*) indicates a possible germinal center origin, which is a feature often associated with BCL6-positive B-cell tumors in humans. (*B*) Precursor T-cell lymphoblastic leukemia/lymphoma. *Upper*: Representative histologic sections. The peripheral blood smear shows many lymphoblasts. The architecture of the spleen is effaced by lymphoma, and the marrow is packed with lymphoma cells. The liver sinusoids are engorged with large numbers of these cells. *Lower*: Representative flow cytometry of the splenic lymphoma cells shows that most are CD3 positive (*Center*) and immature ($CD4^+/CD8^+$; *Right*).

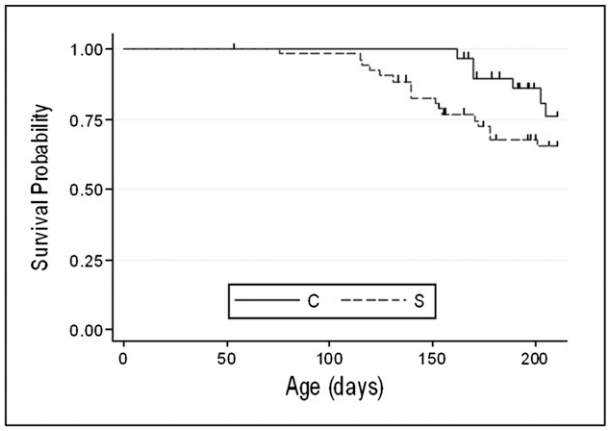

Fig. 2. Lymphoma-related survival, *BCL6* transgenic (i.e., four-copy) line. Cox regression and Fisher exact test show a significantly shorter latency to develop lymphoma for study (S; i.e., transgenic) mice (dashed line) compared with controls (C; i.e., nontransgenic) mice (solid line) at no more than 180 d; the 180-d lymphoma mortality rate was 9.4% in the control group and 30.2% in the study group ($P = 0.03$).

classified as precursor T-cell lymphoblastic lymphoma/leukemia (7), often manifesting with very large thymic masses as well as infiltration in multiple organs.

The 34 lymphomas from transgenic mice (24 from the four-copy line and 10 from the 20-copy line) contained three to 29 insertion sites (average of 11); the 11 neoplasms from the controls contained between eight and 20 insertion sites (average of 12.7). Sequencing of 377 insertion sites identified in the transgenic animals and 140 in the controls led to the identification of 15 insertion sites that were present in at least two transgenic animals but not in any controls. A number of these had been previously reported in the Retrovirus Tagged Cancer Gene Database (July 2007). We elected to focus attention on lymphomas containing retroviral integration sites in or near the *PIM1* gene, which represented the most commonly recurring integration site in our transgenic animals. Specifically, the MOL4070LTR retrovirus had inserted in or near the *PIM1* gene in seven of 34 lymphomas (21%) in transgenic mice, but not in any of the 11 lymphomas that developed in the 32 control (i.e., nontransgenic) animals. Assuming that the retrovirus has an equal likelihood of inserting anywhere within the mouse genome (~25,000 genes), the finding that the lymphomas from seven transgenic mice (but no control animals) contained insertion sites within or near the *PIM1* gene is highly statistically significant ($P < 0.0001$, binomial probability test).

The lymphomas containing insertion sites in or near the *PIM1* gene were identified in four female and three male mice, representing both of our *BCL6* transgenic lines, ranging in age from 4 to 7.6 mo (average, 5.5 mo) at death. The viral integration site was 5′ of the *PIM1* gene in two lymphomas, 3′ in three lymphomas, and within the fifth intron of the *PIM1* gene in two additional cases (Table 1). One was a B-cell lymphoma ($CD19^+$, $B220^+$) with spread to multiple organs, and the others were T-cell tumors (precursor T-cell lymphoblastic lymphoma/leukemia per Bethesda classification system for murine lymphoid neoplasms) (7). They were aggressive malignancies, usually presenting as large thymic masses with spread in multiple organs.

Enhanced PIM1 RNA Expression in Lymphomas Containing Retroviral Insertions in or Near *PIM1*. Total RNA extracted from mouse lymphomas was reverse-transcribed and subjected to real-time quantitative RT-PCR. Each PCR yielded a single product, without nonspecific amplification. Overall, the six T-cell tumors in transgenic mice contained 20.7-fold more PIM1 RNA (mean ± SEM, 0.083 ± 0.044) than the T-cell tumors from three randomly selected controls (mean ± SEM, 0.004 ± 0.0007; $P = 0.04$; Fig. 3). The two T-cell lymphomas containing retroviral inserts

Table 1. Relative PIM1 RNA in lymphomas from transgenic mice

Type	Location of insert	Expression level vs. control
T	Intron 5	63.3-fold
T	Intron 5	46.8
T	92.4 kb 5′ to *PIM1*	3.8
T	92.4 kb 5′ to *PIM1*	3
T	4.4 kb 3′ to *PIM1*	5.8
B	5.1 kb 3′ to *PIM1*	2.2
T	5.2 kb 3′ to *PIM1*	1.3

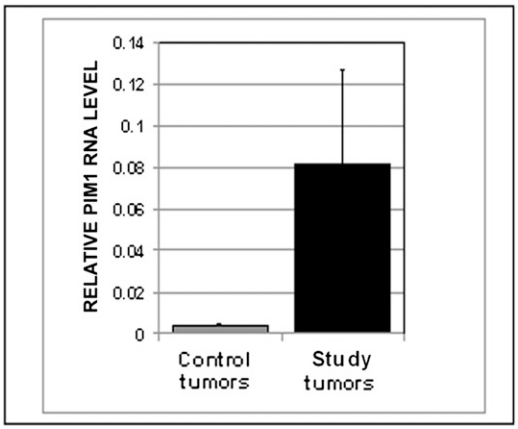

Fig. 3. PIM1 RNA levels in the six T-cell lymphomas from virus-injected *BCL6* transgenic mice (study tumors, black bar) are significantly higher (20.7-fold; $P = 0.04$) than in T-cell lymphomas from virus- injected nontransgenic controls (control tumors, gray bar).

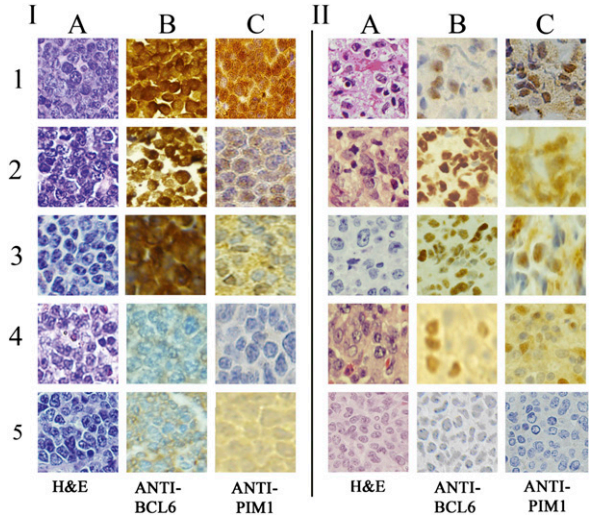

Fig. 4. Concurrent expression of PIM1 and BCL6 in lymphomas, mouse and human, B- and T-cell, by immunohistochemistry. *I*, Representative murine lymphomas. In each case, histologic analysis shows malignant tumor cells (H&E stain in column A). *1–3*, BCL6-positive lymphomas (brown nuclei, *B*) from *BCL6* transgenic mice containing viral insertions within or near the *PIM1* gene (first two rows are T-cell tumors as demonstrated by flow cytometry; Fig. 1*B*), and row *3* is a B-cell tumor (Fig. 1*A*). PIM1 levels are positive in all these lymphomas (brown nuclei, *C*), and the level of staining correlates roughly with the intensity of BCL6 expression. Rows *4* and *5* are lymphomas from control mice (row *4* is T-cell, row *5* is B-cell); BCL6 (*B*) and PIM1 staining (*C*) are negative in both controls. *II*, Representative randomly selected human lymphomas. Histology analysis indicates malignant neoplasm in each case (*A*, H&E stain). *1–3*, Randomly chosen BCL6-positive B-cell tumors previously studied for diagnostic purposes. *4*, Randomly selected BCL6-positive lymphoma previously classified diagnostically as T-cell. BCL6 and PIM1 staining are positive (brown nuclei) in each case. *5*, Previously studied T-cell lymphoma that does not stain for BCL6 or PIM1.

within the *PIM1* gene had the highest PIM1 RNA levels (63.3- and 46.8-fold more, respectively, than the mean level found in the T-cell tumors from the nontransgenic retrovirus-injected mice; Table 1). These two insertion sites were within the fifth intron of the *PIM1* gene (mouse chromosome 17; nucleotide positions 29,631,139 and 29,631,068, respectively). Table 1 shows that three lymphomas (one B-cell, two T-cell) contained inserts 3′ of *PIM1*, a region previously reported to be frequently affected by proviral integrations in murine T-cell lymphomas and associated with enhanced levels of PIM1 RNA (8). The only lymphoma that had a less than twofold elevation in PIM1 RNA level compared with the controls was a T-cell tumor with a 3′ viral insertion site.

PIM1 Protein Levels in Lymphomas from *BCL6* Transgenic Mice and Human BCL6-Positive Lymphomas. PIM1 protein levels in murine B- and T-cell tumors detected by immunohistochemical staining correlated generally with PIM1 RNA levels from murine lymphomas quantified by real-time PCR (Fig. 4). As B5 fixation interfered with anti-PIM1 staining, only formalin-fixed tissues were used. PIM1 protein was detectable in all of 20 randomly selected BCL6-positive human tumors (10 B-cell, 10 T-cell). PIM1 expression was variably cytoplasmic and/or nuclear. Nuclear expression, which is believed to correlate with PIM1 biologic effects (9), was detected at least focally in all the murine and human BCL6-positive lymphomas.

Discussion

Through the use of retroviral insertional mutagenesis in our unique human *BCL6* transgenic mouse model, we identified *PIM1* as a gene that cooperates with *BCL6* to promote the development of lymphomas, and we showed the concurrent expression of the proteins encoded by these two genes in 20 randomly selected BCL6-positive human B- and T-cell lymphomas. It has long been known that *BCL6* and *PIM1* are both protooncogenes that play roles in the pathogenesis of lymphoma, but, in each case, supplemental factors (i.e., further oncogenic "hits") are thought to be necessary for malignant transformation to take place (2, 3, 10). BCL6 expression is believed to become deregulated as a result of mutations that affect binding sites in the first (i.e., noncoding) exon (1) or chromosomal rearrangements in which normal *BCL6* regulatory sequences are replaced by heterologous promoters (11). *PIM1* has been reported as one of the multiple translocation partners that have been identified in rearrangements with *BCL6* (12). *PIM1* and *BCL6* are associated with the somatic hypermutation events that occur in Ig genes (13, 14), a process that, when aberrant, is thought to contribute to malignant transformation (14); and both are expressed predominantly in lymphocytes (9, 15). In humans, overexpression of PIM1 has been noted in B-cell lymphomas (16) and has been associated with genomic instability (17) in prostate cells. However, in *PIM1* transgenic mice, retroviral infection induces T-cell tumors (8). Even in *PIM1* transgenic mice containing a leukemia virus long-terminal repeat supplemented with an upstream Ig enhancer and expressing high PIM1 RNA levels in both B and T cells, development of T-cell lymphomas was noted. Interestingly, these T-cell neoplasms were found to contain activated *MYC* (18), a gene usually associated with B-cell lymphomas in humans. Similarly, in *MYC* transgenic mice (5), and also in *BCL6* (2) transgenic mice, predominantly T-cell lymphomas develop spontaneously (in the case of *MYC*) (5) or, in the case of *BCL6*, after induction with a retrovirus (as described here) or mutagen (2), even though these genes are associated predominantly with B-cell neoplasms in humans. Further, it has long been known that BCL6 is expressed not only in human B-cell neoplasms, but also in certain T-cell lymphomas (19), which are more recalcitrant to current treatment modalities than are B-cell tumors. Recently the role of BCL6 in T-cell development and function has received increasing attention (20–26). It is now believed that, even though BCL6 is not required for B or T-cell development, it is absolutely essential for germinal center B-cell (27, 28) and T follicular helper cell differentiation (20–22) and is important in other stages/subsets as well.

Human *PIM1* encodes a 313-aa serine-threonine kinase that has antiapoptotic activity and is involved in cell survival, prolif-

eration, differentiation, transformation, tumor progression, as well as angiogenesis (29–31). Its protein is expressed in the thymus, liver, and spleen, and in hematopoietic progenitor cells in the bone marrow during development (32), as well as in the central nervous system (33). Although its cellular localization may be nuclear and/or cytoplasmic, nuclear localization is believed to be necessary for its biologic effects (9). In humans, *PIM1* and *c-MYC* appear to have a cooperative role in prostate cancer (34), and increased expression of PIM1 has been noted in gastrointestinal cancers (35), pancreatic adenocarcinoma (36), and oral squamous-cell carcinoma (37). PIM1 also has been noted to be overexpressed in myeloid and lymphoid leukemias (32) and has been associated with B-cell lymphomas (4, 9). Because loss of PIM1 does not adversely affect the fertility or survival of KO mice (38), there has been intense recent interest in small molecules that inhibit PIM1 expression. SGI-1776, a small-molecule inhibitor of PIM1, PIM2, and PIM3 kinases, induced apoptosis in a human mantle cell lymphoma line and acute myeloid leukemia cell lines when studied as mouse xenografts and in acute myeloid leukemia cell lines in cell culture with little toxicity to normal human lymphocytes (39). This drug, which was in a phase I study in patients with refractory prostate cancer and lymphoma, was associated with cardiac toxicity, and further use in human trials was halted (http://clinicaltrials.gov/ct2/show/NCT00848601).

In conclusion, we have identified *PIM1* as a cooperating gene with *BCL6* in lymphomas that develop in retrovirus-injected mice expressing the human *BCL6* transgene, and we have shown in our studies the concurrent expression of PIM1 and BCL6 in a limited number of randomly selected BCL6-positive human lymphomas. Although *PIM1* has long been known to be a protooncogene, its association with BCL6 has not been described to our knowledge. It would be of interest to analyze further the cooperation between PIM1 and BCL6 by breeding Eμ-*pim*-1 transgenic mice (18) with our mice transgenic for human *BCL6* (2) and with the engineered mice of Cattoretti et al. (3). We suggest that study of human lymphomas for PIM1 expression (which is not currently a part of the standard diagnostic lymphoma assessment panel) be added to the routine testing that is currently performed on human B- and T-cell tumors. Our results imply that inhibition of PIM1 kinase in human BCL6-positive and/or PIM1-positive lymphomas may be a useful therapeutic modality in this disease.

Materials and Methods

Retroviral Insertional Mutagenesis. Under approved institutional protocols, we injected 99 *BCL6* transgenic mice (2) and 33 nontransgenic controls [animals of the same background, WT or positive for either (but not both) the *Eμ-tTA* or *BCL6* transgenes] (2) with MOL4070LTR recombinant murine retrovirus (6), 10^5 infectious units intraperitoneally 1 d after birth, and followed them until euthanasia or death, at which time all animals underwent necropsy.

Histology and Flow Cytometry. Tissues were fixed in 10% (vol/vol) buffered formalin, paraffin-embedded, sectioned, stained with H&E, and studied with an experienced hematopathologist who was blinded whether the mouse was transgenic or control. Hematologic malignancies were analyzed by FACS whenever possible. Single-cell suspensions were incubated for 15 min with anti-2.4G2 to inhibit nonspecific binding, then labeled with fluorochrome-conjugated antibodies (BD Biosciences/Pharmingen or Sigma-Aldrich) according to the manufacturer's protocol. Analysis was performed on an LSRII flow cytometer (BD Biosciences). Markers included antibodies directed against murine B and T cells, macrophages, myeloid and erythroid cells, immature cells (e.g., Sca-1, CD117), immunoglobulins, κ-, λ-, and germinal center cells (e.g., peanut agglutinin). Lymphoid neoplasms were classified according to the Bethesda proposals (7).

Inverse PCR and Database Searches. DNA from all analyzable lymphomas was purified and subjected to inverse PCR as described (40), except that 29 cycles of PCR amplification were performed in a 20-μL final volume, with annealing at 61 °C for the 5′ LTR primers and 56 °C for the 3′ LTR primers, and the final extension step was 15 min. Pools of PCR products were cloned and transformed with a TOPO TA Cloning Kit for sequencing (Invitrogen). Purified DNA from ampicillin-resistant colonies was sequenced with a Big Dye Terminator cycle sequencing kit (v3.1; Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems) by using primers from the pCR4-TOPO vector (Invitrogen) in which the inserts had been cloned. Sequences were matched to murine databases of the University of California Santa Cruz (genomic build of July 2007) genome browser and the National Institutes of Health. As it is known that gene expression can be affected over hundreds of kilobases by retroviral integrations (41) and chromosomal translocations (42), we studied potentially affected genes within at least a 100-kb window of an insertion site. Candidate genes identified in this way were considered as possibly relevant if they were noted in at least two transgenic mice but not in any controls (43, 44).

Real-Time Quantitative PCR. Total RNA was extracted from mouse lymphomas, DNase-1-digested, reverse transcribed, and subjected to real-time RT-PCR (along with "no-RT" controls) with the use of SYBR Green Master Mix (Takara Bio) according to the manufacturer's instructions and a LightCycler 480 (Roche Applied Science). Primers included β-actin (Promega) that amplifies 285 bp (2) of cDNA (vs. 396 bp for genomic DNA) and primers that amplify 176 bp of cDNA of the murine *PIM1* gene (5′ primer in exon 5, 3′ primer in exon 6; Real Time Primers). Controls included water template and RNA extracted from randomly selected lymphomas (three T-cell and three B-cell lymphomas) of the comparable organs of retrovirus-injected nontransgenic mice. Results from triplicate wells (for each study animal) or duplicate wells (multiple control mice) were averaged and compared.

Immunohistochemistry. Human lymphoma paraffin-embedded blocks were obtained from the surgical pathology archives under an institutional review board-approved protocol. Immunohistochemical staining on human tissues was performed with mouse monoclonal anti-human BCL6 (clone LN22; Novocastra) at a dilution of 1:20 on the automated Bond system (Leica Biosytems) with Bond Polymer Refine Detection. Antigen retrieval was performed in Epitope Retrieval Solution 2 for 20 min (Leica Microsystems). A modified protocol included antibody incubation for 25 min, post-primary reagent 15 min, polymer 25 min, peroxide block 8 min, 3,3′-diaminobenzidine 10 min, and hematoxylin 8 min. BCL6 staining on mouse tissues was performed as described previously (2). Additionally, paraffin-embedded formalin-fixed human and murine lymphomas were stained with anti-PIM1 (purified rabbit polyclonal antibody, AP7932d at dilution 1:50; Abgent). For anti-PIM1 staining, antigen retrieval was performed in Bond Epitope Retrieval Solution 2 for 20 min; the peroxide block, post-primary rabbit anti-mouse IgG, and hematoxylin applications were omitted, and the antibody was applied twice (25 min each). Images were taken with a BX41 microscope (Olympus), a DP72 digital camera, and cellSens Standard imaging software (Olympus).

Statistical Analysis. Lymphoma-related survival in the study vs. control mice was compared with the use of Cox regression and Fisher exact test (to look further at lymphoma-related survival at specific time points). Kaplan–Meier curves also were generated for each group. The data from real-time PCR in the study and control groups were analyzed by the comparative C_T method (45) and compared by the Wilcoxon rank-sum test. Statistical analyses were performed by using Stata software (version 12; StataCorp).

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