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Whole-Body *Sleeping Beauty* Mutagenesis Can Cause Penetrant Leukemia/Lymphoma and Rare High-Grade Glioma without Associated Embryonic Lethality

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Abstract

The *Sleeping Beauty* (SB) transposon system has been used as a somatic mutagen to identify candidate cancer genes. In previous studies, efficient leukemia/lymphoma formation on an otherwise wild-type genetic background occurred in mice undergoing whole-body mobilization of transposons, but was accompanied by high levels of embryonic lethality. To explore the utility of SB for large-scale cancer gene discovery projects, we have generated mice that carry combinations of different transposon and transposase transgenes. We have identified a transposon/transposase combination that promotes highly penetrant leukemia/lymphoma formation on an otherwise wild-type genetic background, yet does not cause embryonic lethality. Infiltrating gliomas also occurred at lower penetrance in these mice. SB-induced or accelerated tumors do not harbor large numbers of chromosomal amplifications or deletions, indicating that transposon mobilization likely promotes tumor formation by insertional mutagenesis of cancer genes, and not by promoting wide-scale genomic instability. Cloning of transposon insertions from lymphomas/leukemias identified common insertion sites at known and candidate novel cancer genes. These data indicate that a high mutagenesis rate can be achieved using SB without high levels of embryonic lethality or genomic instability. Furthermore, the SB system could be used to identify new genes involved in lymphomagenesis/leukemogenesis. [Cancer Res 2009;69(21):8429–37]

Introduction

Forward somatic cell genetic screens in model organisms are a powerful approach for the identification and validation of tumor suppressor genes (tsg) and oncogenes relevant in human cancer (1–3). Insertional mutagens such as retroviruses and transposable elements are frequently used for this purpose because the mutagen itself serves as a molecular tag, allowing rapid identification of mutagenized genomic loci. Candidate cancer genes are identified by finding regions of the genome that are insertionally mutated in multiple independent tumors, so-called common insertion sites (CIS).

The *Sleeping Beauty* (SB) transposon system has been used as such an insertional mutagen. The SB system is bipartite, consisting of the mobilized piece of DNA, the transposon, and the enzyme that catalyzes the transposition reaction, the transposase (4). Different combinations of SB transposon and transposase transgenics have been used for whole-body somatic cell genetic screens *in vivo* (5, 6). For these studies, different lines of mice harboring multiple copies of the T2/onc transposon in a head-to-tail arrangement in a chromosomally resident concatomer were used. Lines harboring ~25 copies of T2/onc in the donor concatomer were designated as low-copy lines (5), whereas lines harboring >140 copies of T2/onc were designated as high-copy lines (6). Two SB transposase transgenic lines were used to mobilize T2/onc throughout the soma. One transgenic was engineered with the SB11 version of the transposase "knocked" into the *Rosa26* locus (*Rosa26*-SB11; ref. 6), whereas one transgenic expresses the SB10 version of the transposase under the control of the CAGGS promoter (CAGGS-SB10; refs. 5, 7). Mobilizing T2/onc from low-copy lines by CAGGS-SB10 could not generate tumors on an otherwise wild-type genetic background, yet did accelerate sarcoma formation in mice deficient for the tsg *p19Arf* (5). T2/onc mobilization from high-copy lines by *Rosa26*-SB11 on an otherwise wild-type genetic background resulted in high levels of embryonic lethality which limited the number of transposon/transposase doubly transgenic mice that could be generated (6). All mice surviving to birth eventually succumbed to tumors, primarily lymphocytic lymphoma/leukemia, by 120 days. Medulloblastoma and other hyperplasias/neoplasias were also observed at low penetrance. Cloning insertions from 15 lymphoma/leukemias and 1 medulloblastoma identified 33 CISs at known and candidate cancer genes, only a few of which have been previously identified in retroviral screens for lymphoma/leukemia genes (6).

There are two components to the SB system (consisting of both transposons and transposase), so it is possible to modify each component individually to determine its effects on tumorigenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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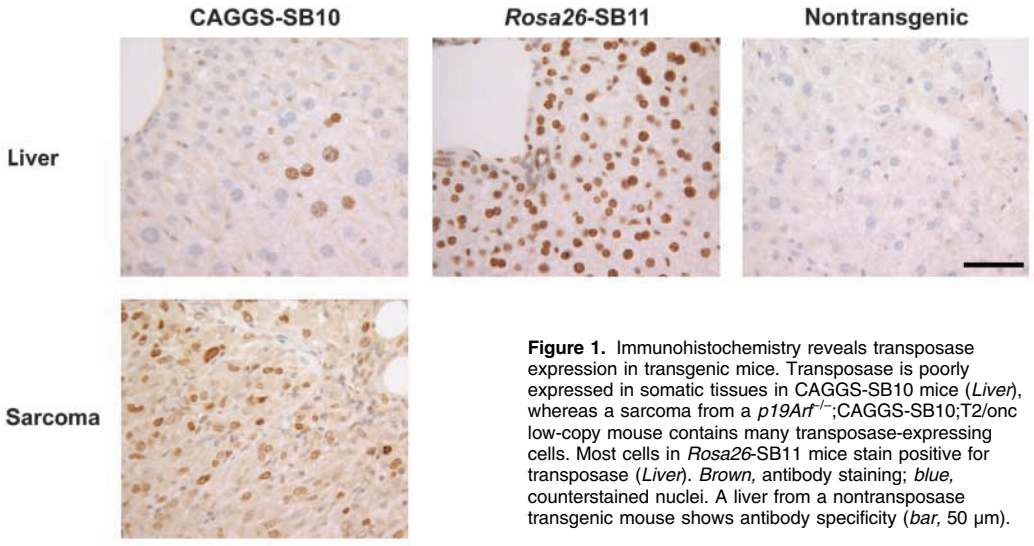

Figure 1. Immunohistochemistry reveals transposase expression in transgenic mice. Transposase is poorly expressed in somatic tissues in CAGGS-SB10 mice (*Liver*), whereas a sarcoma from a *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy mouse contains many transposase-expressing cells. Most cells in *Rosa26*-SB11 mice stain positive for transposase (*Liver*). *Brown,* antibody staining; *blue,* counterstained nuclei. A liver from a nontransposase transgenic mouse shows antibody specificity (*bar,* 50 μm).

To this end, we crossed a T2/onc high-copy line to CAGGS-SB10 and two T2/onc low-copy lines to *Rosa26*-SB11. We have discovered that a rate of mutagenesis sufficient for promoting highly penetrant tumor formation yet insufficient for causing embryonic lethality can be achieved with the SB system. Leukemias/lymphomas predominate the tumor spectrum in mice undergoing whole-body transposon mutagenesis. Gliomas also occur with reduced penetrance, indicating that this tumor type can be modeled using SB mutagenesis. Furthermore, widespread genomic instability is not observed in SB-induced or accelerated tumors, suggesting that transposon insertional mutagenesis and not genomic instability drives tumorigenesis in these models. Transposon insertion sites from SB-induced leukemias/lymphomas identify CISs at both known and candidate novel cancer genes, suggesting that the SB system can reveal a different spectrum of cancer loci than retroviruses.

Materials and Methods

Mice. Mouse work was performed under University of Minnesota Institutional Animal Care and Use Committee guidelines. All strains have been described (5, 6, 8). At necropsy, tissues were snap-frozen for DNA preparation and formalin-fixed/paraffin-embedded for pathologic analysis at the Masonic Cancer Center Histopathology Core and at the Mayo Clinic Tissue and Cell Molecular Analysis Shared Resource. Kaplan-Meier survival analysis was performed using Prism software.

Genotyping. Transposase transgenics were PCR-genotyped using the following primers: 5′GGACAACAAAGTCAAGGTAT3′ and 5′TAACTTGGGTCAAACGTTTC3′. T2/onc mice were genotyped as described (9).

Flow cytometry. Cell staining and flow cytometry techniques were as described (10, 11). Antibodies used were CY5-conjugated anti-CD4, APC-conjugated anti-CD8, FITC-conjugated anti-B220, PE-conjugated anti-TCRβ, PE-conjugated Gr1, and FITC-conjugated Mac1 (BD Biosciences). Data were analyzed using FlowJo software.

Linker-mediated PCR. For tumor DNA, linker-mediated PCR was performed as described (12). PCR products from tumors were shotgun-cloned into pCR4-Topo (Invitrogen). For each PCR, 96 bacterial colonies were robot-picked, prepped, and sequenced on the ABI 3730 platform. For the data set from tail DNA, sequencing was performed on the 454 platform as described (9).

Insertion mapping and CIS analysis. Mapping of insertion sites to the NCBI 36 build of the mouse genome was performed as described (6, 13). CIS analysis was based on published methods (14). Because of the possibility for transposons to local hop after a prior mobilization, insertions from the same animal were not allowed to solely define a CIS. Insertion data was deposited in the Retroviral Tagged Cancer Gene Database (13).

Array comparative genomic hybridization. Tumor DNA samples (1 μg each) were labeled with Cy-3-dUTP and control DNA samples from muscle or spleen tissue (from the same animal when possible and from littermates in all other cases) were labeled with Cy-5-dUTP essentially as described (15), with the omission of the *Dpn*II digest. Samples were combined and mixed with mouse Cot-1 DNA and hybridized to 1,344-element BAC arrays (16) as described. Array images were captured using a CCD camera, and automated spot identification and statistical analysis was carried out using custom software (17) as described (15).

Immunohistochemistry. Immunohistochemistry for transposase was performed using the M.O.M. kit (Vector Laboratories). Anti-transposase antibody (R&D Systems) was used at 1 μg/mL. Immunostain was developed using the ABC Vectastain peroxidase system (Vector Laboratories), and sections were counterstained with hematoxylin. Immunohistochemistry was performed using anti–glial fibrillary acidic protein (polyclonal, dilution 1:4,000; Dako) and anti-synaptophysin antibodies (clone SY38, dilution 1:40; ICN) and showed a similar pattern in 10 gliomas examined. Primary antibody incubation was performed for 30 min, followed by 20 min in the Envision+Dual Link detection system on a Dako autostainer.

Results and Discussion

Combining CAGGS-SB10 with high-copy T2/onc does not result in tumor formation due to limited transposase expression. To determine if mobilization of T2/onc from high-copy lines by CAGGS-SB10 is sufficient to induce tumors, mice doubly transgenic for a T2/onc high-copy concatomer located on chromosome 4 (6) and the CAGGS-SB10 transgene (8) were generated. No evidence of embryonic lethality was observed (data not shown). CAGGS-SB10 only controls (n = 11) and T2/onc high-copy;CAGGS-SB10 experimental mice (n = 9) were aged and monitored for tumor formation for 18 mo. No statistical difference in survival was observed (P = 0.6848, log rank test; Supplementary Fig. S1), indicating that the mutagenesis rate achieved by mobilizing

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Table 1. No evidence for non-Mendelian transgene inheritance in the T2/onc low-copy;*Rosa*26-SB11 cross

Genotype (T2/onc,*Rosa*26-SB11)	+,−	−,+	+,+	−,−
No. of mice	123	138	112	129

NOTE: T2/onc low-copy heterozygous mice were crossed to *Rosa*26-SB11 heterozygous mice and the resulting progeny were genotyped for each transgene. The four possible genotypes and number of mice observed for each genotype are shown.

T2/onc from a high-copy line by CAGGS-SB10 is insufficient for tumor formation on an otherwise wild-type genetic background.

To investigate if transposase expression levels influence mutagenesis rates, immunohistochemistry was performed to detect transposase in CAGGS-SB10 and *Rosa26*-SB11 mice. Normal adult tissues were examined, as was a sarcoma from a *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy mouse (5). Although transposase was detected in the sarcoma, it was absent from most normal somatic tissues in CAGGS-SB10 mice (Fig. 1; Supplementary Fig. S2). When expression was detected, it occurred in a highly variegated pattern (liver in Fig. 1; kidney in Supplementary Fig. S2). In contrast, transposase was robustly expressed in the majority of cell types in *Rosa26*-SB11 mice (Fig. 1; Supplementary Fig. S2). The low level and variegated expression in CAGGS-SB10 mice is potentially due to epigenetic silencing that is often observed in standard transgenics.

The presence of transposase in a *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc sarcoma indicates that transposase is expressed in these mice in an appropriate cell type to promote sarcomagenesis. It could be hypothesized that T2/onc high-copy;CAGGS-SB10 mice could have developed sarcomas on an otherwise wild-type genetic background due to the availability of many T2/onc copies for mutagenesis. However, tumor formation was not observed. In murine models, *p19Arf* is known to play a role in oncogene-induced senescence (18–20). Therefore, in sarcoma-initiating cells in *p19Arf*$^{+/+}$ mice, T2/onc mutagenesis of cancer genes could promote Arf-mediated senescence, providing a block to tumor formation. This experiment suggests that performing SB screens in tumor-predisposed genetic backgrounds may be necessary for robust tumor formation in certain tissue types.

Combining T2/onc low-copy lines with *Rosa26*-SB11 does not cause embryonic lethality but promotes tumor formation in otherwise wild-type mice. To determine if mobilization from low-copy lines is sufficient for tumor formation, two T2/onc low-copy lines (lines 68 and 76; ref. 5) were crossed to *Rosa26*-SB11. χ^2 analysis of the resulting progeny (Table 1) revealed no evidence

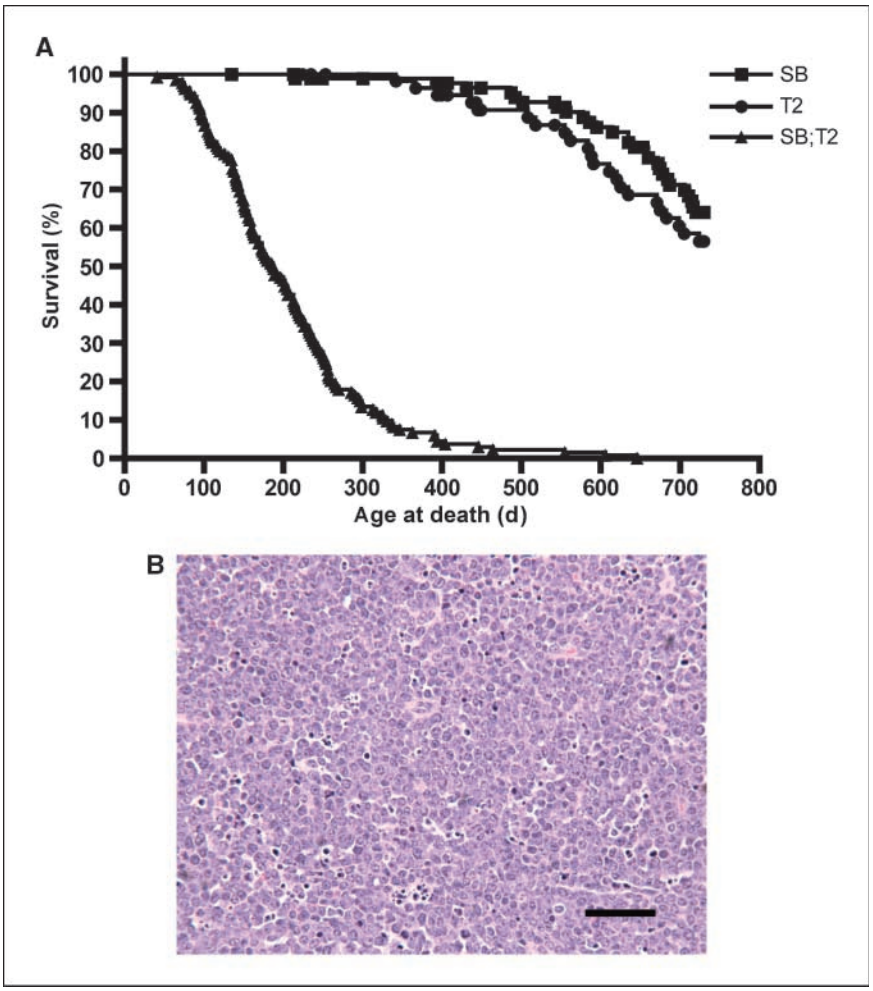

Figure 2. *Rosa26*-SB11;T2/onc low-copy mice are tumor prone. *A,* Kaplan-Meier survival curve for *Rosa26*-SB11;T2/onc low-copy (*SB;T2,* ▲), *Rosa26*-SB11 (*SB,* ■), and T2/onc low-copy (*T2,* ●) mice. *Rosa26*-SB11;T2/onc low-copy mice become moribund more rapidly than controls ($P < 0.001$, log rank test). *B,* H&E-stained example of a lymphocytic leukemia/lymphoma from a *Rosa26*-SB11;T2/onc low-copy mouse (*bar,* 50 μm).

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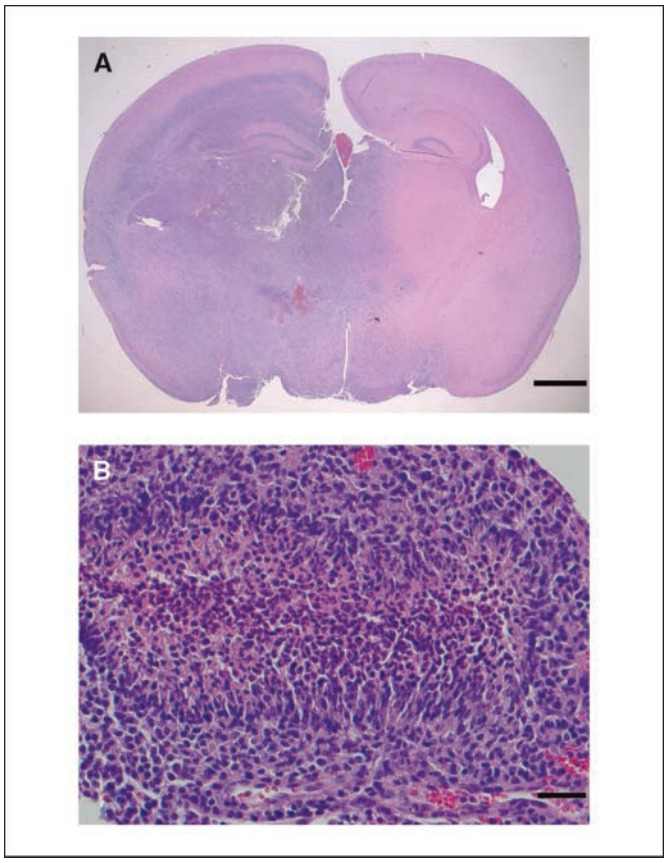

Figure 3. Gliomas are present in *Rosa26*-SB11; T2/onc low-copy mice. *A,* gliomas sometimes involved essentially an entire hemisphere (H&E; *bar,* 1 mm). *B,* pseudopalisading necrosis was evident in a subset of cases which were therefore classified as glioblastoma (H&E; *bar,* 50 μm).

for non-Mendelian inheritance of the transgenes ($P = 0.4153$; 3 *df*). A large cohort of doubly transgenic mice and single transgenic littermate controls were therefore generated. T2/onc low-copy;*Rosa26*-SB11 mice became moribund with an average latency of 187 d, whereas controls had normal life spans (Fig. 2*A*). Separate analysis of each T2/onc low-copy line revealed that T2/onc low-copy line 68;*Rosa26*-SB11 mice develop disease much more rapidly than T2/onc low-copy line 76;*Rosa26*-SB11 mice (Supplementary Fig. S3). However, the tumor spectrum was the same for both lines. At necropsy, 89% (97 of 109) of analyzed doubly transgenic mice had signs of hematopoietic disease including splenomegaly, lymphadenopathy, and/or an enlarged thymus. Twenty-seven mice with hematopoietic involvement were analyzed by veterinary pathologists at the Masonic Cancer Center Comparative Pathology core. Nineteen mice were diagnosed with lymphocytic lymphoma/leukemia (Fig. 2*B*), three with hematopoietic neoplasia of undetermined lineage, four with hematopoietic hyperplasia, and one with myeloid leukemia. Flow cytometry analysis for cell surface markers on 19 tumors verified that the majority of leukemias/lymphomas arising in these mice are phenotypically T-cell lymphocytic disease (Supplementary Table S1).

Several mice presented with neurologic symptoms at morbidity. Medulloblastomas, a tumor of the cerebellum, occurred at low penetrance in T2/onc high-copy;*Rosa26*-SB11 doubly transgenic mice (6). To determine if medulloblastomas also occur in T2/onc low-copy;*Rosa26*-SB11 mice, 82 brains were extensively sectioned for pathology. Fourteen brain tumors were discovered. One tumor was a sarcoma growing on the surface of the brain, whereas one was a glioma of undetermined origin (data not shown). Histopathologic analysis determined that the remaining tumors were high-grade astrocytomas (Fig. 3*A*). Pseudopalisading necrosis was present in three cases that were therefore classified as glioblastomas (Fig. 3*B*). Immunohistochemistry for glial fibrillary acidic protein was performed on a subset of tumors to confirm the diagnosis. Immunoreactivity for glial fibrillary acidic protein was noted at least focally in all tumors examined but they were negative for synaptophysin (data not shown), supporting the diagnosis of astrocytomas. No gliomas were detected in 28 age-matched transposon or transposase-only mice sacrificed for analysis, indicating that T2/onc mobilization induces high-grade astrocytomas. Molecularly defined hyperproliferative lesions were also found in the prostates of moribund T2/onc low-copy;*Rosa26*-SB11 mice (21); but no additional overt tumor types were commonly observed despite the fact that transposase is expressed in most cell types. The aggressive nature of the leukemias/lymphomas and gliomas in these mice limits animal survival, and therefore likely prevents the ability of transposon mobilization by *Rosa26*-SB11 to model more slowly developing tumor types.

In contrast to T2/onc high-copy lines, no embryonic lethality was observed in T2/onc low-copy;*Rosa26*-SB11 mice. Embryonic lethality was proposed to potentially result from unrepaired DNA damage after transposition (6). The lower number of mobilizing transposons in T2/onc low-copy;*Rosa26*-SB11 mice could result in fewer double-strand breaks for cellular machinery to repair. Another explanation for embryonic lethality in T2/onc high-copy;*Rosa26*-SB11 double transgenics could be the generation of concatomer-associated rearrangements which can accompany SB germ line transposition (22). High transposition rates associated with high-copy concatomers could increase the severity of these rearrangements. Whatever the cause of embryonic lethality, the Mendelian inheritance of *Rosa26*-SB11 with T2/onc low-copy transgenes indicates that a mutagenic rate sufficient to promote tumor formation but insufficient to interfere with normal development can be achieved.

Brain tumors have been found in mice in which T2/onc is mobilized by *Rosa26*-SB11. In high-copy lines, the tumors were medulloblastomas (6), whereas in low-copy lines, the tumors were infiltrating gliomas. Differences in tumor latency could contribute to differences in brain tumor type. Medulloblastomas are predominantly found in children, and it is hypothesized that they arise from granule cell precursor cells. It is hypothesized that most granule cell precursors have completed proliferation and differentiation by adulthood, and therefore fewer potential medulloblastoma-initiating cells exist in adults. The high mutagenesis rate in T2/onc high-copy;*Rosa26*-SB11 mice could allow enough mutations in cancer genes to occur prior to terminal differentiation. Conversely, in T2/onc low-copy;*Rosa26*-SB11 mice, the mutagenesis rate may be too low to promote tumor formation prior to terminal differentiation of these cells. This slower mutagenesis rate could still promote mutagenesis in the longer-lived glial precursor cell.

Somatic mobilization of transposons does not cause substantial genomic instability. Transposition of DNA transposons involves double-strand break formation and repair (23). It is possible that somatic mobilization of SB transposons could cause tumor formation by promoting genomic instability due to illegitimate repair of these breaks. To investigate this possibility, BAC-based array comparative genomic hybridization (16) was used

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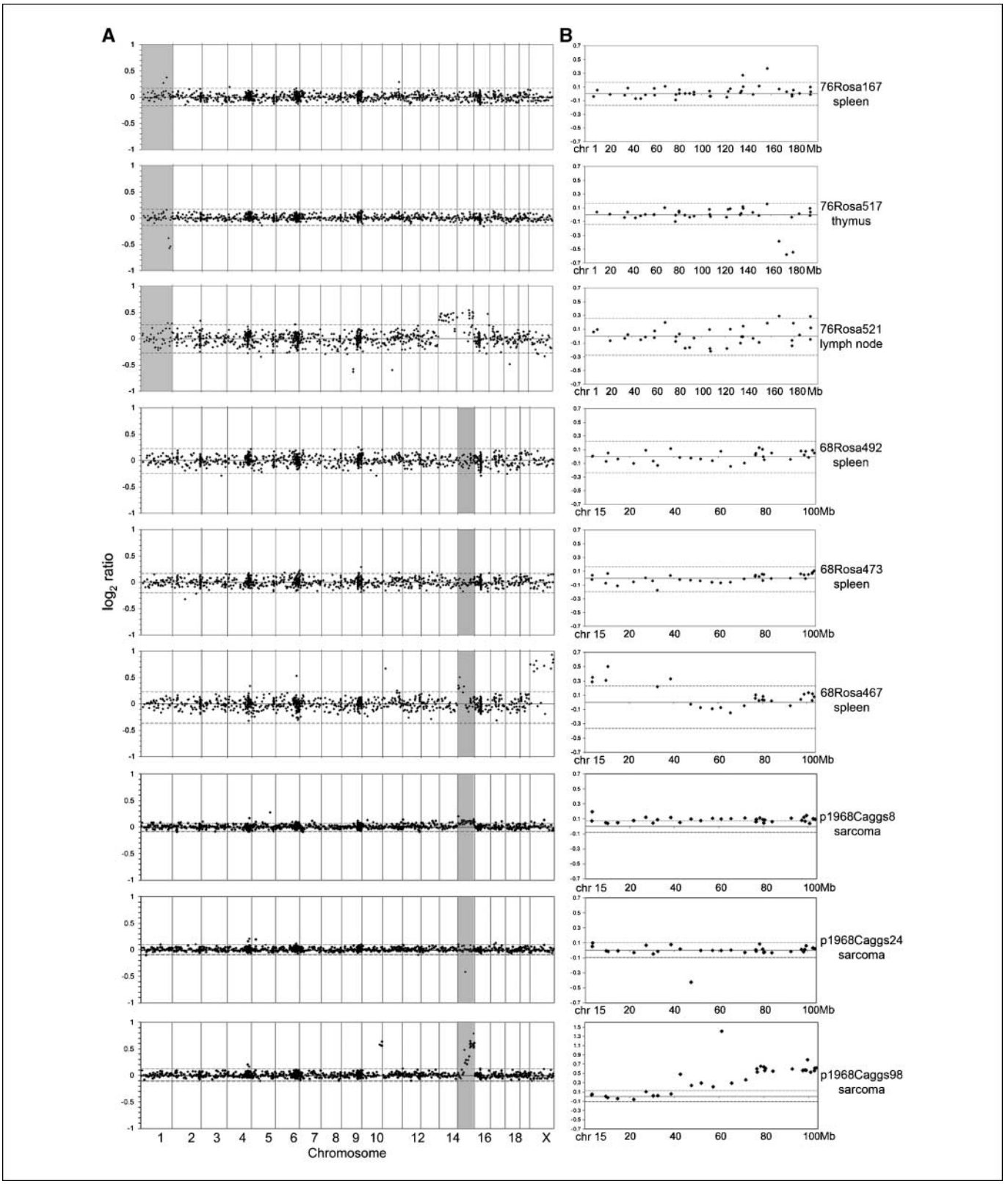

Figure 4. *Rosa26*-SB11;T2/onc low-copy leukemias do not show genome-wide chromosomal amplifications and deletions. Array CGH profiles from six leukemias/lymphomas from *Rosa26*-SB11;T2/onc low-copy mice (76Rosa and 68Rosa) and three sarcomas from *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy mice (p1968Caggs). Each row represents one tumor. *A,* genome-wide $\log_2$ ratios. Dotted lines represent 3 SD from the central mean of all clones genome-wide, indicating cutoffs for gains and losses, respectively. Tumors from 76Rosa167, 76Rosa517, 68Rosa467, p1968Caggs8, p1968Caggs24, and p1968Caggs98 show localized rearrangements in the region surrounding the transposon concatomer on chromosomes 1 and 15 (*shaded areas*). The apparent gain of the X chromosome in the tumor from 68Rosa467 is due to DNA from normal male spleen being used as reference DNA for a tumor from a female littermate mouse. *B,* profiles of clones on the donor concatomer chromosome for the tumors profiled in *A*.

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Table 2. CISs identified in leukemias/lymphomas from T2/onc;*Rosa*26-SB11 mice

CIS name	Chromosome	CIS address start	CIS address end	No. of tumors	No. of independent insertions	Other genes in CIS interval
Myb*	10	20824216	20921673	4	4	
Ube2d1†	10	70669435	70673276	2	2	
Stab2†	10	86412419	86505987	3	3	
Ikzf1*	11	11407716	11749020	14	18	4930415F15Rik, 4930512M02Rik, Q3UW08, Fignl1, Ddc
Csf2†	11	54031828	54569276	4	4	ENSMUSG00000060068, Il3, Acsl6, 4930404A10Rik, Fnip1, Rapgef6, Cdc42se2
Cox10†	11	63785103	63794563	2	2	
Rab5c*,†	11	100370989	100543223	3	3	Ttc25, Cnp1, Dnajc7, Nkiras2, A930006D11Rik, D11Lgp2e, Gcn5l2, Hspb9
Dusp22	13	30572119	30813368	4	4	Irf4
Ibrdc2†	13	47050885	47464428	4	5	Tpmt, Aof1, Dek
H2afy*,†	13	56100124	56645908	4	4	BC027057, Neurog1, Cxcl14, Q8CDW6, AU042651, Il9, Fbxl21, Lect2, Q3U1K8, Tgfbi
Mef2c*	13	83767562	84048492	5	5	
Cenpk	13	105370547	105371252	2	2	
Zmiz1*	14	24269629	24414687	5	5	
Heg1	16	33678449	33679145	2	2	
Btla†	16	44873922	45525600	4	4	Cd200r3, Ccdc80, Q3UVS9, Slc35a5, Atg3, EG547267, Cd200, Gm609, ENSMUST00000060550, Slc9a10
Erg*	16	95072653	95570852	20	10	Kcnj6, Kcnj15
Heatr5b†	17	78655675	78761809	3	3	ENSMUST00000059920, 2310002B06Rik, ENSMUST00000043373, Eif2ak2
Mbd2*	18	70309981	70807502	7	6	2310002L13Rik, ENSMUST00000096551, 4930503L19Rik, Poli, ENSMUST00000031200
Pten	19	32611775	32885582	7	12	Papss2, Atad1, B430203M17Rik
Notch1*	2	26281337	26321310	21	20	
Zbtb34	2	33060035	33919549	5	6	Angptl2, Ralgps1, Lmx1b, C130021I20Rik, ENSMUST00000100174, 2610528K11Rik
Rasgrp1*	2	117030934	117031956	2	2	
Gm414	3	70203770	70205270	2	2	
Ppp3ca†	3	136626657	136865395	2	6	
Bach2*,†	4	32416902	33046612	4	6	XR_001707.1, Q8BQ29, ENSMUST00000093133, Gja10, Casp8ap2, Mdn1
Ptpn12†	5	20125026	21032439	5	5	Magi2, Q3U0Y7, Q8CEH7, Phtf2, Tmem60, Rsbn1l, ENSMUST00000053060, EG626903, A530088I07Rik, 4930528G09Rik, Fgl2, AI847670, Fbxl13
Kit/Kdr*	5	75834997	76133546	4	5	
AB041803	6	31101079	31233559	4	4	
Wnk1†	6	119698409	120263825	4	4	Erc1, 3110021A11Rik, ENSMUST00000036010, Rad52, EG406236, mmu-mir-706, Ninj2, B4galnt3, ENSMUSG00000053059
Etv6	6	134104562	134557768	4	4	Bcl2l14, Lrp6, Q8BPW4
Akt2	7	27305138	27309525	3	3	
Klf13	7	63514893	63864073	4	4	Otud7a, ENSMUST00000003521
Mctp2†	7	72254322	72263587	2	2	
Eed	7	89832779	89844617	2	3	
EG209380†	7	105978304	106615569	4	4	Gvin1, Q922V0, Q9D303, ENSMUST00000071162, Gm1966, Olfr693-701

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Table 2. CISs identified in leukemias/lymphomas from T2/onc;*Rosa*26-SB11 mice (Cont'd)

CIS name	Chromosome	CIS address start	CIS address end	No. of tumors	No. of independent insertions	Other genes in CIS interval
Zfp629	7	127271619	127383373	4	4	Fbs1, Q8C4A9, D030022P06Rik, Phkg2, Gm166, Rnf40
Dcun1d5[†]	9	7186494	7196156	2	2	
Naalad2[†]	9	18088306	18093482	2	2	
Fli1*	9	31723619	32229349	6	5	Grit, Kcnj5, Kcnj1
BC033915	9	45938617	45993423	3	4	Apoa1, Tcea1, Apoc3, Efhc1
4833427G06Rik	9	50485627	50898988	4	4	Dixdc1, 2310030G06Rik, Cryab, Hspb2, 1110032A03Rik, D630004A14Rik, Alg9, Ppp2r1b, Snf1lk2, Layn, tg4, mmu-mir-34b,c
Tcf12*,[†]	9	71786484	71892068	3	3	
Eras*,[†]	X	7019492	7220920	3	3	Otud5, Pim2, Slc35a2, Pqbp1, Timm17b, ENSMUST00000085330, Q3UUQ2, Pcsk1n, Hdac6, Gata1, 2010001H14Rik, EG632013, Suv39h1
Tbl1x[†]	X	73774303	74215530	4	4	EG628893, Prkx, Pbsn

NOTE: The name of the CIS is presented along with the chromosome, the base pair of the first insertion defining the CIS, the base pair of the last insertion defining the CIS, the number of tumors defining the CIS, the number of insertions in independent TA dinucleotides, and additional Ensembl annotated genes found within the bounds of the CIS.
*CIS previously identified in MuLV mutagenesis studies (13, 32).
[†]CIS defined by an expected fraction of 0.005.

to look at genomic copy number changes in six T2/onc low-copy; *Rosa26*-SB11 lymphomas/leukemias and three sarcomas from *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy mice (5) using non–tumor DNA as a reference sample (Fig. 4). Two spontaneously arising sarcomas in *p19Arf*$^{-/-}$ mice served to show the ability of the BAC array platform to detect copy number changes in tumors (Supplementary Fig. S4). Whole chromosome gains or losses were rarely detected in tumors with mobilizing transposons (for example, the gain of chromosomes 14 and 15 in 76Rosa521 lymph node). Deletions or amplifications defined by one or two adjacent probes were occasionally detected. However, for line 76 leukemias/lymphomas, two of three displayed evidence of amplification or deletion on chromosome 1 at probes flanking 166 Mb, the approximate location of the T2/onc concatomer in line 76 (ref. 5; Fig. 4*B*). The exact chromosomal location of the line 68 concatomer has not been determined, but fluorescence *in situ* hybridization and local hopping patterns have placed it at ~45 Mb on chromosome 15. One (68Rosa467 spleen) of three leukemias/lymphomas from T2/onc line 68 and all three sarcomas from *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc line 68 mice showed evidence for amplification or deletion on chromosome 15.

Previously, chromosomal rearrangements flanking a SB transposon donor concatomer on chromosome 11 have been detected as a result of transposition in the germ line and in normal splenocytes (22). The array comparative genomic hybridization reported here indicates that this phenomenon is not limited to the concatomer on chromosome 11, and that they can occur in SB-induced tumors. The data suggest that SB-induced tumorigenesis does not promote genome-wide instability, but does frequently cause amplifications and/or deletions flanking the donor concatomer that could contribute to tumor formation if the donor concatomer happens to reside near a tsg or oncogene. As other DNA elements are known to cause genomic rearrangements (24), it will be important to determine if additional transposons proposed to be used as somatic mutagens including *piggyBac* (25), *Tol2* (26), and *Minos* (27) also promote deletions or amplifications flanking the donor locus.

T2/onc local hops in somatic cells. Cancer gene identification using insertional mutagens relies on performing CIS analysis to identify chromosomal regions where transposons have inserted in tumors at a rate greater than that expected by random chance. *In vivo*, SB is known to have a preference for reinserting at loci linked to the starting integration site, a phenomenon termed local hopping (28), which complicates CIS analysis. Previously, an unselected insertion set (n = 490) obtained from T2/onc high-copy; *Rosa26*-SB11 embryos was used to examine local hopping rates from chromosomal concatomers in somatic cells *in vivo* (6). Although only 6% to 11% of insertions were found in the 25 mb region surrounding donor concatomers, 38.6% of insertions were found on the same chromosome as the donor concatomer. However, it is unclear if transposon copy number or the chromosomal location of a concatomer influences local hopping rates.

To determine how local hopping from low-copy concatomers may influence the analysis of SB transposon tumor insertion sites, a data set of unselected SB transposon insertion sites from tail biopsy DNA isolated from 14- to 21-day-old T2/onc low-copy; *Rosa26*-SB11 mice was generated (9, 29). As no leukemias have been observed in mice this young, the insertions cloned from this material likely represent the SB transposon insertion site profile under unselected conditions. Sixteen thousand, four hundred and eleven unique SB insertion sites were cloned from 88 doubly transgenic mice using linker-mediated PCR and 454 pyrosequencing methods

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(Supplementary Table S2). Of these, 22.8% were located in the 25 Mb surrounding the donor concatomer, indicating that transposons in low-copy concatomers local hop in somatic cells. Furthermore, 49.3% of insertions were located on the same chromosome as the donor concatomer (8,095 of 16,411). The percentage of insertions on the donor chromosome contrasts with T2/onc high-copy line embryo insertions in which 38.6% of insertions reside on the same chromosome as the donor concatomer. This could potentially be explained by differences in local hopping rates from different copy number concatomers. Nevertheless, both of these data sets indicate that in somatic cells, SB transposons have a local hopping interval that encompasses the whole chromosome on which the concatomer resides.

CIS analysis was performed on this control data set after removal of insertions mapping to the donor chromosomes (n = 8,316 insertions) using criteria described by Mikkers and colleagues (14) at an expected fraction of 0.001 (two insertions in 0.325 kb, three insertions in 14.75 kb, and four insertions in 62 kb), which is predicted by Monte Carlo simulations to result in ~25 CISs being identified by random chance alone. Forty-three CISs were identified in the control data set. Clustering criteria using an expected fraction of 0.005 was also applied (two insertions in 1.625 kb, three insertions in 33.75 kb, and four insertions in 109.75 kb), which is predicted to result in a total of 124.5 CISs identified based on random chance alone. Using these criteria, 134 CISs were identified (of which 43 also met the criteria used above for an expected fraction of 0.001; Supplementary Table S3).

More CISs were identified in this control data set than would be predicted by Monte Carlo simulations. This observation could actually be due to random chance as Monte Carlo simulation predictions of false-positive clustering rates are based on averages for an infinite number of experiments. Therefore, 50% of the time, a random data set of 8,316 insertions is generated, the number of CISs identified at an expected fraction of 0.005 would be ≥124.5, and 50% of the time, the number of CISs identified would be ≤124.5. Alternatively, SB is known to have some insertion preferences for specific sequences or DNA conformations (30, 31), and preferred SB insertion sites may not be randomly distributed through the genome. Five of the CISs in the control data set are also CISs in leukemias (see below), supporting the hypothesis that there are genomic "hotspots" for SB integration. Interestingly, CISs are found in the control data set on proximal regions of chromosomes 5, 11, 12, 13 and 18, indicating the possibility that SB transposons have an affinity for inserting into centromeric regions. The generation and analysis of additional data sets under unselected conditions will help refine the statistical methods used for CIS analysis in SB-induced tumors.

T2/onc identifies candidate genes involved in lymphoma/leukemia formation not identified by MuLV. To determine if T2/onc identifies new lymphoma/leukemia cancer genes, 2,296 independent T2/onc insertion sites were cloned from 59 lymphomas/leukemias from 58 T2/onc low-copy;*Rosa26*-SB11 animals (Supplementary Table S4). Local hopping was observed as 13.1% of insertions from line 68 occurred within the 25 Mb surrounding the donor concatomer, 18.6% within the 40 Mb surrounding the donor concatomer, and 33.6% on the entire donor chromosome. For line 76, the local hopping percentages were similar at 11.2%, 15.6%, and 31.5%, respectively.

The local hopping rate in T2/onc low-copy;*Rosa26*-SB11 tumors is intermediate between those reported for sarcomas from *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy mice (23% of insertions within the 40 Mb surrounding the donor concatomer; ref. 5) and that reported for T2/onc high-copy;*Rosa26*-SB11 leukemias/lymphomas ("little local hopping"; ref. 6). The chromosomal location and environment of the concatomers could influence local hopping rates. Transposase levels could also influence local hopping rates as differences were observed between *p19Arf*$^{-/-}$;CAGGS-SB10;T2/onc low-copy sarcomas and *Rosa26*-SB11;T2/onc low-copy leukemias/lymphomas. The lower local hopping rates in *Rosa26*-SB11;T2/onc low-copy leukemias/lymphomas compared with weaning tail biopsies from the same cohort of mice could be due to the increased time for transposons to remobilize in tumors from older mice compared with tissue from young mice.

CIS analysis was performed on insertions cloned from 59 *Rosa26*-SB11;T2/onc low-copy induced leukemias/lymphomas after removal of insertions residing on the donor concatomer chromosome (n = 1,547 insertions) to identify candidate leukemia/lymphoma genes. Analysis was performed using criteria described by Mikkers and colleagues (14) at an expected fraction of 0.001 (two insertions in 1.95 kb, three insertions in 88.5 kb, and four insertions in 371.5 kb), which is predicted to result in 4.5 CISs being identified by random chance alone. This resulted in the identification of 28 CISs in the leukemia data set. Clustering criteria of insertions using an expected fraction of 0.005 was also applied (two insertions in 9.75 kb, three insertions in 202 kb, and four insertions in 658.5 kb), which is predicted to result in a total of 22.5 CISs identified based on random chance alone. Using these criteria, a total of 49 CISs were identified (of which 28 also met the criteria used above for an expected fraction of 0.001). Five of these CISs were also in the unselected data set, indicating that they likely do not tag a locus important for cancer formation. Removal of these resulted in a final total of 44 CISs in leukemias/lymphomas (Table 2).

Of the 44 CISs identified, 15 are CISs in the Retroviral Tagged Cancer Gene Database of retroviral screens for lymphoma/leukemia genes, or in a recent report analyzing MuLV insertions from more than 500 tumors (Table 2; refs. 13, 32). Therefore, the majority of CISs identified by SB have not been previously identified in retroviral screens. Notably, in lymphomas/leukemias resulting from mobilization from T2/onc low-copy lines, a CIS is found in *Pten*. Although an important tsg in the hematopoietic system and other cancers (33, 34), *Pten* has not been previously identified as a CIS in retroviral screens (13, 32). This supports the hypothesis that SB could identify cancer genes that are not readily tagged by retroviruses, including tsgs. Only seven CISs were common between the leukemia/lymphoma data set described here and the data set from the 15 leukemias/lymphomas generated using high-copy lines (6), indicating that cloning insertions from a larger cohort of tumors increases the identification power of CISs.

In summary, by combining T2/onc low-copy lines with *Rosa26*-SB11, we have achieved whole-body mobilization rates that are sufficient to promote penetrant tumorigenesis without the complication of embryonic lethality or genomic instability. Although lymphomas/leukemias predominate the tumor spectrum, whole-body mobilization of T2/onc could also promote glioma formation including glioblastoma, a tumor type in humans with an extremely poor prognosis. In lymphomas/leukemias, T2/onc tags both known and candidate novel cancer genes. Recent reports have shown the ability of T2/onc mobilization by tissue-specific transposase expression to generate liver tumors and intestinal tumors useful for candidate cancer gene discovery (9, 29). In these models, true carcinoma/adenocarcinoma on a wild-type genetic background

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occurred with long latency and incomplete penetrance, indicating that additional improvements to increase mutagenesis rates are still needed for the SB system. Our data indicate that such mutagenesis rates can be obtained without undesired consequences such as lethality or genome-wide instability, and that further development of the SB system is warranted for cancer gene discovery in a wider range of cell types.

Disclosure of Potential Conflicts of Interest

In the past, SB technology was exclusively licensed to Discovery Genomics, Inc. (DGI), which is co-founded by D.A. Largaespada. D.A. Largaespada has an equity interest in and is an unpaid scientific advisor to DGI. DGI is pursuing the use of SB for human gene therapy. Neither DGI money nor personnel were involved in the work reported here. The University of Minnesota has a pending patent on the process of using transposons such as SB for cancer gene discovery. D.A. Largaespada, L.S. Collier, A.J. Dupuy, N.A. Jenkins, and N.G. Copeland are named inventors. The other authors disclosed no potential conflicts of interest.

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