ARTICLES

Mammalian mutagenesis using a highly mobile somatic *Sleeping Beauty* transposon system

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Transposons have provided important genetic tools for functional genomic screens in lower eukaryotes but have proven less useful in higher eukaryotes because of their low transposition frequency. Here we show that *Sleeping Beauty* (SB), a member of the Tc1/mariner class of transposons, can be mobilized in mouse somatic cells at frequencies high enough to induce embryonic death and cancer in wild-type mice. Tumours are aggressive, with some animals developing two or even three different types of cancer within a few months of birth. The tumours result from SB insertional mutagenesis of cancer genes, thus facilitating the identification of genes and pathways that induce disease. SB transposition can easily be controlled to mutagenize any target tissue and can therefore, in principle, be used to induce many of the cancers affecting humans, including those for which little is known about the aetiology. The uses of SB are also not restricted to the mouse and could potentially be used for forward genetic screens in any higher eukaryote in which transgenesis is possible.

Transposon-tagged mutagenesis has proven invaluable for functional genomic screens in organisms such as *Drosophila melanogaster*[1,2], *Caenorhabditis elegans*[3] and plants[4], but the lack of active elements in higher eukaryotes has precluded their use for mammalian functional genomics. This problem was partly solved when molecular reconstruction was used to produce a transposon, *Sleeping Beauty* (SB), that transposes in higher eukaryotic cells[5]. There is much interest in these transposons as insertional mutagens because they have a small target site for integration (for SB, this is TA) and require few host cell factors for their activity. SB is a bipartite transposition system consisting of the transposase and transposon vector, which is flanked by the binding sites for the SB transposase, the so-called inverted repeats/direct repeats (IRDRL (left) and IRDRR (right)). Transposons are mobilized by SB transposase supplied *in trans*. SB is active in the mouse germ line[6–8] (one or two transpositions per animal born) and mouse somatic cells[7,9] but the transposition frequency is too low to be useful for most genetic screens[10].

Analysis of SB transposition integration sites cloned from the mouse germ line indicates that SB has fewer transposition site biases than retrotransposons, increasing its potential as an insertional mutagen[9,11]. However, SB does show a small but significant bias towards genes and their upstream regulatory sequences, although this bias is much less than that observed with retroviruses[12]. SB elements are also not locked in place after transposition and can continuously transpose to new sites. These qualities would make SB an attractive insertional mutagen for functional genomic screens in higher eukaryotes if the frequency of transposition could be increased.

A limitation of SB is that transposed elements tend to reintegrate at sites linked to the donor site. Previous studies showed that 50–80% of germline SB transpositions are located within 10–25 megabases of the donor site[7–9]. This problem can also be overcome by increasing SB transposition frequencies to levels in which local transposition is no longer rate limiting for genome-wide insertional mutagenesis.

Creating a highly active SB mutagenesis system

To develop a more active eukaryotic SB transposition system we made several enhancements to the SB transposition system used previously. We generated a mutagenic transposon vector, T2/Onc2 (Fig. 1a). This transposon is similar to that described by an accompanying paper (ref. 13) but contains a larger fragment of the *engrailed-2* (*En2*) splice acceptor (SA) and is flanked by optimized SB transposase binding sites that increase SB transposition[14]. It is also smaller than other SB transposons used previously (about 2.0 kilobases (kb)) and approaches optimal size for transposition[15]. T2/Onc2 contains two splice acceptors and a bi-directional poly(A) and can terminate transcription when integrated in either orientation in a gene. It also contains a murine stem cell virus (MSCV) long terminal repeat (LTR) and a splice donor (SD) and can promote gene expression when integrated upstream or within a gene. Thirty T2/Onc2 transgenic founders were generated after microinjection. Because SB transposes by a cut-and-paste mechanism, the number of transposons in the transgene concatomer can initially limit the number of transposition events. Any methylation present on the transposon could also be transferred to new sites within the genome. Methylation of the MSCV promoter might therefore inhibit its ability to affect expression of neighbouring genes. With this in mind, founder transgenic animals were screened to determine their transposon copy number and the methylation status of the MSCV promoter (Fig. 1b, Supplementary Fig. 1). Three founder transgenic animals containing a high copy number of unmethylated transposons (6057, 6070, 6113) were used to establish transgenic lines (Fig. 1b). Transposon concatomers from each line were transmitted

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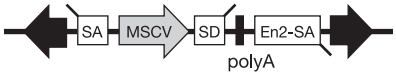
a



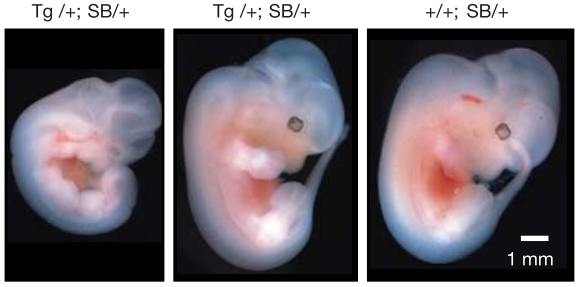
b

Founder no.	Copy no.	Percentage methylated	No. of unmethylated copies
5985	84	71	24
5993	38	78	8
6044	34	95	2
6057	148	80	30
6065	65	88	8
6070	214	83	36
6113	358	79	77
6132	37	22	29
6136	57	63	21

c

Time point	No. of embryos	Double-transgenic
E10	34	9 (26.5%)
E16	22	3 (13.6%)
Live born	334	31 (9.3%)

d



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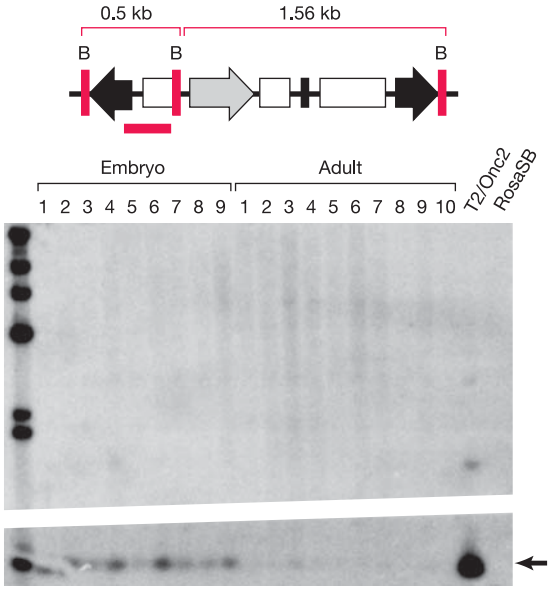


Figure 1 | Analysis of double-transgenic embryos and adults. a, Structure of the T2/Onc2 transposon. **b**, Estimates of transgenic transposon copy number, and percentage of methylated transposons determined after digestion with *Dra*I–*Msp*I or *Dra*I–*Hpa*II. **c**, Reduced number of E16 double-transgenic embryos and adults. **d**, Double-transgenic embryos (left panel) were often smaller than control littermates. **e**, The 500-bp *Bam*HI concatemer fragment (arrow) is reduced in intensity in double-transgenic embryos and adults relative to the T2/Onc2 heterozygous transgenic control. For adult tissues, brain DNA is in the odd-numbered lanes and kidney DNA is in the even-numbered lanes.

at normal mendelian frequencies, and heterozygous mice showed no obvious phenotype.

Next, we generated a transposase knock-in allele to avoid the epigenetic silencing often seen with transgenes. To increase SB transposition we generated the knock-in by using the SB11 transposase[15]. This transposase contains four amino acid substitutions that increase its activity above that of the SB10 transposase used previously. An expression cassette consisting of a splice acceptor site upstream of the SB11 complementary DNA followed by an SV40 polyadenylation signal was targeted to the *Rosa26* locus to generate the RosaSB allele (Supplementary Fig. 2). This site was chosen because genes targeted to this locus are expressed ubiquitously during development and in adult mouse tissues. Western blotting confirmed the expression of the SB11 transposase in RosaSB mice (data not shown), and quantitative polymerase chain reaction (PCR) indicated that the RosaSB allele is equally expressed in all tissues tested (brain, spleen, skin and lung) (data not shown). Heterozygous RosaSB mice were aged for more than a year and showed no obvious phenotype.

RosaSB mice were then crossed to each T2/Onc2 transgenic line to generate a cohort of mice harbouring both elements. Unexpectedly, intercross offspring showed a non-mendelian inheritance pattern with a significant decrease in progeny inheriting both the RosaSB transposase and the T2/Onc2 transgene. All three T2/Onc2 lines produced fewer double-transgenic progeny than expected, although the frequency varied between the lines (TG6070, 17/136 (12.5%); TG6057, 5/89 (5.6%); TG6113, 9/109 (8.3%)). We proposed that this decrease in viability was due to lethality induced by SB transposition and/or DNA damage that was not repaired after SB excision. Previous studies with mice deficient for proteins involved in nonhomologous end joining indicate that lymphocytes and neurons are particularly sensitive to double-strand breaks during development[16,17]. To test this hypothesis, we characterized embryos at various developmental time points. Normal frequencies of double-transgenic embryos were observed at embryonic day 10 (E10), whereas a significant decrease was seen by E16 (Fig. 1c). Embryos at both time points seemed grossly normal, although many double-transgenic embryos seemed smaller than control littermates (Fig. 1d). Histopathological examination of double-transgenic embryos showed various developmental abnormalities unique to each embryo (data not shown).

To determine whether SB transposition occurs in double-transgenic embryos, we asked whether SB transposons had been excised from transposon concatomers in double-transgenic embryos, because excision is the first step in transposition. *Bam*HI sites are located within the plasmid sequences that flank each transposon in the concatomer and in the transposon itself (Fig. 1e). Consequently, any transposon in the concatomer will generate a 500-bp fragment by using the probe indicated. It is unlikely that *Bam*HI sites will immediately flank a transposon after transposition. Reintegrated transposons will therefore primarily generate *Bam*HI fragments that are larger than 500 bp. Analysis of nine double-transgenic embryos showed that most transposons were excised from the concatomer by E10 (Fig. 1e). Analysis of the brain and kidney of ten adult double-transgenic animals showed that transposon excision continues in the adult, until by postnatal day 45 virtually all of the transposons within the concatomer have been excised (Fig. 1e). Excision therefore begins early in development and continues into the adult, affecting virtually all cell types.

Previous studies showed that 75% of excised transposons re-integrate into the mouse genome[10]. To confirm that excised transposons reintegrate into the mouse genome we used ligation-mediated PCR (LM-PCR)[18] to amplify SB junctions from ten double-transgenic embryos. LM-PCR is a powerful new amplification method that makes it possible to amplify and sequence thousands of SB transposition sites rapidly (Supplementary Methods). Ninety-six SB junction fragments were picked randomly and sequenced from each amplified embryo library (Supplementary Table 1). BLAST

searches of the 490 independent transposon junctions showed that most junctions were rare and represented only once among the clones analysed (Supplementary Table 1), indicating that each junction is present in a limited number of cells. This is consistent with Southern blot data, which showed no detectable newly acquired SB transposons in double-transgenic embryos (Fig. 1e). SB transposons are therefore reintegrating into the mouse genome at many sites in double-transgenic mice.

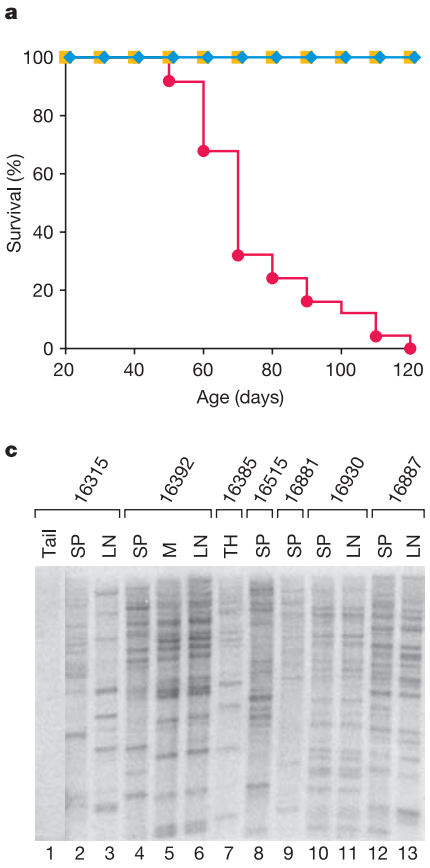
T2/Onc2 concatemer integration sites are located on chromosomes 1, 4 and 6 (data not shown). As expected, we saw an increased frequency of transposons reintegrated on these chromosomes in double-transgenic embryos. The percentage of local transposition within a 25-megabase region varied from 6% to 11% in double-transgenic embryos (data not shown) in contrast to germline transpositions reported by others, in which 50–80% of the transpositions were local[7–9]. Even when transposons landed on the same chromosome as the transgene concatemer, the transposon integrations were well distributed across the chromosome and there was no easily defined local hopping interval. We speculate that the higher SB transposition frequencies obtained with our system permit secondary and tertiary rounds of transposition, which masks local transposition. This high rate could be attributed to a more optimal expression of the SB11 transposase from the RosaSB allele. Previous work has indicated that even moderate changes in SB transposase expression can have a significant impact on transposition frequency[15].

SB transpositions in the embryo are fairly well distributed across the genome. When T2/Onc2 integrated in or near a gene there was little preference for a gene region or orientation relative to the nearest gene (Supplementary Tables 1 and 5). Only four regions in the genome (less than 30 kb in size) contained two SB transposon integrations in independent embryos (Supplementary Table 2), which is similar to the number (3) predicted by Monte Carlo simulations for random integration, and no region (less than 100 kb in size) contained three SB transposon integrations. The embryo data therefore seem to represent a population of unselected transposon integrations.

Double-transgenic mice are tumour-prone

Twenty-four double-transgenic mice that survived to weaning were monitored for tumour development. At 7 weeks of age the mice began to show signs of illness, and by 17 weeks all the mice had died from cancer (Fig. 2a). Multiple tumour types were identified: the most common tumour was T-cell lymphoma (Fig. 2b). Tumour cells were frequently found in all tissues of the animal and in some cases a single animal developed two or even three different cancer types (Fig. 2b). Haematopoietic tumours predominated, possibly reflecting the large pool of haematopoietic stem cells present in mice. Medulloblastoma, a solid tumour of the cerebellum, was also observed in two mice; intestinal and pituitary neoplasia was seen in other animals. Thus, unlike retroviral insertional mutagenesis, SB mutagenesis is not limited to the haematopoietic system. Stained sections of the medulloblastoma from animal TG6057-17106 (Fig. 3a, c) and a corresponding normal cerebellum (Fig. 3b, d) showed that the normal morphology of the cerebellum was disrupted, with tumour cells invading the molecular layer (Fig. 3a). Tumour tissue also extended down the brain stem and could be seen adjacent to the spinal cord (Fig. 3c). This is similar to what is observed in human medulloblastoma.

*Bam*HI-digested tumour DNAs were subsequently analysed by



b

Mouse ID	Age (days)	Tumour type
TG6057-16315	114	T-cell
TG6057-17106	101	Medulloblastoma
TG6057-17306	84	T-cell, non-T/non-B
TG6057-18137	45	B-cell
TG6070-16392	57	B-cell
TG6070-16515	65	T-cell (thymic), non-T/non-B (disseminated)
TG6070-16881	58	Non-T/non-B
TG6070-16887	62	T-cell
TG6070-17221	60	T-cell
TG6070-17223	87	B-cell, hyperplasia in duodenum
TG6070-17503	102	Non-T/non-B
TG6070-17900	68	T-cell (thymic), non-T/non-B (disseminated)
TG6070-17902	68	T-cell
TG6070-18533	73	T-cell
TG6070-18665	53	T-cell
TG6070-18732	47	T-cell, hyperplasia in duodenum
TG6070-18804	58	Non-T/non-B, medulloblastoma, intestinal neoplasia
TG6113-16835	67	T-cell
TG6113-16927	96	T-cell, pituitary hyperplasia
TG6113-16930	71	T-cell
TG6113-17548	52	T-cell, non-T/non-B
TG6113-18019	59	Non-T/non-B
TG6113-18022	62	T-cell, non-T/non-B
TG6113-18521	62	T-cell

Figure 2 | Adult double-transgenic mice die from cancer. **a**, Survival curves showing decreased viability of double-transgenic mice: yellow, RosaSB; blue, T2/Onc2; red, double-transgenic. **b**, Age at death and tumour type of double-transgenic mice. **c**, Southern analysis of *Bam*HI-digested tumour DNA. Each band represents a separate SB transposon integration. LN, lymph node; M, mass; SP, spleen; TH, thymus.

Southern blotting to determine whether they contained clonal or subclonal SB transpositions. As expected, Southern blotting failed to identify clonal, somatically acquired transposon integrations in tail DNA (Fig. 2c, lane 1) or in DNA from normal brain and kidney (Fig. 1e). In contrast, numerous clonal and subclonal transposon integrations were seen in lymph nodes, spleen and thymus in tumour DNA (Fig. 2c, lanes 2–13). The pattern of transposon integrations in different tumour tissues from the same animal was similar but not identical (Fig. 2c, lanes 4–6, 12 and 13), indicating that some transpositions are lost while others are gained during tumour development. These results are consistent with insertional mutagenesis of cancer genes as the disease-inducing mechanism.

Analysis of SB integration sites in tumour DNA

To confirm that these tumours are induced by insertional mutagenesis, we cloned and analysed 781 SB junctions from 16 tumours (Supplementary Table 3). In contrast to the results from embryos (Supplementary Table 2), we identified multiple genes that were mutated by SB integration in two or more tumours (Supplementary Table 4). These results are unlikely to have occurred by chance. Seven of these genes are validated human cancer genes, and a further seven are mutated by retroviral integration in mouse leukaemias (http://rtcgd.ncifcrf.gov). We also identified four genes that were mutated more than once in the same tumour (two *Notch1* integrations (TG6057-16315), two *Jak1* integrations (TG6070-16887), two *Csf3r* integrations (TG6070-17306), two *Erg* integrations (TG6070-17900) and three *Erg* integrations (TG6070-16881)) (Supplementary Table 4). This could reflect tumour microheterogeneity, with the different integrations occurring in different subpopulations of tumour cells during tumour progression. We also identified integrations in several genes that have not yet been examined for a role in human cancer but which represent excellent disease gene candidates (Supplementary Table 4).

Like the embryo integrations, tumour integrations were widely distributed across the genome with little local hopping (data not shown). However, integrations in tumour DNA located upstream or within genes showed an orientation bias that was not found in embryo integrations (Supplementary Table 5). In tumours, 65% of transposons located 5′ of genes are in the same transcriptional orientation as the gene, compared with 52% for integrations in embryos ($P < 0.001$). In addition, 62% of transposons located within genes are in the same orientation, compared with 54% for integrations in embryos ($P < 0.001$). Unlike retroviruses, which have strong enhancer activity and can activate gene expression over large distances, T2/Onc2 seems to have little enhancer activity. This is supported by our failure to identify common integration sites in which transposons are integrated downstream of the gene (Supplementary Table 4) and by recent data showing that SB transposons that lack viral LTR and corresponding SD sequences fail to increase the expression of nearby genes significantly[12]. Consequently, T2/Onc2 primarily activates gene expression by integrating upstream of a gene or in an upstream intron and promoting the expression of the gene from the MSCV LTR, or by integrating into the coding region and either promoting the expression of a truncated protein or truncating the transcript prematurely (Supplementary Table 4; data not shown). This lack of enhancer activity greatly simplifies the identification of cancer genes mutated by SB.

Activating *Notch1* transpositions

Activating *NOTCH1* mutations have been identified in more than 50% of human T-cell acute lymphoblastic leukaemia (T-ALL) cell lines[19]. Among ten SB-induced T-cell lymphomas analysed, six contained SB integrations in intron 27 of *Notch1* (Fig. 4a, Supplementary Table 4). These transposon integrations mapped to three different sites in intron 27, indicating that transposition is not totally random or that integration at these sites is selected because of their effect on *Notch1* expression. All six integrations are oriented in the same transcriptional direction as *Notch1* and induce the expression of a *Notch1* fusion transcript containing the MSCV promoter and the

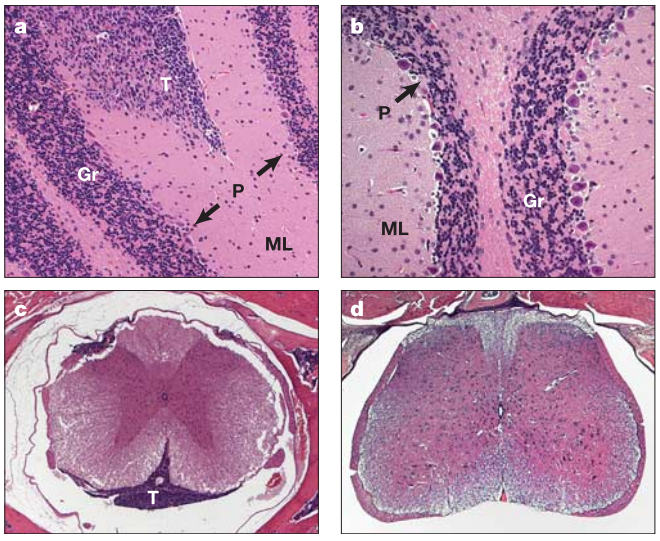


Figure 3 | Medulloblastoma pathology. Sections of an SB-induced medulloblastoma and control cerebellum, stained with haematoxylin and eosin. **a**, Section of the cerebellum from animal TG6057-17106 showing normal morphology, with the Purkinje cell layer (P) adjacent to the granule cell layer (Gr). Tumour cells (T) have invaded the molecular layer (ML). **b**, Comparable section for a normal cerebellum. **c**, Tumour (T) has grown down the brain stem adjacent to the spinal cord. **d**, Comparable section for a normal spinal cord.

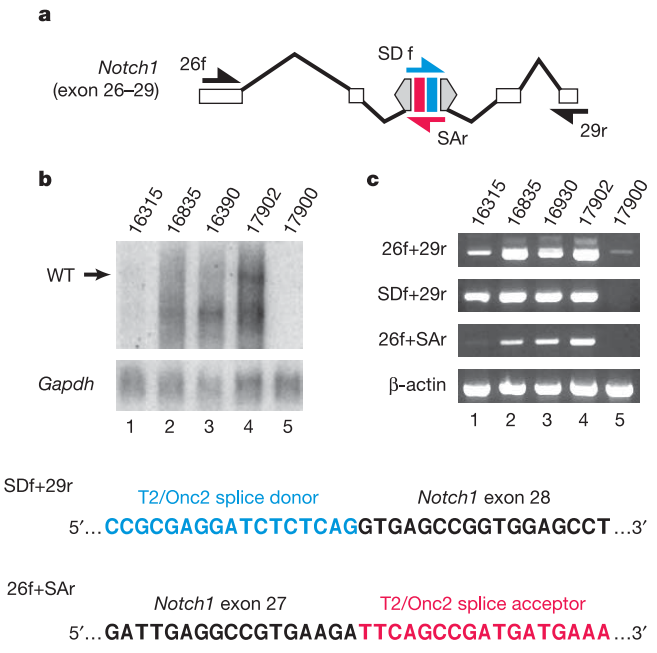


Figure 4 | Analysis of *Notch1* integrations. **a**, Structure of mutated *Notch 1* allele in SB-induced T-cell leukaemias. White, exons; grey, transposon IRDRs; red, transposon splice acceptor; blue, splice donor; arrows, primer binding sites (f, forward; r, reverse). **b**, Northern analysis using a 3′ *Notch1* cDNA probe showed that all tumours with *Notch1* integrations (lanes 1–4) express a truncated *Notch1* transcript. The transcript in tumour 16315 is less intense but can be seen on longer exposure. WT, wild type. **c**, RT–PCR shows that only tumours with *Notch1* integration express a truncated *Notch1* transcript. The sequence of the SB-*Notch1* splice junction in tumours is shown below.

3′ end of *Notch1* (Fig. 4b, c). This fusion transcript mimics that seen in human T-ALL patients with t(7;9), in which the translocation drives expression of an activated *NOTCH1* carboxy-terminal protein fragment[20]. Furthermore, transgenic mice overexpressing a similar fragment of *Notch1* develop T-cell lymphoma[21]. These results confirm that SB-induced tumours are induced by insertional mutagenesis.

Cooperating cancer genes and pathways

Among the six tumours with activating integrations at *Notch1*, three also had activating integrations upstream of *Rasgrp1* (Fig. 5, Supplementary Table 4), a gene that positively regulates *Ras* signalling. The probability of finding two tumours with integrations in the same two pair of genes by chance is low ($P = 9.2 \times 10^{-5}$; Supplementary Methods). Integrations in *Rasgrp1* were seen only in tumours with *Notch1* integrations, indicating that *Ras* signalling might cooperate with *Notch1* in tumour induction. Two tumours with *Notch1* and *Rasgrp1* integrations also had activating integrations upstream of *Sox8*, an uncharacterized member of the Sox family of *SRY*-related high-mobility-group (HMG)-box DNA-binding proteins (Fig. 5). The probability of finding two tumours with integrations in *Notch1*, *Rasgrp1* and *Sox8* by chance is exceedingly low ($P = 2.2 \times 10^{-7}$) and indicates that *Sox8* could represent another signalling pathway that cooperates with *Notch1* in tumour induction. Finally, two *Notch1* tumours also have activating integrations upstream of *Runx2* (Fig. 5), indicating that *Runx2* might represent yet another *Notch1*-cooperating gene.

Although most genes mutated by SB transposition in tumours were identified in only one tumour, several belong to related signalling pathways. Careful annotation identified seven pathways that were commonly disrupted in SB-induced tumours (Supplementary Table 6). Similar analysis of the integration sites cloned from embryos did not reveal any similar trends. Integrations in most cases are predicted to affect a given pathway in a similar manner, but they accomplish this through the disruption of different genes. For example, six tumours have transposon-induced mutations that are predicted to result in decreased TNF signalling. Similarly, decreased rates of receptor recycling, increased signalling through the Ras superfamily, increased Jak/Stat signalling and increased Wnt signalling are all common pathways affected by SB transposition in tumours (Supplementary Table 6). The identification of genes and signalling pathways that cooperate to induce cancer will make it possible to develop better combinatorial therapies for treating human cancer.

a

Tumour ID	Gene	Clonal integration
16315	*Notch1*	Yes
16835	*Notch1*	Yes
	Rasgrp1	No
	Runx2	No
16887	*Notch1*	Yes
	Rasgrp1	Yes
	Sox8	No
16930	*Notch1*	Yes
	Runx2	Yes
17902	*Notch1*	Yes
	Rasgrp1	No
	Sox8	Yes

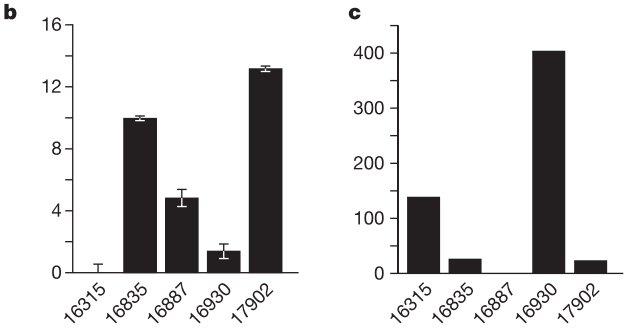


Figure 5 | *Notch1* cooperating genes. a, Clonality of *Notch1*, *Rasgrp1*, *Sox8* and *Runx2* integrations in *Notch1* tumours was determined by Southern analysis. **b**, **c**, Quantitative PCR was used to measure the expression levels of *Rasgrp1* (**b**) and *Runx2* (**c**) in tumours relative to a glyceraldehyde-3-phosphate dehydrogenase gene, *Gapdh* control. Results are means ± s.d. for three independent assays. Error bars in **c** are too small to show. Quantitative PCR could not be reliably performed on *Sox8*; *Sox8* expression in tumours with *Sox8* integrations was therefore monitored by RT–PCR (data not shown).

Discussion

We describe here a non-viral insertional mutagen that efficiently induces tumours in wild-type mice. SB transposition can easily be controlled to mutagenize a specific target tissue by simply restricting the site of SB transposase expression. In principle, SB transposition can be adapted to generate virtually any kind of cancer by restricting the sites or timing of transposase expression. This should also reduce or eliminate the embryonic death observed in our studies, in which the SB transposase was widely expressed. The high frequency of transposition possible with our system might also prove valuable in generating tumours in other cell types and in wild-type genetic backgrounds that do not carry a predisposing cancer mutation. This will make it possible to model various types of human cancer without any knowledge of the causative events and in a more unbiased manner. Together with high-throughput LM-PCR, cancer genes and their pathways associated with tumorigenesis can be rapidly identified, providing insight into human cancer through the use of mouse models. Given the unexpectedly high somatic SB transposition frequencies achieved in our studies, there is no theoretical reason why SB transposition frequencies cannot be increased in the mouse germ line to levels that would permit efficient forward genetic screens with SB. Because SB tags the mutated gene, the gene is much easier to clone than one mutated by a point mutagen such as ethylnitrosourea. Finally, the uses of SB are also not restricted to the mouse. SB was originally isolated from fish and has already been shown to function in zebrafish[22] and medaka[23]. SB could therefore be used in forward genetic screens in any higher eukaryote in which transgenesis is possible.

METHODS

Generation of the RosaSB allele. An expression cassette consisting of an *En2* splice acceptor, SB11 cDNA and SV40 poly(A) was cloned upstream of a floxed PGKneo cassette. This cassette was then recombined into a plasmid containing a TK selection cassette as well as the promoter region, exon 1 and a portion of the single intron of the *Rosa26* locus by using the recombineering strategy described previously[24]. This recombination introduced the knock-in cassette into the *Xba*I site of the *Rosa26* intron that has been used in previous *Rosa26* knock-in alleles[25]. The targeting plasmid was then linearized and introduced into ES cells. After selection, ES cell colonies were picked and DNA was extracted and digested with *Spe*I to screen the 5′ region of *Rosa26* and *Bgl*I for the 3′ region. Southern blotting was performed on the 5′ region with the use of a 908-bp *Sac*I fragment of the *Rosa26* promoter region. A 667-bp *Ssp*I fragment derived from the intron of *Rosa26* was used to confirm the 3′ recombination site by Southern blot analysis. Three independent clones were injected into blastocysts to derive three RosaSB knock-in lines. Mice were genotyped by PCR with primers specific for the SB11 cDNA: 5′-ATGGGAAAATCAAAAGAAATCAGCCAAG-3′ and 5′-GCCAAA CAGTTCTATTTTTGTTTCATCAGACCA-3′. One line was subsequently maintained by backcrossing to C57BL/6 mice.

Generation of T2/Onc2 transgenic mice. The T2/Onc2 transposon was made by replacing the *Hpa*I/*Bgl*II fragment containing the *En2* splice acceptor from pT2/Onc with a fragment containing a larger portion of the *En2* exon. In addition to this change, the overall size of T2/Onc2 was reduced (2,050 bp, compared with 2,163 bp for T2/Onc) but was otherwise identical to T2/Onc. The

pT2/Onc2 plasmid was linearized using *Sca*I and prepared for microinjection into (B6C3)F_2 hybrid embryos with standard techniques. Tail biopsy DNA from founder animals was screened by Southern blotting using an *En2* splice acceptor probe. Transgenic lines were established by crossing to C57BL/6. Offspring were genotyped by PCR using primers 5′-CAGTTGAAGTCGGAAGTTTA-3′ and 5′-GGAATTGTGATACAGTGAAT-3′.

Calculation of expected number of common sites. A Java program was created to simulate the random SB transposon insertions in the mouse genome. The program randomly selected 491 (number of sites cloned from embryos) or 782 (number of sites cloned from tumours) TA motifs from the whole mouse genome by using a random number generator. The program then counted the number of common integration sites by calculating distances between the integration sites. After repetition of this procedure 10,000 times, the average expected number of common integration sites was determined.

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