Somatic Mutagenesis with a 
*Sleeping Beauty* Transposon System Leads to Solid Tumor Formation in Zebrafish

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Abstract

Large-scale sequencing of human cancer genomes and mouse transposon-induced tumors has identified a vast number of genes mutated in different cancers. One of the outstanding challenges in this field is to determine which genes, when mutated, contribute to cellular transformation and tumor progression. To identify new and conserved genes that drive tumorigenesis we have developed a novel cancer model in a distantly related vertebrate species, the zebrafish, *Danio rerio*. The *Sleeping Beauty* (*SB*) *T2/Onc* transposon system was adapted for somatic mutagenesis in zebrafish. The carp β-actin promoter was cloned into *T2/Onc* to create *T2/OncZ*. Two transgenic zebrafish lines that contain large concatemers of *T2/OncZ* were isolated by injection of linear DNA into the zebrafish embryo. The *T2/OncZ* transposons were mobilized throughout the zebrafish genome from the transgene array by injecting SB11 transposase RNA at the 1-cell stage. Alternatively, the *T2/OncZ* zebrafish were crossed to a transgenic line that constitutively expresses SB11 transposase. *T2/OncZ* transposon integration sites were cloned by ligation-mediated PCR and sequenced on a Genome Analyzer II. Between 700–6800 unique integration events in individual fish were mapped to the zebrafish genome. The data show that introduction of transposase by transgene expression or RNA injection results in an even distribution of transposon re-integration events across the zebrafish genome. SB11 mRNA injection resulted in neoplasms in 10% of adult fish at ~10 months of age. *T2/OncZ*-induced zebrafish tumors contain many mutated genes in common with human and mouse cancer genes. These analyses validate our mutagenesis approach and provide additional support for the involvement of these genes in human cancers. The zebrafish *T2/OncZ* cancer model will be useful for identifying novel and conserved genetic drivers of human cancers.

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Introduction

In sporadic human cancers the initiating event is proposed to be an oncogenic mutation that activates cell growth pathways and leads to cellular transformation. Subsequent cancer progression and metastasis are associated with genomic instability and defective DNA repair, leading to the accumulation of large numbers of somatic mutations [1,2,3]. Large scale sequencing of human cancer genomes has revealed a vast array of somatic mutations in breast, colon, lung, glioma, ovarian, pancreatic, and prostate cancers [4,5,6,7,8,9]. A subset of these somatic mutations, termed drivers, are thought to provide a selective growth advantage in cancer cells, promoting cellular transformation and progression to metastasis. The majority of mutations, termed passengers, are carried along as the cancer cell population expands. Distinguishing driver and passenger mutations within the human cancer genome has been a major hurdle in identifying causal mutations in different cancers. A major goal in the field is to correlate cancer genotype with clinically relevant phenotypes in order to develop effective therapies for specific cancers.

One approach to identify new cancer genes and associated causal mutations is to compare the mutation profiles between human cancers and animal cancer models. A successful strategy for cancer gene identification has been developed in mice using the DNA transposon system *Sleeping Beauty* (*SB*) for insertional somatic mutagenesis [10,11,12,13]. The *SB* system was originally reconstituted from inactive elements present in the genomes of salmonid species [14]. Because *SB* shows minimal site specificity aside from the presence of a TA dinucleotide at the integration site [15], it is an effective tool for random mutagenesis. In the mouse somatic mutagenesis cancer models the mutagenic *SB* transposon, *T2/Onc*, was designed to create loss- and gain-of-function mutations similar to those that drive tumorigenesis in sporadic human cancers. The system was shown to induce primarily hematopoietic tumors in adult mice and solid tumors at a lower frequency [10,11]. Recent modifications to the system using an alternative *T2/Onc*...
transposon and inducible, tissue-specific transposase sources have been shown to produce a wide range of epithelial-derived carcinomas [16] and tissue-specific cancers including hepatocellular carcinoma [17] and gastrointestinal neoplasms [18]. Analyses of common integration sites across multiple tumor samples have identified putative cancer genes associated with the various types of neoplasms in the mouse T2/Onc-induced cancers. Comparative studies with the genes identified in mice and human cancer genomes have revealed a set of mutated genes in common with human cancers [17,16,19]. These studies underscore the importance of model systems for validating known cancer genes and identifying novel genes and signaling pathways mutated in sporadic human carcinogenesis.

The development of cancer models in the more distantly related vertebrate species, zebrafish, has demonstrated the conservation of gene pathways underlying cancer [20,21,22,23]. Zebrafish models of human cancers have been created in which tissue-specific over-expression of oncogenes causes soft tissue cancers and solid tumors [24,25,26,27,28,29]. Loss of function tumor suppressor lines have been identified that result in solid tumors at varying frequencies [30,31,32]. Application of the mouse SB-induced mutagenesis strategy [10,11] would provide a new method to genetically screen for novel cancer genes in zebrafish. Given the high fecundity and inexpensive cost of zebrafish compared to mammalian model systems, large-scale screens approaching saturation mutagenesis of the cancer genome are a possibility in fish. Here we report the adaptation of the SB T2/Onc system for somatic mutagenesis in zebrafish. The T2/Onc transposon was modified by addition of the carp β-actin promoter[33], which is widely expressed in zebrafish tissues. We isolated stable transgenic lines that have large copy number concatamers of T2/Onc genes. Introduction of a transient or constitutive source of SB11 transposase activates T2/Onc excision from the concatamer and genome-wide re-integration in somatic tissues. Somatic mutagenesis leads to solid tumor induction in ~10% of adult fish. Comparative analysis of the T2/Onc integration sites from the zebrafish tumor samples reveals a many genes in common with human and mouse cancer genes. This study demonstrates the capability of the Sleeping Beauty T2/Onc system for genome-wide insertional mutagenesis in somatic tissues in zebrafish and its potential for identifying novel cancer genes.

**Results**

A modified T2/Onc transposon system for somatic mutagenesis in zebrafish

The T2/Onc transposon used in previous mouse models contains elements to terminate transcription and promote overexpression after the transposon inserts in or near a gene [10,11]. The MSCV 5’ LTR promoter in T2/Onc drives high-level expression in hematopoietic tissues in mouse. However, it was unknown how well this promoter would be expressed when integrated into the zebrafish genome. In addition, we wanted to build a T2/Onc transposon that contains a promoter that is widely expressed in various tissues and cell types. We altered T2/Onc by addition of the carp β-actin promoter [33], which has been used extensively to drive constitutive and ubiquitous expression in fish species [34]. The promoter is defined by 2.5 Kb of upstream sequence, the first exon which is non-coding, and the first intron which contains elements necessary for high level expression from the promoter [33]. The splice acceptor sequences (SA) at the 3′ end of intron 1 were removed to force splicing out of the transposon and into downstream exons after insertion into a gene. The modified β-actin promoter was cloned into T2/Onc behind the MSCV 5’ LTR to create T2/Onc-(β-actin)(Fig. 1 A).

To isolate transgenic fish carrying large arrays of T2/Onc we used a standard transgenesis method of injecting linear DNA fragments into the 1-cell zebrafish embryo. A 4.9 Kb linear fragment containing T2/Onc was released from the plasmid vector and mixed with a linear fragment containing a widely expressed β-actin:RFP reporter cassette [36] before injection (Fig. 1 A). After injection into the 1-cell embryo the T2/Onc and RFP reporter fragments ligate via the non-homologous end-joining pathway to form a concatamer that inserts randomly into the genome. We used the RFP reporter to screen for transgenic progeny and to follow the concatamer through subsequent generations. Out of 54 founder F0 adults screened by outcrossing with wild type zebrafish, we identified 7 F0s that produced from 3–10% RFP+ embryos in the F1 generation (Fig 1 B). The 7 independent F1 lines were raised and outcrossed to create an F2 generation. The transgenic embryos are easily distinguished by ubiquitous RFP expression, and adults appear red due to high levels of RFP expression from multiple β-actin:RFP reporter cassettes in the concatamer (Fig. 1 B).

The copy number of the T2/Onc transposon in the concatamer lines was determined by genomic Southern blot analysis of DNA isolated from F2 adults (Fig. 1 C). Plasmid DNA containing the T2/Onc transposon was loaded in amounts corresponding to 1, 10 or 100 copies/ genome and used as a reference to estimate the transposon copy number in each line by densitometry. The number of transposons in the concatamers ranged from ~5 to >100 copies (Fig. 1 C). Additional Southern blot analyses consistently indicated that the number of transposons in the Tg(T2/Onc-β-actin:RFP) lines far exceeded 100. To clarify this issue, we again examined the estimated copy number of transposons in the line 5 and line 6 concatamers in the F3 generation (Fig. 1 D). The results indicate that line Tg(T2/Onc-β-actin:RFP) contains 70–100 transposons, while Tg(T2/Onc-β-actin:RFP) contains >500 transposons. While our analysis provides only an estimate of the actual number of T2/Onc transposons in the concatamers, the important point, as demonstrated below, is that both concatamer lines have a large number of transposons that are capable of robust transposition and reintegration in somatic tissues. We have maintained lines Tg(T2/Onc-β-actin:RFP) and Tg(T2/Onc-β-actin:RFP) and observed that the concatamers appear stable, behave like a single copy locus, and show a Mendelian pattern of inheritance through 5 generations to date (data not shown).

To isolate a transgenic line that constitutively expresses SB11 transposase [37], a transgenesis vector was assembled inside the SB Tol2 transposon [38] for Tol2 transposase-mediated integration into the genome. The carp β-actin promoter [33] was used to express SB11 cDNA from within the transposon (Fig. 2 A). For a marker of transgenesis, a heart-specific reporter cassette containing the cardiac myosin light chain 2 (cmlc2) promoter [39,40] driving EGFP was cloned into the miniTol2 vector 3′ to the SB11 expression cassette. To isolate transgenic lines carrying single copies of the β-actin:SB11, cmlc2:EGFP transposon, the vector and Tol2 mRNA were co-injected into 1-cell embryos. Founder F0 embryos were raised to adulthood and screened for transgenic progeny by outcrossing with wild type zebrafish and scoring EGFP expression in the heart. Nine independent lines were isolated that carry a single Tol2:<β-actin:SB11, cmlc2:EGFP> transgene insertion and two were maintained (Table 1). Expression of the SB11 transposase was confirmed in line Tg(Tol2:<β-actin:SB11, cmlc2:EGFP>) by western blotting of 5-hour post fertilization (hpf) embryos (Fig. 2 B).
Figure 1. Isolation of transgenic T2/OncZ concatemer lines. (A) T2/OncZ transposon vector and RFP reporter used to isolate concatemers. IRL and IRR, left and right transposon inverted repeats; SA, splice acceptor from intron 1 of β-actin gene; MSCV 5’ LTR, murine stem cell virus 5’ long terminal repeat; β-actin, β-actin promoter minus splice acceptor at 3' end; SD, splice donor; En2-SA, splice acceptor from mouse engrailed 2 gene; pA, SV40 polyadenylation sequence; AFP, ocean pout antifreeze protein 3’ UTR; black bar represents probe used on genomic Southerns shown in panel C. Grey box represents probe used on genomic Southerns shown in panel D. Linear DNA fragments of T2/OncZ and the β-actin:RFP reporter gene were mixed and co-injected into 1-cell zebrafish embryos. (B) Adult F0 founders were outcrossed to wild type and transgenic F1 embryos identified by ubiquitous RFP fluorescence. (C) Genomic Southern blots to estimate transposon copy number in Tg(T2/OncZ, β-actin:RFP)fluorescer and Tg(T2/OncZ, β-actin:RFP)fluorescer adults. Plasmid pT2/OncZ was loaded as reference in copy #: control lanes.

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These results demonstrate that the SB11 transposase expressed in transgenic fish is capable of excising the concatemer and repairing the excision site. In addition, the results show that the SB11 transposase expressed in transgenic fish is capable of excising the concatemer from either an mRNA or a transgene can actively promote mobilization of transposons out of the concatemer. 

Figure 2. Isolation of transgenic zebrafish expressing constitutive SB11 transposase. (A) Diagram of miniTol2 vector containing a constitutive β-actin promoter: SB11 cDNA cassette. 5’ and 3’, Tol2 inverted terminal repeats; zf β-a 3’UTR, zebrafish β-actin 3’UTR; cmlc2, zebrafish cardiac myosin light chain 2 promoter. (B) Western blot demonstrates the expression of SB11 in Tg[Tol2<β-actin:SB11, cmlc2:GFP>] transgenic embryos. The blot was stripped and re-probed with an anti-β-actin antibody for loading control. doi:10.1371/journal.pone.0018826.g002

To demonstrate the activity of the SB11 transposase and the ability of the T2/OncZ transposon to excise from the concatemer, we used a PCR-based assay as described previously [10]. We used two methods for introducing SB11 transposase into the Tg[T2/OncZ, β-actin:RFP] transgenic fish. In the first method, in vitro transcribed SB11 mRNA was injected into 1-cell embryos from Tg[T2/OncZ, β-actin:RFP] fish (Fig. 3 A). In the second method, double transgenic embryos were recovered by crossing the Tg[T2/OncZ, β-actin:RFP] line to the Tg[Tol2<β-actin:SB11, cmlc2:GFP>] line to the Tg[T2/OncZ, β-actin:RFP] line. The excision PCR product, which indicates mobilization of transposons out of the concatemer, is expected to amplify a 200 bp product only if the transposon with SB11 transposase cassette contains heart-specific GFP reporter cassette. This assay was performed to test whether the T2/OncZ transposon integration after transient or constitutive exposure to SB11 transposase

Demonstration of genome-wide T2/OncZ transposon integration after transient or constitutive exposure to SB11 transposase

To test whether the T2/OncZ system is effective at producing genome-wide somatic insertion mutations, we examined the extent of re-integration following injection of SB11 mRNA into 1-cell stage embryos that had either the Tg[T2/OncZ, β-actin:RFP] (~500 copies) or Tg[T2/OncZ, β-actin:RFP]* (~70 copies) concatemer (Fig. 3 A, Table 2, Experiments 1 and 2). At one year of age, three fish (1a, 1b, and 6) that had developed large tumors (described below) were sacrificed and DNA was isolated separately from muscle tissue (C, control) and tumor tissue (T, tumor). Junction fragments from transposon integration sites were amplified using a ligation-mediated PCR protocol [16] with barcoded primers adapted for next generation sequencing on the Illumina/Solexa platform (Table 3). The sequences were trimmed and the transposon–genomic DNA junction fragments were mapped to the zebrafish v8 RefSeq genome (http://www.sanger.ac.uk/resources/zebrafish). We recovered 1630, 717, and 2479 unique integration sites in control 1aC, 1bC, and 6C samples, respectively (Table 4 and Dataset S1), that mapped to TA dinucleotides in the zebrafish genome. These data indicate that the short burst of transposon expression from mRNA injection in the embryo was enough to trigger widespread transposon mobilization and re-integration.

The T2/OncZ re-integration site data for individual samples was plotted and revealed that in each sample the integration events mapped to every chromosome (Fig. 4 A–C, black line plots). The average number of integration sites per Mb across each chromosome was ~0.5, 1 (samples 1bC and 1aC), or 2 (sample 6C) and appeared evenly distributed across the genome with two exceptions. First, in each sample a large number of re-integration sites mapped to chromosome 3 (Figure 4, A–C, arrows), where the zebrafish β-actin gene is located. These are most likely re-integration events in the β-actin promoter present in the T2/OncZ transposon (Dataset S1). Second, in each sample one chromosome has a higher frequency of re-integration events than the rest (Figure 4 A–C, asterisks). For example, in Tg[T2/OncZ, β-actin:RFP]* sample 1a the peak on chromosome 16 indicates there are ~1.5 times as many events as on the other chromosomes (Fig. 4 A asterisk). In Tg[T2/OncZ, β-actin:RFP]* sample 6C, the highest frequency of re-integration events mapped to chromosome 5 (Fig. 4 C asterisk). The cluster of integration sites most likely represents transposon excision and re-integration to locations linked to the concatemer, a previously reported phenomenon [10,11] that is discussed further below. Interestingly, in control tissue from fish 1b the distribution of integration sites was relatively even across each chromosome, without an obvious peak representing linkage to the predicted concatemer location on chromosome 16 (Fig. 4 B). However, analysis of the integration sites from the tumor tissue isolated from fish 1b shows a peak on chromosome 10 (Fig. 4 B asterisk). One explanation for this

Table 1. Transgenic zebrafish lines isolated in this study.

| Transgenic line | Transgene copy number | Notes |
|-----------------|-----------------------|-------|
| Tg[T2/OncZ,β-actin:RFP]* | ~70 copy concatemer | RFP reporter integrated into concatemer |
| Tg[T2/OncZ,β-actin:RFP]* | ~500 copy concatemer | RFP reporter integrated into concatemer |
| Tg[Tol2<β-actin:SB11, cmlc2:GFP>] | single Tol2 transposon with SB11 transposase cassette | contains heart-specific GFP reporter cassette |
| Tg[Tol2<β-actin:SB11, cmlc2:GFP>] | single Tol2 transposon with SB11 transposase cassette | contains heart-specific GFP reporter cassette |
discrepancy is that a genomic rearrangement or translocation of part of the concatemer occurred in the developing embryo after injection of SB11 transposase mRNA. Subsequently, the cells that inherited the rearrangement went on to contribute to the tumor in the adult. This is a reasonable explanation, given that transposition from a concatemer has been previously shown in mice to cause genomic rearrangements at the concatemer chromosomal site [41].

The experiments described above demonstrated that supplying a transient source of SB11 transposase by injection of mRNA into...
the embryo results in a robust level of transposition. We next compared the extent of transposon mobilization after transient transposase expression with the constitutive SB11 transgene. To do this we cloned and analyzed the distribution of transposition integration sites in the genomes of four three-month-old fish that were doubly transgenic for the Tg(T2/OncZ)is6/1 concatemer and the Tg(Tol2<β-actin:RFP)>1/48 transgene (Figure 3 B, Table 4 Experiment 3). Between 4446–6042 unique integration sites were identified per tissue sample (Table 4 and Dataset S1). The average number of integration sites per Mb was between 2 and 6 in samples 1C, 2C, 3C and 5C (Fig. 4 D), at least twice the number seen after supplying SB11 transposase transiently in the embryo (Fig. 4 A–C). This could reflect the continued transposition driven by the constitutive transposase through adult stages.

In each of the four Tg(T2/OncZ)is7/8, Tg(Tol2<β-actin:RFP)>0/20 fish (sample 5C) the number of integration events that are in or near a transposon-genomic DNA junction fragments.

### Table 2. Somatic mutagenesis experiments performed in this study.

| Experiment | Parental Genotype | Transposase Source | Mutant Class | Control Siblings |
|------------|-------------------|--------------------|--------------|-----------------|
| 1          | Tg(T2/OncZ)is7/8  | SB11 mRNA injection | Tg(T2/OncZ)is7/8 | Tg(T2/OncZ)is6/1 |
| 2          | Tg(T2/OncZ)is7/8  | SB11 mRNA injection | Tg(T2/OncZ)is7/8 | Tg(T2/OncZ)is6/1 |
| 3          | SB11 mRNA injection | constitutive transgene | Tg(T2/OncZ)is7/8 | Tg(T2/OncZ)is6/1 |
| 4          | SB11 mRNA injection | constitutive transgene | Tg(T2/OncZ)is7/8 | Tg(T2/OncZ)is6/1 |

Tumor incidence measured at 1–1.5 years.  
TP53M214K allele previously described in Berghmans et al., 2005.

### Table 3. 5′-3′ Sequence of barcoded oligos for ligation-mediated PCR of T2/OncZ transposon-genomic DNA junction fragments.

| Primer | Illumina Adaptor Sequence | Barcode | 72 transposon arm sequence |
|--------|---------------------------|---------|---------------------------|
| BC1    | AGGAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC2    | AGGAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC3    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC4    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC5    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC6    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC7    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC8    | GGCAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC9    | ACTAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC10   | ACTAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC11   | ACTAGT                   | GTTATGAAACTTCGACTCTAAGCTG |
| BC12   | ACTAGT                   | GTTATGAAACTTCGACTCTAAGCTG |

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Table 4. Analysis of T2/OncZ integration events in control tissue and tumor tissue after exposure to transient or constitutive SB11 transposase.

| Sample | T2/OncZ concatemer line | Transposase Source | Integrations/Genome | Integrations in Genic Regions* | # genes with ≥1 integration | # genes with >2 integrations | # genes with >3 integrations | # genes with >4 integrations | % genes with multiple integrations |
|--------|-------------------------|---------------------|----------------------|-----------------------------|--------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------------|
| 1aC    | ~500 copy               | SB11 mRNA           | 1630                 | 1340                        | 1169                     | 135                         | 19                          | 3                           | 11.5%                            |
| 1bC    | ~500 copy               | SB11 mRNA           | 717                  | 589                         | 491                      | 54                          | 16                          | 5                           | 11%                              |
| 6C     | ~70 copy                | SB11 mRNA           | 2479                 | 2035                        | 1626                     | 269                         | 71                          | 18                          | 16.5%                            |
| 1aT    | ~500 copy               | SB11 mRNA           | 2596                 | 2143                        | 1679                     | 302                         | 81                          | 28                          | 18%                              |
| 1bT    | ~500 copy               | SB11 mRNA           | 1831                 | 1497                        | 1220                     | 194                         | 44                          | 11                          | 15.9%                            |
| 6T     | ~70 copy                | SB11 mRNA           | 1818                 | 1480                        | 1256                     | 163                         | 29                          | 11                          | 13%                              |
| 1C     | ~500 copy               | Tg(β-αSBI I)        | 4446                 | 3567                        | 2769                     | 571                         | 142                         | 32                          | 20.6%                            |
| 2C     | ~500 copy               | Tg(β-αSBI I)        | 5363                 | 4280                        | 3232                     | 762                         | 182                         | 56                          | 23.6%                            |
| 3C     | ~500 copy               | Tg(β-αSBI I)        | 6842                 | 5469                        | 3849                     | 1092                        | 333                         | 102                         | 28.4%                            |
| 5C     | ~500 copy               | Tg(β-αSBI I)        | 4470                 | 3666                        | 2850                     | 599                         | 142                         | 22                          | 21%                              |
| 2T     | ~500 copy               | Tg(β-αSBI I)        | 2306                 | 1848                        | 1598                     | 214                         | 21                          | 5                           | 13.4%                            |
| 8T     | ~500 copy               | Tg(β-αSBI I)        | 2307                 | 1868                        | 1599                     | 213                         | 40                          | 11                          | 13.3%                            |

C = control muscle tissue from tumor born fish (1a, 1b, 6) or from tumor free control fish (1C, 2C, 3C, 5C).
T = tumor tissue from fish 1a, 1b, 6, 2 and 8.
*Integrations in Genic Region is defined as an integration in the 5’, 3’, intronic or exonic region of a gene.
The number of individual genes with one or more independent integration events.
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events that mapped to the genome (Table 4). Further analysis revealed that in each double transgenic sample 22–28% of the genes have multiple independent insertion sites (Table 4). The number of independent integrations in a gene ranged from 1 to 4 in each sample, with 22, 32, 56 and 102 genes in the different samples having 4 or more independent integration events (Table 4, samples 5C, 1C, 2C, 3C). Surprisingly, multiple independent insertions were also detected in 11–16% of the genes after transient expression of transposase by SB11 mRNA injection into the embryo (Table 4). Multiple independent events in a gene in one tissue sample could represent intragenic excision and re-integration, as described previously in mouse [10,11,17,18]. The ability to detect these multiple events may result from the massive sequencing capability of the Illumina/Solexa platform, compared with previous high throughput methods. In summary, both methods we tested for introducing transposase into the \( T2/OncZ \) concatemer lines resulted in transposition and genome-wide re-integration.

\( T2/OncZ \) mutagenesis leads to tumor induction after transient or constitutive exposure to \( SB11 \) transposase

To determine if transposon re-integration in the zebrafish \( T2/OncZ \) system would lead to tumorigenesis, we monitored the \( Tg(T2/OncZ, \beta\text{-actin:RFP})^{is6} \) zebrafish exposed to transient or constitutive \( T2/OncZ \) transposase for gross evidence of neoplasms or tumors. In a wild type background, 10% (3/29) of the \( \geq 500 \)-copy number concatemer \( Tg(T2/OncZ, \beta\text{-actin:RFP})^{is6} \) adults that had been injected with \( SB11 \) mRNA at the 1-cell stage developed gross tumors beginning at about 1 year of age (Table 2, Experiment 1). Twenty control siblings that were injected and reared alongside the \( Tg(T2/OncZ, \beta\text{-actin:RFP})^{is6} \) positive fish showed no evidence of neoplasms. Naturally occurring neoplasms have been reported in zebrafish with varying frequencies [42,43,44], however, we have not observed spontaneous tumors in the \( T2/OncZ \) transgenic lines or in the wild type populations of fish raised in our facility. We harvested the tumor from one of the mutagenized fish (1a) and performed both histopathology and transposon re-integration site analysis. In fish 1a a mesenchymal neoplasm was located in the abdominal body wall (Fig. 5A). The neoplasm consisted of densely packed and highly cellular cords of spindle shaped cells within a fine fibrous stroma; a microscopic appearance consistent with spindle cell sarcoma (Fig. 5E). Neoplastic cells invaded extensively into and separated the skeletal muscle of the abdominal wall. Together, these results indicate that \( SB \) transposon mutagenesis induces tumors in zebrafish adults.

We examined whether predisposing fish to cancer in a tumor suppressor mutant background would accelerate tumor onset and increase the frequency of tumors after \( T2/OncZ \) mutagenesis. Homozygous \( \delta53^{M214K} \) mutant zebrafish are susceptible to development of peripheral nerve sheath tumors [31]. \( SB11 \) mRNA was injected into embryos from a cross between the 70 copy background.
number Tg(T2/OncZ; β-actin:RFP<sup>red</sup>) concatemer line and homozygous <sup>tp53<sub>M214K</sub></sup> zebrafish, so that all progeny were heterozygous for the <sup>tp53<sub>M214K</sub></sup> allele (Table 2, Experiment 2). Transgenic and non-transgenic progeny were monitored for evidence of neoplasms (Table 2). At 9 months of age, one adult zebrafish (1/48–2%) that was injected with <sup>SB11</sup> mRNA developed a large neoplasm rostral and ventral to the pectoral fin (Fig. 5 C). None of the 55 non-transgenic siblings developed tumors. The neoplasm in the fish was composed of densely packed nests of cuboidal to polygonal cells that had moderate anisocytosis and anisokaryosis. In multiple foci neoplastic cells were arranged into tubular/acinar structures (Fig. 5 G). The morphology of neoplastic cells suggests an epithelial origin, indicating a carcinoma, which is different from the typical MPNSTs that arise due to <sup>p53</sup> heterozygosity. The <sup>tp53<sub>M214K</sub></sup> genotype of DNA isolated from the tumor and control muscle tissue of the fish was determined by amplification and sequencing of a 290 bp fragment surrounding the <sup>M214K</sup> lesion. The results indicate that the control muscle tissue and tumor tissue were heterozygous for the <sup>tp53<sub>M214K</sub></sup> allele [data not shown]. It is possible that DNA from stromal tissue in the tumor contributed to the presence of the wild type allele in the genotyping data. However, given the absence of tumor induction in 55 non-transgenic control siblings, it is less likely that the tumor appeared after spontaneous loss of the wild type <sup>tp53<sub>M214K</sub></sup> allele. The lower frequency of tumor induction observed in these experiments (2%), compared to the frequency for the high copy number concatemer (10%), suggests transposon copy number positively correlates with tumor induction frequency. Predisposing fish with a single copy of mutant <sup>tp53</sup> likely did not compensate for the lower transposon dosage in the lower copy number concatemer line.

The introduction of transposase by mRNA injection was able to induce tumors in wild type and <sup>p53</sup> heterozygous adult <sup>T2/OncZ</sup> fish, albeit at a low frequency. We expected that a continuous, transgenic source of <sup>SB11</sup> transposase would result in an increase in tumor frequency and a decrease in latency, particularly in the predisposed <sup>p53</sup> background. However, zero out of 40 double transgenic <sup>T2/OncZ</sup>; <sup>β-actin:SB11<sup>red</sup></sup> adults developed tumors by 1 year of age (Table 2, Experiment 3). Additionally, in crosses between the constitutive <sup>β-actin:SB11</sup> transposase and the ~500 copy number <sup>T2/OncZ</sup> concatemer line that was heterozygous for the <sup>tp53<sub>M214K</sub></sup> allele, only three out of approximately 20 double transgenic progeny survived (Table 2, Experiment 4). However, at 1.5 years two of the three adults developed gross neoplasms (Fig. 5 B, D), while 0/18 siblings that did not inherit the <sup>β-actin:SB11</sup> transgene showed signs of tumors (Table 2, Experiment 4). These data are consistent with the idea that mutant <sup>p53</sup> predisposes fish to increased tumorigenesis and further supports the observation that transposon dosage in the concatemer correlates with tumor incidence. In fish 2 we identified a neoplasm in the dorsal cervical region (Fig. 5 B, D). This neoplasm appeared to be composed of two neoplastic cell populations each with distinct morphology. The first cell type was characterized by loosely packed sheets of round cells within a fibrous stroma (Fig. 5 F). The second neoplastic population consisted of densely packed and highly cellular spindle shaped cells within a fibrous stroma (Fig. 5 F). Neoplastic cells in both populations had moderate anisocytosis and anisokaryosis.

Our interpretation of these findings was that this was a mixed neoplasm containing two neoplastic cell phenotypes, mesenchymal and round cell. These morphologies are consistent with spindle cell sarcoma and histiocytic sarcoma, respectively. In fish 8 there was a mesenchymal neoplasm located in the abdominal cavity with abundant fibrous tissue that may indicate further differentiation and classification as fibrosarcoma. Neoplastic cells had moderate anisocytosis and anisokaryosis (Fig. 5 H). The morphology of this neoplasm is consistent with spindle cell sarcoma. We determined the <sup>tp53</sup> genotype of control muscle tissue and tumor tissue from both fish 2 and fish 8 using PCR and sequencing. Like control tissue, the tumor DNA remained heterozygous for the <sup>tp53<sub>M214K</sub></sup> allele. These results are consistent with <sup>T2/OncZ</sup> mutagenesis driving tumorigenesis in these neoplasms, as opposed to loss of heterozygocity at the <sup>tp53</sup> locus.

**T2/OncZ transposon integration site analysis in neoplastic tissue**

We examined the profile of transposon sites from neoplastic tissues (Table 4, Dataset S1) and compared the gene insertion list with human and mouse cancer genome databases. We isolated

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**Figure 5. Histopathologic features of solid tumors in T2/OncZ mutagenized fish.** (A–D) Gross images of neoplasms in fish 1a, 2, 6, and 8 respectively. (E–H) Histopathology of zebrafish neoplasms: Hematoxylin and Eosin stained sections at 1000× magnification. (E) Spindle cell sarcoma from fish 1a, note entrapped skeletal muscle fibers (arrow). (F) Mixed neoplasm from fish 2, neoplastic round cells (arrow) were intermixed with neoplastic spindle shaped cells (arrow head). (G) Carcinoma from fish 6, neoplastic cells were arranged into multiple acinar structures (arrow). (H) Spindle cell sarcoma from fish 8, note the mitotic figure (arrow). Scale bar A–D, 0.5 cm; E–H, 50 μm.

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DNA from tumor tissues 1aT, 1bT, 6T, 2T and 8T. A list of all integration sites for every tumor and control tissue is provided in Dataset S1. The integration site list was filtered according to a method previously used in the analysis of T2/Onc integration sites from mouse [16]. This analysis is designed to create a conservative estimate of the genes that contribute to cellular transformation based on their representation in the Illumina sequence data set. Using this method we identified in each tissue sample the integration sites two standard deviations above the mean, which potentially represent transposon integrations that were clonally expanded during tumor growth (Table 5, Dataset S2). The number of annotated genes for all zebrafish tumor samples (T) after filtering is 149. Interestingly, insertions at different locations in the GALNTL6 gene that encodes N-acetylgalactosaminyltransferase-like 6 were observed in two tumor samples, 2T and 8T.

We then compared the gene list from the zebrafish tumors with the mouse retroviral tagged cancer gene database (RTCGD: retroviral tagged cancer gene database, http://RTCGD.nicicrf.gov) [45]. Retroviral insertions were present in the mouse homologs of 10 genes tagged in the zebrafish tumor samples. Bub2, Carb3, Fgfb, Hexim 1, Mapi 2K3, Mapi 14, Ncoa 2, Scl 30A 5, Sox 4, and Sox 5 (Table 6). Additionally, a T2/OncZ transposon insertion in Snapc3 was observed in tumor 8T, a gene previously identified with a T2/Onc insertion in mouse tumor samples [11]. We also compared the zebrafish tumor integration gene list with the Cancer Genome Project Cancer Gene Census (http://www.sanger.ac.uk/genetics/CGP/Census/) [46]. This revealed that eleven of the zebrafish genes (11/149; 7.4%) have human homologs that are causally implicated in various cancers (Table 6; CBFb, CBL, ETV1, GATA2, MAP4K3, NCOA2, NCOA4, PXB1, PRDX3, SMARC B1, and TSHR) (Table 6). We performed the same analyses using the integration data from all control samples. Twelve of the 602 genes (2%) from the control samples were represented in the Cancer Gene Census list (BRD3, CBFb, CRLF2, ETV1, GPHN, FANCG, JAZF1, KTN1, MAF, NCOA2, NRAS, SFPQ, and nineteen (3.2%) were present in the mouse RTCGD database (Cen2, Dlx, Fanog, Ghr, Igfb1, Inadl, Jazf1, Map2K3, Nkx2, Pax6, Ppp1r14b, Pri, Rras2, Rm4ghl, Tum006a, Vac14, Zdb1c10). The observed frequencies of integration into known cancer genes are significantly higher in the tumor samples than expected by chance (chi-square test P<0.002 and P<0.02 when comparing representation in Cancer Census List and RTCGD, respectively). Together, the results indicate the zebrafish tumors induced by T2/OncZ mutagenesis have an increased frequency of mutations in the homolog of known human and mouse cancer genes. This analysis validates our mutagenesis approach and provides additional comparative support for the involvement of these genes in human cancer.

**Discussion**

The SB T2/OncZ system presented in this study provides a robust strategy for insertional mutagenesis in somatic tissues in zebrafish. The results demonstrate that transient or constitutive sources of SB11 transposase effectively mobilize transposition from a stable T2/OncZ concatemer followed by genome-wide re-integration. T2/OncZ re-integration was randomly distributed across the genome and evenly distributed along each chromosome. The exceptions were a high number of re-integration events on chromosome 16 in concatemer line Tg(T2/OncZ; β-actin:RFP) <sup>αα</sup> and on chromosome 5 in line Tg(T2/OncZ; β-actin:RFP) <sup>αα</sup>, which indicates linkage of the concatemer to these chromosomes.

The somatic mutagenesis strategy described here results in highly mosaic tissues with respect to transposon integration sites. In each sample there was at least 1 integration event per Mb on each chromosome, and in some samples the number was as high as 6. Variation in the average number of integrations could represent differences in the density of genes along each chromosome, since the plotted data represents integration sites that map to unique locations in the genome. Remarkably, we were able to detect multiple independent integration events in 21–28% of tagged genes from control fish when the constitutive β-

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**Table 5. Number of T2/OncZ re-integration sites/sample after filtering.**

| Sample | # of sites | # of mapped reads | Avg. read # | standard deviation | Cut off value | # of sites above Cut off value | # annotated genes |
|--------|------------|-------------------|-------------|-------------------|--------------|-------------------------------|------------------|
| 1aC    | 1621       | 51475             | 31          | 388               | 806          | 10                            | 7                |
| 1bC    | 693        | 43805             | 62          | 347               | 758          | 14                            | 10               |
| 6C     | 2452       | 182394            | 74          | 526               | 1126         | 51                            | 45               |
| 1aT    | 2578       | 239283            | 93          | 1061              | 2215         | 34                            | 26               |
| 1bT    | 1826       | 194852            | 107         | 2027              | 4160         | 5                             | 4                |
| 6T     | 1811       | 74780             | 41          | 529               | 1099         | 11                            | 9                |
| 1C     | 4220       | 345805            | 78          | 305               | 688          | 155                           | 127              |
| 2C     | 5353       | 520800            | 78          | 359               | 824          | 247                           | 185              |
| 3C     | 6834       | 718548            | 105         | 359               | 730          | 238                           | 193              |
| 5C     | 4461       | 215711            | 48          | 345               | 738          | 70                            | 47               |
| 2T     | 2304       | 205197            | 89          | 355               | 800          | 90                            | 72               |
| 8T     | 2308       | 228567            | 99          | 571               | 1243         | 55                            | 49               |

Samples 1aC, 1bC, and 6C are from normal muscle tissue from fish 1a, 1b, 6.
Samples 1aT, 1bT, and 6T are from tumor tissue from fish 1a, 1b, 6.
Samples 1C, 2C, 3C, and 5C are from β-actin:SB11/T2/OncZ<sup>αα</sup> double transgenic control fish.
Samples 2T and 8T are from tumor tissues from β-actin:SB11/T2/OncZ<sup>αα</sup> double transgenic fish.

Reads that mapped to the β-actin promoter were removed from each sample before calculating values.

Cut off value is equal to two standard deviation units above the average read # in that sample.

*# annotated genes is the # of sites within an annotated gene or positioned 5’ or 3’ to an annotated gene.*

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actin:SB11 transgene was used as the transposase source. Multiple integration sites within a gene were also detected in 11–16% of tagged genes in DNA samples from fish that were injected with SB11 mRNA. Intragene excision and re-integration has previously been reported in mouse T2/Onc tumors [10,11,17,18] and was interpreted to represent the selection of integrations that create mutations favorable for tumor cell survival and maintenance. The data presented here suggest that similar events occur with the zebrafish T2/Onc system, not only in tumor tissues, but in control tissues as well. The β-actin:SB11 transgene appears to provide a constant level of transposase. This is evident in the absence of a large number of re-integration events surrounding the concatamer linkage site on chromosome 16 in tumors from double transgenic Tg(β-actin:SB11)iso; Tg(T2/Onc)iso; β-actin(RFP)iso fish aged 1–1.5 years and the increased frequency of multiple transposon insertions in single genes. The data are consistent with continuous mobilization and dispersal of T2/Onc transposons across the genome as the fish age.

Given the robust transposition and transposon re-integration we observed in somatic tissues, the transgenic T2/Onc system could be an effective tool for germline mutagenesis screens. SB was first shown to promote germline transposition and transgenesis in mice [47,49] and was further developed as a germline mutagenesis tool [47,49]. In zebrafish SB has been used for transgenesis and germline mutagenesis screens by injection of transposase RNA and transposon DNA into the embryo [50,51,52,53]. Similar strategies for SB-mediated transgenesis in the germline have also been demonstrated in medaka [54] and Xenopus [55]. We show in the current study that the T2/Onc system promotes a high level of transposition and genome-wide integration. Germline mutagenesis in the mouse starting with transgene concatemers has previously been shown for both the T2/Onc transposon [41] and a gene trap T2 vector [56]. Adopting the T2/Onc system for germline mutagenesis might increase the efficiency of insertional mutagenesis screens compared to previous transgenic methods in zebrafish [57].

Introducing a transient source of transposase into the high copy number T2/Onc concatemer line by RNA injection resulted in a tumor frequency of 10%. The occurrence of tumors was surprising, given that transposase expression is transient and the embryo is mosaic for integration sites in individual cells. Although 2 out 3 double transgenic Tg(β-actin:SB11)iso; Tg(T2/Onc)iso; β-actin(RFP)iso developed tumors, the small number of fish that survived to adulthood prevents an estimate of the true frequency of tumor induction using constitutive transposase. We are currently testing an alternative Tg(β-actin:SB11) line to optimize SB11

| Zebrafish Gene* | Human Gene* | COSMIC Cancer Gene Census% | Mouse RTCGD Cancer Gene% |
|-----------------|-------------|-----------------------------|---------------------------|
| brd2b           | BRD2        | X                           | X                         |
| ebf             | CBFB        | X                           | -                         |
| cbl             | CBL         | X                           | -                         |
| cirbp           | CIRBP       | X                           | -                         |
| ext1c           | EXT1        | X                           | -                         |
| fgfa            | FGFB        | X                           | -                         |
| gata2           | GATA2       | X                           | -                         |
| gzc:162976      | HEXIM1      | X                           | -                         |
| gzc:172137      | MAP2K5      | X                           | -                         |
| mmp14b          | MPP14       | X                           | -                         |
| msi2b           | MSI2        | X                           | -                         |
| ncoa2           | NCOA2       | X                           | -                         |
| gzc:55307       | NCOA4       | X                           | -                         |
| pbx1a           | PBX1        | X                           | -                         |
| prdx5           | PRDX5       | X                           | -                         |
| slc30a5         | SLC30A5     | X                           | -                         |
| smarcb1b        | SMARCB1     | X                           | -                         |
| snapc3          | SNAPC3      | X                           | -                         |
| zic3key-76p14.4 | SOX4        | X                           | -                         |
| sox5            | SOX5        | X                           | -                         |
| LOC560609       | TSHR        | X                           | -                         |

*Zebrafish genes with T2/OncZ insertion in tumor tissue in the present study.

1Human Gene: BRD2, bromodomain containing 2; CBFB, core-binding factor, beta subunit, CBL, Cas-Br-M (murine) ecotropic retroviral transforming sequence; EXT1, exostosin 1; FGFB, fibroblast growth factor 8 (androgen-induced); GATA2, GATA binding protein 2; HEXIM1, hexamethylene bis-acetamide inducible 1; MAP2K5, mitogen-activated protein kinase kinase 5; MPP14, matrix metallopeptidase 14 (membrane-inserted); MSI2, musashi homolog 2 (Drosophila); NCOA4, nuclear receptor coactivator 4; PBX1, pre-B-cell leukemia homeobox 1; PRDX5, peroxiredoxin 5; SLC30A5, solute carrier family 30 (zinc transporter), member 5; SMARCB1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1; SNAPC3, small nuclear RNA activating complex, polypeptide 3, 50 kDa; SOX4, SRY (sex determining region Y)-box 4; SOX5, SRY (sex determining region Y)-box 5; TSHR, thyroid stimulating hormone receptor.

2Gene identified as a Human Cancer Gene in the Cancer Gene Census List.

3Gene identified as a CIS in mouse tumor tissues by retroviral or T2/Onc transposon insertion. X, present in Human Cancer Gene Census or mouse RTCGD: -, absent.

4PBX1 is mutated by translocation in human pre B-ALL, myeopithelioma.

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Table 6. Comparison of zebrafish and human and mouse cancer genes.
expression levels, as well as additional tumor suppressor genetic backgrounds, in order to increase the frequency of tumor induction.

This study demonstrates the capability of the Sleeping Beauty T2/OncZ system for genome-wide insertional mutagenesis in somatic tissues in zebrafish and the potential for identifying novel cancer genes. The comparison of T2/OncZ integration sites in the five zebrafish tumors to the human Cancer Gene Census and mouse Retrivial and Transposon Cancer Genome Database revealed overlapping genes. Three of the five tumors had a T2/OncZ insertion in one gene (CBL, TSHR, or SMARCB1) known to be highly mutated in human cancers [46]. While these insertions could be interpreted as the activating mutations responsible for initiating cellular transformation and tumor progression, similar insertions from multiple tumors are necessary to support that conclusion. This comparative approach among zebrafish, mouse and human provides additional evidence in support of putative cancer genes and the conservation of genetic pathways mutated in cancer.

Future studies examining integration events in large numbers of zebrafish tumors will be necessary to identify novel cancer genes as has been done previously in mouse SB-induced cancer models [10,11,17,18]. Modification of the system using alternative T2/OncZ vectors, or combining transposase injection and transgenic sources, could increase tumor frequency. However, the ability to scale up the mutagenesis strategy in order to recover significant numbers of tumor samples is straightforward using zebrafish. For a standard zebrafish laboratory it is feasible to inject 500 embryos, or recover 500 double transgenic fish, and raise the fish to adulthood. One important future use of the T2/OncZ system is to combine it with other zebrafish models of human cancers to screen for enhancers of tumor onset, progression and possibly metastasis. These studies will provide a novel strategy for elucidating the genetic pathways that drive cellular transformation and tumorigenesis in human cancer.

Methods

Ethical Statement

Animals were reared and euthanized according to protocol #06-D-029-A approved by the Iowa State University IACUC Committee. All efforts were made to minimize suffering.

Zebrafish strains

Zebrafish were reared and housed in an AHAB system [Aquatic Ecosystems, Inc.] and kept under a 14 hour light/10 hour dark photoperiod at 27°C. Wild type zebrafish were obtained from 3-D Tropical Inc. (Florida), WIK, TU, and the yf53M2148 lines were obtained from the Zebrafish International Research Center (ZIRC).

Isolation of transgenic Tg(T2/OncZ, β-actin:RFP) concatemer lines

The SB T2/OncZ transposon was built by modifying the previously characterized T2/Onc vector [10] by inserting the carp β-actin promoter [33] (detailed cloning steps are provided in Methods S1). To isolate zebrafish lines carrying T2/OncZ concatemer arrays linear DNA fragments containing T2/OncZ and the β-actin:RFP reporter cassette were co-injected into embryos. The pT2/OncZ vector was digested PstI and BsaXI to release a ~4.9 Kb fragment containing the transposon flanked by 160 bp and 280 bp of vector sequence at the 5’ and 3’ ends, respectively. The 4.9 Kb T2/OncZ fragment was mixed at a 2:1 ratio with a 3.1 Kb fragment containing a β-actin:RFP reporter cassette to a final concentration of 250 pg/µl. The DNA was diluted 1× in Danio buffer and 125–250 pg injected into 1-cell WIK or wild type embryos. Founders were raised to adulthood and screened by outcrossing with WIK or wild type and examining F1 embryos for ubiquitous RFP expression at 24 hpf. Out of 54 founders screened, seven independent F1 concatemer lines were recovered. Tg[T2/OncZ, β-actin:RFP]50K (>500 copy number) and Tg[T2/OncZ, β-actin:RFP]\mathbf{\times}7 (75 copy number) were kept and the remaining 5 lines were not maintained.

Genomic Southern blotting, Western blotting, transposon excision PCR assay, and isolation of transposase mRNA are described in Methods S1.

Isolation of S811 transposase transgenic lines by Tol2-mediated transgenesis

Details of the molecular construction of Tol2<β-actin:SB11, cmlc2:GFP> are provided in Methods S1. To isolate S811 transposase expressing transgenic fish, 50 pg Tol2 mRNA and 100 pg Tol2<β-actin:SB11, cmlc2:GFP> plasmid was coinjected into the 1-cell stage WIK embryos. At 24 hpf injected embryos were screened for mosaic GFP expression in the heart and positive embryos were selected and raised to adulthood. Adult founders were outcrossed with wild type and the F1 embryos screened for GFP expression in the heart. Nine independent F1 Tg[Tol2<β-actin:SB11, cmlc2:GFP>] lines were recovered after screening 22 F0 founder adults. Lines Tg[Tol2<β-actin:SB11, cmlc2:GFP>]10 and Tg[Tol2<β-actin:SB11, cmlc2:GFP>]11 were maintained.

Somatic Mutagenesis Strategies

Heterozygous transgenic Tg[T2/OncZ, β-actin:RFP] adult males were crossed to heterozygous Tg[Tol2<β-actin:SB11, cmlc2:GFP>] females. Embryos were collected and aged to 24 hpf or 48 hpf and sorted into 4 progeny classes based on ubiquitous RFP expression and heart-specific GFP expression. Approximately, 50 individuals in each progeny class were reared in separate tanks and examined for macroscopic tumors before sacrificing and harvesting tissues for DNA isolation and pathology. For transposase mRNA injections, Tg[T2/OncZ, β-actin:RFP] males were outcrossed to WIK females and ~50 pg of SB11 mRNA injected into 1-cell stage embryos. At 24 hpf embryos were separated into RFP+ and RFP- classes and reared in separate tanks.

Histopathology

Tissue and tumors were dissected from anesthetized adults and fixed in 10% buffered Formalin (Fisher). All tissues were embedded in paraffin and processed for routine sectioning (4 μm) and hematoxylin and eosin staining (H&E) at the Histopathology Laboratory, Department of Veterinary Pathology, in the College of Veterinary Medicine at Iowa State University.

Identification of transposon integration sites

Sequences flanking transposon integration sites were PCR amplified using a previously described adapter ligation-mediated PCR protocol [16] that was modified for use with the Illumina/ Solexa Genome Analyzer II. Bar-coded primers used for amplification are listed in Table 3 and additional methods are found in Methods S1.

Computational Analyses

Illumina sequences were trimmed to remove primer and transposon sequences and mapped to the zebrafish genome assembly v8 using BLAT. Graphical plots of transposon distribution and density across the genome were created using
the R program. Chi-square tests were performed at http://people.
ku.edu/~preacher/chisq/chisq.htm [30] and http://www.graphpad.
com/quickcalcs/chisquare1.cfm?Format=C.

Comparative Genomic and Network Analyses

Filtered datasets of integration sites were analyzed for cross-
contamination between samples processed on the same day and
sequenced in the same Illumina run. The identical location for 6
integration sites in the tumor samples was also present in one or
more control samples. The corresponding genes were removed
from the tagged gene list before performing comparative genomic
analyses.

Supporting Information

Dataset S1 Tabs are provided for all T2/OncZ integration
sites from SB11 mRNA injected tumor and control
zebrafish (all sites SB11 injected), and all
T2/OncZ integration sites from tumor and control zebrafish with
Tg[Tol2<ß-actin:SB11, cmld2:GFP>] (all sites BaSB11
transgene).

(XLS)

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Dataset S2 Tabs are provided for all T2/OncZ integration
sites remaining in control samples and tumor samples after filtering the lists to remove sites that fall
below two standard deviations above the mean.

(XLS)

Methods S1

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Author Contributions

Conceived and designed the experiments: MM JJE. Performed the
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