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Identification of Tf1 integration events in *S. pombe* under nonselective conditions

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Abstract

Integration of retroviral elements into the host genome is a phenomena observed among many classes of retroviruses. Much information concerning integration of retroviral elements has been documented based on *in vitro* analysis or expression of selectable markers. To identify possible Tf1 integration events within silent regions of the *S. pombe* genome, we focused on performing an *in vivo* genome-wide analysis of Tf1 integration events from the nonselective phase of the retrotransposition assay. We analyzed 1000 individual colonies streaked from four independent Tf1 transposed patches under *nonselection conditions*. Our analysis detected a population of G418^S/*neo*⁺ Tf1 integration events that would have been overlooked during the *selective phase* of the assay. Further RNA analysis from the G418^S/*neo*⁺ clones revealed 50% of clones expressing the *neo* selectable marker. Our data reveals Tf1's ability to insert within silent regions of *S. pombe*'s genome.

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1. INTRODUCTION

Long terminal repeat (LTR) retrotransposons are retrovirus-like transposons that replicate by reverse transcription of an RNA intermediate producing cDNA. The life cycle is similar to some higher order retroviruses in that the final stage of the life cycle includes insertion of the cDNA –using a viral encoded integrase protein – into the host chromatin (Boeke and Devine, 1998); (Koonin et al., 2006). Because the eukaryotic cell chromatin is composed of euchromatic (regions of the chromatin that contains actively transcribing genes) and heterochromatic (regions of the chromatin referred to as “silent” for the transcription of genes) regions, careful target selection for integration is critical for propagation of the virus and survival of the host.

Integration of retroviral elements into the host genome is a phenomena observed among many classes of retroviruses. Much information concerning integration of retroviral elements has been documented based on *in vitro* analysis or expression of selectable markers (Asante-Appiah and Skalka, 1997). The epigenetic environment of the eukaryotic cell’s genome creates many challenges for target site selection. In order to fully analyze the cellular interactions of target site selection among retrotransposons it is critical to observe the impact of integration within the host environment.

Tf1 is an endogenous and active LTR retrotransposon of the fission yeast *Schizosaccharomyces pombe*. Tf1 contains a single open reading frame (ORF) that encodes 1,331 amino acids. The ORF is composed of capsid, protease, reverse transcriptase, and integrase proteins (Levin et al., 1993; Hizi and Levin, 2005). As a member of the Metavirus genus (formerly called Ty3/Gypsy family) (Malik and Eickbush, 1999; Jern et al., 2005) the integrase protein contains a conserved module called a chromodomain (CHD) located within the carboxyl terminal (C-terminal) domain of the protein. Chromodomains are found in several eukaryotic proteins. The regions are believed to be essential for methylation of histones as well as interaction with heterochromatic regions (Eissenberg, 2001). The presence of a CHD region within the C-terminal domain of Tf1 integrase suggests possible interaction with heterochromatic regions. Previous work has shown that mutations within the CHD of Tf1 affect transposition (Chatterjee et al., 2009).

Introduction of an *in vivo* assay system to monitor the life cycle of retroviral elements in prokaryotic (Naumann and Reznikoff, 2002) and eukaryotic (Boeke et al., 1985; Boeke and Corces, 1989; Sandmeyer et al., 1990; Curcio and Garfinkel, 1991; Jensen and Heidmann, 1991; Yang et al., 2007) cells has greatly advanced the field of retrovirology.

Retrotransposition assays allow phenotypic monitoring of retroviral integrations *de novo*. The ability to isolate retroviral insertions is based on the cell’s ability to express a selectable marker within the genome. The presence of a selectable marker could create a selection bias based on integrations into genomic regions that could suppress transcription (Francis and Spiker, 2005). The retrotransposon assay used to monitor the life cycle of Tf1 consists mainly of two phases. Phase I is a *nonselective phase* that selects against the donor plasmid carrying a copy of Tf1 (Levin and Boeke, 1992). Phase II is the *selective phase* that selects for genomic integrated copies of Tf1 based entirely on expression of a selectable neomycin (*neo*) marker tagged to Tf1. Because Tf1 elements have never been observed to integrate

into heterochromatic regions (Behrens et al., 2000; Singleton and Levin, 2002) it is unknown if a population of cells containing genomic Tf1 insertions exists within silent regions of the genome. Integration events within heterochromatic regions would go undetected in the *selective phase* of the retrotransposon assay. Previous reports have demonstrated this selection bias with T-DNA in the genome of *Arabidopsis* (Francis and Spiker, 2005; Kim et al., 2007; Gelvin, 2012).

Because Tf1 has been reported to harbor a chromodomain located within the C-terminal of the integrase protein, and because it is well documented that chromodomains are known to interact with heterochromatic regions of the genome, we anticipate possible Tf1 targeting to heterochromatic regions of *S. pombe*. Previous reports have isolated Tf1 insertions from the *nonselective phase* but bacteria were used to identify the insertion events (Singleton and Levin, 2002). In this study we report an *in vivo* genome-wide analysis of Tf1 transposition events. To identify possible Tf1 integration events within silent regions of the genome we focused on performing an *in vivo* analysis of Tf1 integration events from the nonselective phase of the retrotransposition assay. Our analysis detected a population of G418^S/*neo*⁺ Tf1 integration events that would have been overlooked during the *selective phase* of the assay. Further RNA analysis from the G418^S/*neo*⁺ clones revealed expression of the *neo* selectable maker. Because silencing in *S. pombe* has an essential role in the formation of heterochromatin at the centromeres, telomeres, the mating-type locus, and ribosomal DNA (rDNA) (Hansen et al., 2005), the use of this organism in understanding how cells may silence viral infections is critical in advancing the design of therapies used to treat viral infections and diseases in mammals and humans.

2. Materials and methods

2.1. Media

S. pombe liquid and agar plate media were composed according to Singleton and Levin, 2002. Plasmid propagation plates were composed of Edinburgh Minimal Medium (EMM) (Forsburg and Rhind, 2006) lacking uracil and supplemented with appropriate amino acid, and Vitamin B1 (Thiamine) to suppress the *nmtl* promoter. Nonselective plates were composed of EMM supplemented with 1mg/ml 5-Fluoroorotic acid (5-FOA) (Boeke et al., 1987) (Zymo Research) and uracil at a final concentration of 50ug/ml. Selective plates consisted of YES, supplemented with 500ug/ml Geneticin (G418) (Agilent) and 1mg/ml 5-FOA (YES/G418/5-FOA).

2.2. Construction of strains

The yeast strains and plasmids used in this study are described in Table 1. Yeast strain YHL6488 is a diploid *S. pombe* strain carrying the Tf1-ori/*neo* donor plasmid pTS1559-9 (Singleton and Levin, 2002). *S. pombe* strain YHL1282 is a haploid strain carrying the Tf1-*neo* donor plasmid pHL449-1 (Levin, 1995). *S. pombe* strain YTS7078 is a haploid strain carrying the Tf1-ori/*neo* donor plasmid pTS1559-9.

2.3. Tf1 Transposition Assay

The Tf1 retrotransposition assay was performed as described previously (Singleton and Levin, 2002). Individual colonies were selected and used to grow patches on EMM lacking uracil supplemented with Vitamin B1 agar plates for 3 days at 30°C. Patches were replica-printed to EMM agar plates lacking uracil and Vitamin B1 to induce transcription of Tf1. Patches were grown for 4 days at 30°C then replica-printed to EMM agar plates supplemented with uracil and 5-FOA and allowed to grow for 3 days. This *nonselective phase* only allowed selection against cells containing the Tf1-*neo* and Tf1-ori/*neo* donor plasmids. Next, a *selective phase* consisted of replica-printing patches to YES/G418/5-FOA agar plates. Confluent cell growth was indicative of expression of the *neo* gene thereby creating G418-resistant (G418^R) colonies. The lack of cells' growth on YES-G418 is due to sensitivity to G418, and is G418-sensitive (G418^S).

2.3.1 Phenotypic analysis of Tf1 transposed colonies—Tf1 transposed patches (YTS6716, YTS6932, YTS7084, and YTS7133) from the nonselective phase of the assay were streaked to obtain single colonies. Two hundred-fifty (250) individual colonies were patched on EMM agar plates supplemented with uracil, Vitamin B1, and 5-FOA. Patches were replica-printed to YES agar plates containing G418 to determine the number of G418^S and G418^R colonies in the population. G418^S colonies were further analyzed by Southern blot hybridization.

2.4. Preparation of Digoxigenin (DIG)-labeled DNA probes for Southern and Northern analysis

All oligonucleotides (MWG Biotech) used for PCR are listed in Table 2. The primers selected for PCR are complementary to regions within the 5' and 3' genes adjacent to Tf1 targets. DIG-labeled DNA probes were amplified by incorporating a DIG-11-dUTP alkali-labile nucleotide (Roche Biochemicals) according to the manufacturer protocol. Long Amp enzyme (New England BioLabs) was used for all reactions. Cycle parameters for amplification: hot start denaturation at 95°C for 5 min, followed by 30 cycles of amplification (95°C for 1 min; 60°C, 1 min; and 65°C, 3 min), and a final extension at 65°C for 10 min. The amplified DNA probes were analyzed on a 1.5% agarose gel in Tris-Borate-EDTA buffer. DIG-labeled probes ranged in size from 214–500bp.

2.5. Genomic DNA preparation and Southern Blot Hybridization

G418-sensitive cell cultures (10ml) were grown in YES with vigorous shaking overnight at 30°C. Cells were pelleted at 5,000 rpm for 10min and resuspended in 0.1M NaCl. After pelleting, cells were resuspended in Solution A (1M sorbitol, 50mM Na₂PO₄ [pH 7.5]). Betamercaptoethanol (Sigma Aldrich) and Zymolyase 100T (ICN) was added with an incubation of 37°C for 1 hr. Spheroplasts were collected and gently resuspended in Solution B (50mM Tris-HCl [pH 8.0], 50mM EDTA [pH 8.0], 1% sarkosyl). RNase A (10mg/ml; 5ul) was added with an incubation of 37°C for 1 hr. Proteinase K (10mg/ml; 5ul) was added with an incubation of 65°C for 1 hr. Two chloroform:isoamyl alcohol (24:1) and one phenol:chloroform:isoamyl alcohol (25:24:1), and one final chloroform:isoamyl alcohol

(24:1) extractions were performed. Genomic DNA was precipitated and resuspended in 1X TE (10mM Tris-HCl, 1mM EDTA) buffer.

Genomic DNA (10ug) was digested with *Sna*BI restriction enzyme, electrophoresed on a 0.7% agarose gel in 0.5X TBE, and subjected to capillary transfer to positively charged nylon membranes (Roche Biochemical) in Alkaline Transfer Buffer (0.4N NaOH, 1M NaCl). Nylon membranes were hybridized with an 851bp DIG-labeled *neo* probe in DIG Easy Hybridization Buffer (Roche Biochemical) at 42°C for 12–16 hrs. Chemiluminescent detection assay (Roche Biochemical) was performed according to the manufacturer. Membranes were exposed to x-ray film and analyzed using the ChemiDoc XRS Photodocumentation System (BioRAD).

2.6. Determination of Tf1-*neo* insertion sites by Inverse PCR

To determine the sequence of the genomic regions flanking the Tf1 integrations, 1ug of genomic DNA from all G418^S/*neo*⁺ clones was digested with *Hin*P1 restriction enzyme (Fig. 2A). Self-ligation reactions were performed using T₄ DNA ligase (New England BioLabs) and incubated at 16°C for 16 hrs. After heat inactivation at 65°C for 10 min the *Hin*P1 self-ligated DNA was subjected to Inverse PCR (iPCR) using the Long Amp Taq PCR system (New England BioLabs). Self-ligated constructs were amplified using primer set TS612/TS614 and primer set TS586/TS587 (Fig. 2A). Long Amp cycling conditions: 94°C for 30 seconds; 94°C for 30 seconds, 60°C for 1 min, and 65°C for 1 min (30 cycles); and a final extension cycle at 65°C for 10 min. Amplifications were analyzed on a 0.7% agarose gel. PCR products were purified (Qiagen PCR purification kit) and sequenced (Wake Forest DNA Sequencing Core, Winston-Salem NC) using primers TS612 and TS614. Tf1 genomic target site locations were determined using the *S. pombe* genome database of the Sanger Centre (<http://www.sanger.ac.uk>). Sequences were further analyzed using Lasergene software (DNASar).

2.7. Total RNA preparation and Northern Blot Hybridization

Total cellular RNA was isolated from 1x10⁹ G418^S/*neo*⁺ cells using the Qiagen RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. All solutions were treated with Diethylpyrocarbonate (DEPC) or prepared in DEPC treated water. For RNA blot analysis 20ug of total RNA from G418^S/*neo*⁺ clones were resuspended in 2X loading buffer (50% formamide, 6% Formaldehyde, 1X MOPS, 10% glycerol, 0.05% bromphenol blue) and heated at 65°C for 10 min and cooled. Samples were electrophoresed on a 1% agarose-formaldehyde gel in 1X MOPS buffer for 8 hours under 50 volts. Total RNA was transferred to a positively charged nylon membrane (Roche Biochemical) by capillary transfer in DEPC-treated 20X SSC for 24 hrs. Hybridizations were performed in DIG Easy Hybridization solution with designated DIG-labeled probes. Membranes were analyzed using the DIG Detection system (Roche Biochemicals) and analyzed using the ChemiDoc XRS Photodocumentation System (BioRAD).

2.8. Trichostatin A (TSA) treatment

G418^S/*neo*⁺ clones YTS6932-14, YTS6932-38, YTS6932-45, YTS6932-121, YTS6932-203, YTS6932-215, YTS6932-230, and YTS6716-68 were treated with the following

concentrations of TSA (Sigma Aldrich): 0.6ng, 1.2ng, 2.0ng, 6.0ng, 91.0ng, 0.5ug, 1.0ug, 2.0ug, 3.0ug, 4.0ug, 5.0ug, 10.0ug, 35.0ug, 50.0ug, and 100.0ug/ml. Overnight cultures were diluted to an OD₆₀₀ of 0.1 in YES broth and grown for 6 days at 30°C. Growth plots were taken at days 3 and 6. Cells were washed in 200ul YES and the whole culture was spotted onto YES-G418 agar plates. Plates were incubated for 3 days at 30°C. Control cultures (Wild type: 972; and G418^S/*neo*⁺ clones) were grown in YES in the absence of TSA.

3. Results

3.1. In vivo Genome-wide analysis of Tf1 integration events under nonselective conditions

The retrotransposition assay developed to monitor and detect yeast genomic Tf1 integration is based on cell resistance to Geneticin (G418) due to expression of the neomycin (*neo*) selectable marker during the selective phase of the retrotransposition assay (Levin et al., 1993; Singleton and Levin, 2002). During the *nonselective phase* cells expressing the URA3 gene located on the donor plasmid are sensitive to 5-FOA present in the media and are therefore, eliminated from the cell population. The *selective phase* selects for Tf1 chromosomal integration events via expression of the *neo* selectable marker. To determine if Tf1 suppressed insertions can be identified, we chose to isolate and phenotypically analyze individual colonies isolated from Tf1 transposed patches growing under *nonselective* conditions. *S. pombe* strain YTS6932 was transposed with the Tf1-*neo* donor plasmid pHL449-1 (Levin et al., 1995). *S. pombe* strains YTS6716, YTS7133, and YTS7084 were transposed with the Tf1-ori/*neo* donor plasmid pTS1559-9 (Singleton and Levin, 2002). We isolated 250 individual colonies from individual Tf1 transposed patches YTS6932, YTS6716, YTS7084, and YTS7133 from the nonselective phase of the assay. All colonies were exposed to 5-FOA conferring 5-FOA-resistance (5-FOA^R) and therefore, are void of the Tf1 donor plasmid. To determine resistance to G418, all individual colonies were replica-printed onto YES/G418 agar plates. We obtained 6 (2.4%) individual G418^R colonies from patch YTS6932, 7 (2.8%) individual G418^R colonies from patch YTS6716, 7 (2.8%) individual G418^R colonies from patch YTS7133, and 4 (1.6%) individual G418^R colonies from patch YTS7084 as shown in Table 3. Because Tf1-*neo* retrotransposition frequencies of 1.0 to 4.0% (Levin and Boeke, 1992) have been reported, our retrotransposition efficiency percentages reported in this study are well within range with patch YTS7084 more on the low end of the efficiency range. The remaining 244 (95% of the colony population), 243 (97.2% of the colony population), 243 (97% of the colony population), and 246 (98% of the colony population) G418^S individual colonies derived from patches YTS6932, YTS6716, YTS7133, and YTS7084, respectively, represent cells possibly void of Tf1 insertions or Tf1 insertions suppressed by position effect variegation due to insertion within silent regions of the genome.

To investigate the possibility that the remaining 976 G418^S clones could harbor silent Tf1 insertion events, we analyzed the genomes using Southern blotting (Fig. 1). The genomes of the 976 total G418^S clones were screened by blot hybridization using an 851bp DIG-labeled *neo* probe generated by PCR. The probe is complementary to approximately 90% of the *neo* gene sequence. Surprisingly, blot analysis of all 976 G418^S clones identified ten G418^S clones signaling the presence of a *neo* gene as detectable using the 851bp DIG-labeled *neo*

probe (Fig. 1B). We were not able to detect the presence of the *neo* gene in the genomic DNA of the 250 individual colonies patched from strain YTS7084. We also noticed that patch YTS7084 produced the lowest transposition frequency, which may explain our inability to detect any silent integration events. Our results revealed that approximately 1.0% of the total cell population in the *nonselective phase* of the transposition assay would have been overlooked under selective conditions. We clearly show the presence of a Tf1 insertion in *S. pombe* colonies that are 5-FOA^R/G418^S. The G418^R clones YTS6716-2 and YTS7084-4 both produced strong positive signals for the presence of *neo*, as seen in Fig. 1B. Genomic DNA from wild type strain 972 gave no signal when hybridized with the DIG-labeled *neo* probe revealing the absence of any endogenous copies of Tf1-*neo*. The inability of the G418^S/*neo*⁺ clones to phenotypically display G418^R, hints to possible Tf1 insertion within silent regions of the genome.

3.2. Genomic locations of G418^S/*neo*⁺ Tf1 insertion events

Identification of genomic Tf1 target sites allows analysis of transcriptional activity within the targeted region. To identify the genomic location of all ten G418^S/*neo*⁺ clones, we used inverse PCR (iPCR) and the *S. pombe* sequence database. Previous methods used to identify Tf1 genomic insertions (Singleton and Levin, 2002) relied on genomic DNA restriction, recircularization of Tf1-ori/*neo* insertions with flanking genomic DNA, and bacterial transformations that could introduce selection biases. We employed the iPCR technique to identify the genomic location of all ten Tf1 insertion targets and to reduce any selection biases. Genomic DNA from the G418^S/*neo*⁺ clones were restricted with *Hin*P1 to produce two detectable fragments containing sections of the *neo* gene and flanking yeast genomic DNA, Fig. 2A. Because the 851bp DIG-labeled *neo* probe is complementary to 90% of the *neo* gene, two distinct *Hin*P1 fragments were detectable in most of the clones. We observed that clones YTS7133-90 and YTS6932-121 both produced only one detectable *Hin*P1 fragment, Fig. 2B. Oligonucleotides TS612 and TS614 and TS585/TS588 were used to sequence the self-ligated insertion events (Fig. 2A). Sequencing data concluded that Tf1 insertions occurred among the three chromosomes of *S. pombe* (Table 4). All insertions among the G418^S/*neo*⁺ clones occurred within intergenic regions. Intergenic regions ranged in size from 871bp (YTS6932-230) to 4263bp (YTS6932-203). In Table 4 we report Tf1 insertion events as close as 114bp to the nearest ORF - observed in clone YTS6932-38 - or as far as 1375bp from the nearest ORF - observed in clone YTS6932-203. We were not able to obtain DNA sequence from clone YTS7133-90 possibly due to chromosomal recombination among existing solo LTRs. DNA sequencing of clones YTS6716-68 and YTS6716-102 revealed insertions that occurred at the same exact nucleotide. Interestingly clone YTS6716-68 was found to have inserted 443bp near the MAT1-MC gene, Table 4. The Mat1-MC gene is a known constricted silent region. We also noticed that majority of Tf1 insertions occurred between genes with divergent orientation, Table 4. Chromosomal plotting of all insertion events via contig location is represented in Fig. 3. Strains YTS6932-230, YTS6932-215, and YTS6716-68 contain Tf1 insertions near the centromere, (p7G5) on chromosome 1; the telomere (c212), on chromosome 1; and the matingtype locus (c23G7) on chromosome 2, respectively.

3.3. RNA analysis of G418^S/neo⁺ clones reveals possible mechanisms for gene silencing

Because isolation of Tf1 insertions in this study was not based on expression of the *neo* gene, we asked whether the *neo* genes in G418^S/neo⁺ clones are transcriptionally active. The inability of cells to establish resistance to G418 is due to the lack of *neo* gene expression or absence of the *neo* gene. To determine if the *neo* gene is transcriptionally active, we analyzed the eight G418^S/neo⁺ clones by Northern blot hybridization. RNA blots of all eight G418^S/neo⁺ insertion events were hybridized with the 851bp DIG-labeled *neo* probe. Surprisingly, the 851bp DIG-labeled *neo* probe was able to detect a 956bp signal in clones YTS6932-14, YTS6932-203, YTS6932-215, and YTS6932-230 representing expression of the *neo* gene transcripts Fig 4. The inability of the G418^S/neo⁺ clones to grow in the presence of G418 hints to possible silencing of the *neo* gene. G418^R clones YTS6716-2 (diploid) and YTS7084-4 (haploid) both produced strong 956bp signals when hybridized with the 851bp DIG-labeled *neo* probe, Fig. 4. We were not able to detect *neo* transcripts from clones YTS6932-38, YTS6932-45, YTS6932-121, and YTS6716-68 when hybridized with an 851bp DIG-labeled *neo* probe. To determine if the *neo* gene is active in clones YTS6932-38, YTS6932-45, YTS6932-121, and YTS6716-68, we chose to excise and self-ligate each insertion event and transform into bacteria. We were able to detect Kanamycin resistance in all G418^S/neo⁺ stains. Our inability to detect *in vivo* transcripts in clones YTS6932-38, YTS6932-45, YTS6932-121, and YTS6716-68 hints to a possible different mechanism of silencing used by *S. pombe*.

To observe transcriptional activity in genes surrounding Tf1's target site, we analyzed expression of adjacent genes upstream and downstream from each target site using Northern blot hybridization (Table 5). Of the 22 genes analyzed using DIG-labeled PCR probes only three transcripts: SPCC1223.10 (*eaf1*), SPAC3H5.04 (*aar2*), and SPBC119.03 (conserved hypothetical protein) were detected (Table 5).

3.4. TSA treatment restores G418-resistant phenotypes in G418^S/neo⁺ clones

The loss of *neo* gene silencing, via the loss of G418-sensitivity as measured by the ability of cells to grow in the presence of G418, is related to possible alterations of histone modifications indicative of nonsilent chromatin. In order to study any relationship between genomic silencing of Tf1 integration events and possible histone deacetylation, G418^S/neo⁺ clones YTS6932-14, YTS6932-45, YTS6932-121, YTS6932-203, YTS6932-215, YTS6932-230, and YTS6716-68 were grown in the presence of increasing concentrations of the histone deacetylase inhibitor Trichostatin A (TSA). All G418^S/neo⁺ clones were treated with increasing concentrations of TSA (0.6, 1.2, 2.0, 6.0, 91.0ng/ml, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 35.0, 50.0, and 100.0ug/ml). To determine the effect of TSA on the growth of G418^S/neo⁺ clones, we chose to measure cell growth rate in liquid cultures prior to spotting the cultures onto YES/G418 agar plates. Figure 5B reveals the cellular growth curves of various concentrations of TSA and no TSA treated cultures. No significant defect in growth rates of cells treated with TSA as compared to growth rates without TSA treatment (Figure 5B) was observed. We did notice a higher cell count in cultures treated with 0.6ng and 1.2ng TSA compared to no TSA treatment. After TSA treatment whole culture suspensions (200ul) were spotted on YES-G418 agar plates. Surprisingly, G418-resistant colonies were observed among strains YTS6932-14, YTS6932-203, YTS6932-215, and YTS6932-230 Fig.

5A. Because undiluted whole cultures were spotted the population of colonies conferring G418-resistance could be observed. Strains YTS6932-14 resulted in more G418-resistant colonies at TSA concentration 3.0ug/ml. YTS6932-203 and YTS6932-215 produced more G418-resistant colonies within 1.0–5.0ug/ml TSA with 2.0ug/ml resulting in more colonies. Strain YTS6932-230 produced more G418-resistant colonies at the lower TSA concentrations of 91ng/ml, 0.5ug/ml, 1.0 and 3.0ug/ml. No colonies were observed at 100ug TSA concentration. Interestingly, we were not able to observe any G418-resistant colonies from TSA treated cultures: YTS6932-38 YTS6932-45, YTS6932-121, and YTS6716-68. Cultures YTS6932-14, YTS6932-203, YTS6932-215, and YTS6932-230 at TSA concentrations of 3.0ug/ml, 2.0ug/ml, 4.0ug/ml, and 0.5ug/ml, respectively, were spotted using a 5-fold dilution on YES/G418 agar, Fig. 6. Interestingly, we were able to draw a sharp correlation between G418^S/*neo*⁺ clones void of an RNA transcript (Fig. 4B) and TSA treated G418^S/*neo*⁺ clones (Fig 5A). It is clear that clones (YTS6932-38 YTS6932-45, YTS6932-121, and YTS6716-68) not expressing a *neo* transcript were also not able to produce G418-resistant clones after treatment with increasing concentrations of TSA,

3.5. The 3' flanking region of Tf1-*neo* donor plasmid reveals preexisting integration within a silent region of the genome

A genomic library of *S. pombe* NCYC132 was originally used to isolate Tf1-107 clone (Levin et al., 1990). The cloned *Xho*I-*Bam*HI fragment contained flanking genomic DNA from the 3' LTR. The flanking genomic DNA not only reveals the genomic location of the target site of Tf1-107 clone, but also revealed information about the genomic region chosen for integration. To identify the target site region chosen for this primitive Tf1 insertion, we chose to analyze genomic DNA sequence flanking the 3'LTR of the Tf1-ori/*neo* donor plasmid pTS1559-9. Plasmid pTS1559-9 contains the plasmid backbone for cloned Tf1-107 (Levin et al., 1990; Levin et al., 1993). Using sequencing primers TS612, and TS621, we were able to identify the genomic location chosen for Tf1-107 clone, Fig. 7. Surprisingly, Tf1-107 was found to have inserted 59kb upstream from the right end of chromosome 2. Insertion is within a 2.406kb intergenic region between genes SPBPB2B2.09 and SPBPB2B2.10. Tf1-107 inserted 2bp from the SPBPB2B2.09 ORF. More amazing is the presence of three solo LTRs downstream from the target site, indicating a favorable region for previous integrations. The most proximal anchored cosmid to the telomere region on chromosome 2 is pT2R1. We found that Tf1-107 inserted within cosmid pB2B2, which is located upstream from cosmid pT2R1 (ftp://ftp.ebi.ac.uk/pub/databases/pombase/pombe/Cosmid_assembly_data/SP_chr2_dump.text). Cosmid pT2R1 is defined as the telomeric cosmid for chromosome 2. Because telomeric regions are heterochromatic regions and defined as silent, we chose to analyze the genes adjacent to the Tf1-107 target site. We were not able to detect transcripts from genes SPBPB2B2.09 and SPBPB2B2.10. This further defines this area of chromosome 2 targeted for Tf1 integration as silent.

4. Discussion

In this study we report an *in vivo* genome-wide analysis of Tf1 integration events from the nonselective phase of the retrotransposition assay. In this study we identified ten G418-sensitive clones harboring a genomic copy of Tf1-*neo* and Tf1-ori/*neo*. The ten G418^S/*neo*⁺

clones were isolated from four independent transposed patches. Tf1 targets were sequenced by iPCR and found to span all three chromosomes of *S. pombe*. Analysis of each target site revealed a target preference for intergenic regions of the genome with insertion distances ranging from 100-400 nucleotides from the closest 5' ORF as previously reported (Brehens et al., 2000; Singleton and Levin, 2002). The most surprising results came from the RNA analysis. In our experiments we were able to detect transcription activity from the *neo* gene in 50% of the G418^S/*neo*⁺ clones (Fig. 3).

The techniques used in this study were designed to: (1) identify targets that may have occurred in silent regions of the genome that otherwise would have gone undetected, and (2) analyze the genome *in vivo* using the S. blot to eliminate any possible selection biases when transformed into bacteria. Because our previous report of Tf1 insertion events were based on self-ligation and selection using bacteria, (Behrens et al., 2000; Singleton and Levin, 2002) we were unable to analyze a cohort of Tf1 insertions in the parental cell. The techniques used in this study address the direct *in vivo* analysis of Tf1 insertion events thereby, allowing possible identification of insertions within silent regions of the genome.

The ability of cells to protect themselves upon viral infection is clearly demonstrated in bacteria via the restriction/modification system (Vasu and Nagaraja, 2013). Fission yeast cells have in place mechanisms such as DNA methylation, and histone modification, to control retroviral activity (Goto and Nakayama, 2011). There are increasing reports suggesting that RNAi may play a role in silencing virally infected eukaryotic cells (Haddad et al., 2011; Houzet and Jeang, 2011b; Vasu and Nagaraja, 2013). RNAi was also shown to be involved in the inhibition of viruses and silencing of viruses in plants, insects, fungi, and nematodes (Voinnet, 2001; Waterhouse et al., 2001; Wilkins et al., 2005; Wang et al., 2006; Segers et al., 2007). Our data suggests *S. pombe* is utilizing different silencing mechanisms that are observed in 50% of G418^S/*neo*⁺ clones. Our inability to detect the *neo* transcript or produce G418-resistance in G418^S/*neo*⁺ clones YTS6932-38, YTS6932-45, YTS6932-121, and YTS6716-68 (Fig. 3), suggest yet another silencing mechanism possibly not related to histone deacetylation. This mechanism could involve interactions of Tf1's chromodomain and proteins involved in the establishment of heterochromatin. Future studies would clarify the mechanisms involved.

It is plausible that TSA-sensitive deacetylases in fission yeast are different based upon the area of chromatin chosen for silencing. Based on our findings, that G418^S/*neo*⁺ clones are able to obtain G418-resistance at various TSA concentrations, indicates the existence of various TSA-sensitive histone deacetylase complexes. This explains the differences of G418-resistant clones obtained after various TSA concentration treatments.

Analysis of the Tf1 donor plasmid p1559-9 revealed essential information concerning integration of Tf1. Tf1-107 was originally isolated from *S. pombe* library NCYC132 (Levin et al., 1990). The flanking DNA was analyzed and found not to be tRNA-like sequences but location of the insertion was not determined at that time because sequencing was very limited. We chose to analyze the target site of Tf1-107 and, surprisingly, we identified the chromosomal location to be very close to the telomeric region on chromosome 2. Analysis of the available *S. pombe* genome database identified the target location on chromosome 2

within cosmid pB2B2. Amazingly, the insertion site is 59 kilobasepairs from the end of chromosome 2 and upstream from cosmid pT2R1 that contains the telomere. The presence of 3 solo-LTRs (one Tf2-type, and two Tf1 type) within the intergenic region suggests a favorable region for Tf1 insertion. The use of a library allowed isolation and cloning of Tf1-107. This insertion event would have gone undetectable under selectable conditions of the retrotransposition assay.

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Abbreviations

TSA	Trichostatin A
HDAC	Histone deacetylase
IPCR	Inverse Polymerase Chain Reaction
G418	Geneticin
Neo	neomycin

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Research Highlights

- A genome-wide analysis of Tf1 integration events under nonselective conditions.
- Southern blot analysis reveals the presence of Tf1 insertion events in G418s clones.
- RNA analysis of G418-sensitive clones detects a *neo* transcript.

Figure 1. G418^S clones isolated under nonselective conditions contain Tf1 insertion events
(A) Of the 976 G418^S clones analyzed, ten G418^S clones are represented here on YES media and replica-printed to YES plates containing G418. Lack of cell growth is due to non-expression of the Tf1-*neo* gene. Control strains YTS6716-2 and YTS7084-4 are G418^R as seen on YES/G418 media plates due to expression of the *neo* gene. **(B)** Southern blot analysis of the ten G418^S clones are represented. Genomic DNA was isolated from all G418^S clones. The DNA was digested with *Bam*HI and *Bgl* II (which does not restrict Tf1, ori, or *neo* sequences), separated by gel electrophoresis in a 0.7% agarose gel in 0.5X TBE.

Genomic DNA was hybridized with an 851bp DIG-labeled *neo* probe. Yeast genomic DNA is represented by dashed lines. LTRs are represented by full dark triangles. Arrows depict *Bam*HI and *Bgl* II restriction sites. The 851bp DIG-labeled *neo* probes are represented as a thick black horizontal bar above the *neo* gene.

Figure 2. Determination of genomic Tf1-*neo* insertions in G418^S/*neo*⁺ clones using Inverse PCR (iPCR)

(A). Genomic DNA from G418^S/*neo*⁺ clones were digested with *HinPI*, blotted, and hybridized with an 851bp DIG-label *neo* probe. Genomic locations were identified using IPCR and the *S. pombe* sequencing database. (B). G418^S/*neo*⁺ clones YTS6932-121 and YTS7133-90 hybridized with the 851bp DIG-labeled *neo* probed produced one single *HinPI* restriction fragment. DNA Marker is a 1kb DNA ladder. Dashed lines represent yeast

genomic DNA. Solid bar represents the 851bp DIG-labeled *neo* probe. Arrows represent restriction sites and PCR primers. Large full triangles represent LTRs.

Figure 3. Chromosomal locations of all G418^s/neo⁺ Tf1 integration events

Integration events are bold and underlined. Contigs are represented in parenthesis.

Telomere, centromere, mating-type locus, and ribosomal RNA regions are represented by black triangles, red circles, teal hexagons, and purple squares, respectively.

Figure 4. Northern analysis of total RNA isolated from G418^S/neo⁺ clones
(A) Tf1 insertion clones were cultured in YES liquid and spotted onto YES agar plates. After 3 days the patches were replica-printed to YES agar plates supplemented with G418. All plates were incubated at 30°C. Colonies growing on YES/G418 agar plates represent G418^R, and lack of growth represents G418^S. (B) Re-hybridization of the RNA blot with an Actin DIG-labeled probe indicates approximately equal loading of RNA in each lane. Northern Blot analysis of G418^S/neo⁺ clones utilized an 851bp DIG-labeled *neo* probe. Transcripts are detected in strains YTS6932-14, YTS6932-203, YTS6932-215, and YTS6932-230. No expression of *neo* was detected in strains YTS6932-38, YTS6932-45, YTS6932-121, and YTS6716-68. G418^R strains YTS6716-2 and YTS7084-4 were grown in

YES (Y), and YES supplemented with G418 (YG) prior to total RNA isolation. *S pombe* wild type strain 972 and a diploid wild type strain YHL5661 (Singleton and Levin, 2002) were grown in YES prior to total RNA isolation.

Figure 5. TSA treatment of silent Tf1 insertions results in the loss of G418-sensitivity
(A). Effects of TSA on the growth rates of G418^s/*neo*⁺ clones. All cells were synchronized at an OD₆₀₀ of 0.1. Growth rates at day 6 of TSA treated cells in increasing concentrations were plotted. The concentrations of TSA used in this study are presented. Wild-type (972) was also cultured in medium of increasing concentrations of TSA or lacking TSA. **(B).** Whole cultures (200ul TSA treated) were washed in YES media and spotted onto YES/G418 agar plates and incubated for 3 days at 30°C.

Figure 6. G418^S/neo⁺ clones treated with Trichostatin A (TSA) results in a loss of *neo* silencing G418^S/neo⁺ clones were cultured in medium containing TSA and allowed to grow for 6 days at 30°C. These colonies were obtained from the whole culture spots (Fig. 5B). Cell numbers were adjusted to OD₆₀₀ of 1.0. Cells were washed in YES and serially diluted 1:5. In all cases, 3ul of each cell suspension was spotted onto a YES-G418 agar plate. Strain YTS6932-14, YTS6932-203, YTS6932-215, and YTS6932-230 were the only strains isolated from YES/G418 agar plates.

TS612
 5' gatgatatat TTTTTatctgtgcaatg TAAATCTTGGGGAGGGCAA ***TGTCAGCAATACTACACTACGCTATGATACACTACGTTGCGTATCACTA***
TATGTCATATGTTGGAATACTAGCTAAGATCCGTTTATTGATAATTACGGGTAAGTATCCGCTATATGCATAGATACCCGTTGATAACAATAATTT
AGAAATATTATAAATAGTGTACAACCTGAACCTCGTTCCTCAGTTCAGTTATGAGCTATATAAATGATAGGTAACATTATAACCCGTGTTAATACAAT
ACCTATACTCAGTTGTTACTCATACAACCTGTGTATTGTAATATAATAGATCGCAAGGAACTCACCGCAGTTCACGTATCCTTAAATCAAATAC
CAAACTGCGTAGCTAACA gaagtaaatgaataacgatcaacttc ATATCAAACGTTAGCAGGACACTGAATCGTGACTGTCCTTATCAAAATAATT
AGTGCTGTAGCAAGCAGCGTTAAAGACTGCATAATAATATCATCTTATCGAGTTTATTCATGGAATGTTTCATCCTCTTATGTTTACTAACATGAATT
 TACATCATC TTTTTTTTAGAAATATTTTCGCA ATATTTCTAGCTGTTAACAAATTTTAAATGCATTATGTTTCGATACATTGAAGTAAAAGGAAGAA
TTGAGGGTCCTTGAAATTTTCGTTTAAATCAAATTGATACCAACCATATACTCAAGTATTTAAATGTATTGTAAATAATAATTCTGTCTCTCTAAAT
ATGACAGGAATCCATACTTCACAGTTTCGTGTTTCTTCACAGAACATACCAAAATATCCAGCGGTTCAAGATAAATTTCTCAAATATATATATGTAC
TCGCAAAATGACACTGCAATAGATTAGTATGGATAGGGTTGAAAACAAAAAAGTTCGCGAGTTAACCTCCTGCTACTATACGGTGTTTTTAATTTTG
TATTATCCTATGACTGAATAATGAATTCTCTCAATGAACCTTTAACTCAGAGGATTTTATAAGGATTAGATATTCGGTCACTACTGTATTTTCTA
TGAAATATTAGCAGTTAAACACCCAAGCTTGATATCGAATTCCTGCAGCCCGGGGATCCAGCTGGCTAGCAGCAGCCATAGTGACTGGCGATG
CTGTCGGAATGGACGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAGCCTATGCCTACAGCATCCAGGGTGACGGTGCCGAGGATGACGA
TGAGCGCATTGTTAGATTTATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAAGCTTTTCAATTCATCATTTTTTT
TTTTATTCTTTTTTTGATTTCGGTTTCCTTGAAATTTTTTTGATTTCGGTAATCTCCGAACAGAAGGAAGAACGAAGGAT ***TS612*** AGCACAGACTTAGAT
TGGTATATATACGCATATGTAGTGTGAAGAAACATGAAATTGCCAGTATTCTTAACCAACTGCACAGAACAAAACTGCAGGAAACGAAGATA
AATC ***ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCTGTTGCTGCGCAAGCTATTTAATATCATGCACGAAAAGCAACAAAC***
TTGTGTGCTTCATTGGATGTTTCGTACCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACACATGTGGATA
TCTTGACTGATTTTCCATGGNGGGCAC 3'

Figure 7.

Bold italic sequence represents the 3' LTR. Boxed sequences represent DNA primers.
 Single underlined sequence represents genomic flanking the 3' LTR. Double-underlined
 region represents the *Ura* gene present on pTS1559-5.

TABLE 1

Yeast strains and plasmids used in this study.

Parental Yeast Strains	Genotype Parent/plasmid	Reference
972	Wild type	ATCC ^a
YHL912	<i>h⁻ ura4-294 leu1-32</i>	Singleton and Levin, 2002
YHL5661	<i>Stable diploid ura4-D18/ura4-D18 leu1-32::nmt1-lacZ-leu1-32::nmt-lacZ-leu1 ade6-M210/ade6-M216</i>	Singleton and Levin, 2002
Nontransposed Yeast Strains		
YHL1282	YHL912/pHL449-1	ATCC ^a
YTS7078	YHL912/pTS1559-9	This study
YHL6488	YHL5661/pTS1559-9	This study
Transposed Yeast Strains		
YTS6716 [†]	YHL5661/pTS1559-9	This study
YTS6932 [†]	YHL912/pHL449-1	This study
YTS7084 [‡]	YHL912/pTS1559-9	This study
YTS7133 [†]	YHL912/pTS1559-9	This study
Plasmid		
pTS1559-9	Tf1-ori/ <i>neo</i>	Singleton and Levin, 2002
pHL449-1	Tf1- <i>neoAI</i>	Levin, 1995

^a American Type Culture Collection[†] Transposition using solid media.[‡] Transposition using liquid media.

Table 2

Oligonucleotides

Oligonucleotide	Sequence (5' to 3')	Use	Ref
TS217	GTCAAGTCAGCGTAATGCTCT	5' oligo used to create <i>neo</i> gene probe	This study
TS218	GGAAACGTCTTGCTCGAGGC	3' oligo used to create <i>neo</i> gene probe	This study
TS858	GTACCACCAGACATAACAACGT	5' oligo for PCR probe to ORF Actin gene	This study
TS859	ATGGCTCTGGTATGTGCAAAGC	3' oligo for PCR probe to ORF Actin gene	This study
TS586	GGAGTACGGATAAAATGCTT	5' oligo for IPCR to neo upstream of <i>HinPI</i>	This study
TS587	CGGTTTGGTTGATGCGAGTG	3' oligo for IPCR to neo upstream of <i>HinPI</i>	This study
TS760	CCACTGCCAGTACTCTTGAGAAC	5' oligo for PCR probe to ORF SPCC1223.09	This study
TS761	ACCAAGACTCACACTGTCTACGA	3' oligo for PCR probe to ORF SPCC1223.09	This study
TS764	CGTCTCGTGGAATTAGTCGTGAC	5' oligo for PCR probe to ORF SPCC1223.10	This study
TS765	CCAGGAATGTGGACTGTGTACTA	3' oligo for PCR probe to ORF SPCC1223.10	This study
TS851	CTGATCCTCCTTATAGGCAGTATT	5' oligo for PCR probe to ORF SPCC1919.03	This study
TS852	CTCGAGGTGGAAGCTAATGAGA	3' oligo for PCR probe to ORF SPCC1919.03	This study
TS855	TGGACTGTAGTCAGAGCTGTAGT	5' oligo for PCR probe to ORF SPCC1919.04	This study
TS856	ATGAATCTTGAGGCGCACGAA	3' oligo for PCR probe to ORF SPCC1919.04	This study
TS833	GAGTGCATCCACCTGGTCTTT	5' oligo for PCR probe to ORF SPBC776.12	This study
TS834	GTTGACTTTGGATTAGCAGAGCG	3' oligo for PCR probe to ORF SPBC776.12	This study
TS837	CTGAGTATGCTGCTGATCTTGTT	5' oligo for PCR probe to ORF SPBC776.13	This study
TS838	TCTATGATAGCGCATCTCAGAG	3' oligo for PCR probe to ORF SPBC776.13	This study
TS796	GTGCTTCCTCGACATCAGCTG	5' oligo for PCR probe to ORF SPAC4A8.03	This study
TS797	CGTGGGTGGATACATGGCGG	3' oligo for PCR probe to ORF SPAC4A8.03	This study
TS800	TTCTACTGCCTGTGCTGCACCA	5' oligo for PCR probe to ORF SPAC4A8.04	This study
TS801	TGTTGCTCAAATCAGGCTGCA	3' oligo for PCR probe to ORF SPAC4A8.04	This study
TS813	TAGACGATCTCTCCAGCAGCTT	5' oligo for PCR probe to ORF SPAC57A7.05	This study
TS814	TCGAAGTCATAAGAGTCGTCTTC	3' oligo for PCR probe to ORF SPAC57A7.05	This study
TS817	GCATCATGTGGTCTCAACGTGACC	5' oligo for PCR probe to ORF SPAC57A7.04	This study
TS818	TAGCAGCATTGGCAGACTCGAC	3' oligo for PCR probe to ORF SPAC57A7.04	This study
TS780	CGATAGTGGCAGCAGAGGTGTGT	5' oligo for PCR probe to ORF SPAC13A11.02	This study
TS781	CGCTGACTTGTACCATGACCTTGA	3' oligo for PCR probe to ORF SPAC13A11.02	This study
TS784	TGGCACTAGCAATTACTACTGGTC	5' oligo for PCR probe to ORF SPAC13A11.03	This study
TS785	AGCTATCAAAGTATGGATTTGATCGGT	3' oligo for PCR probe to ORF SPAC13A11.03	This study
TS786	CCACACGCTTTGGACGAGTCTT	5' oligo for PCR probe to ORF SPAC3H5.04	This study
TS787	AGACACTTCAACGTATGTCAACGC	3' oligo for PCR probe to ORF SPAC3H5.04	This study
TS792	GCTTCTACTCATGAATCTAATTAC	5' oligo for PCR probe to ORF SPAC3H5.03	This study
TS793	TAGCTTGATCACTCTCGTAGGCT	3' oligo for PCR probe to ORF SPAC3H5.03	This study
TS544	CTGAGCTCCTGCACGTTGTAAAG	5' oligo for PCR probe to ORF SPCC74.03	This study
TS545	GGACCGCGTTGGACAGTCTCCG	3' oligo for PCR probe to ORF SPCC74.03	This study
TS546	GTACTGCAGCAATGCTTCTCGGC	5' oligo for PCR probe to ORF SPCC74.04	This study
TS547	ATTGAAGCCGTGATTCTTGCTAGAC	3' oligo for PCR probe to ORF SPCC74.04	This study
TS845	AGCTACCTGTTGGATGGGAATTC	5' oligo for PCR probe to ORF SPBC23G7.09	This study

Oligonucleotide	Sequence (5' to 3')	Use	Ref
TS846	CTCCACATCTCTCCAACCAGCT	3' oligo for PCR probe to ORF SPBC23G7.09	This study
TS849	ACCAGAGCTAACATCTATCAAGTC	5' oligo for PCR probe to ORF SPBC23G7.10	This study
TS850	GTCAACAGTTAGTCCTGCCACT	3' oligo for PCR probe to ORF SPBC23G7.10	This study
TS839	TTCACGTATCCAGACGGATCTG	5' oligo for PCR probe to ORF SPBC119.03	This study
TS840	TGACTATTTCATCCAGACCGGC	3' oligo for PCR probe to ORF SPBC119.03	This study
TS843	GCTGGATCGGTACATAGCGTTC	5' oligo for PCR probe to ORF SPBC119.04	This study
TS844	TCTCGTTAGGTCGAGGAACATGA	3' oligo for PCR probe to ORF SPBC119.04	This study
TS827	CGCATGGCGTTTCAGCTGCTT	5' oligo for PCR probe to ORF SPBPB2B2.09	This study
TS828	CAAGGAGTAATCTCACATGGGTG	3' oligo for PCR probe to ORF SPBPB2B2.09	This study
TS831	GGTGCTCTTTAGCTATCAGTAATG	5' oligo for PCR probe to ORF SPBPB2B2.10	This study
TS832	TATTCCTTCGGTTGTGCTCAAG	3' oligo for PCR probe to ORF SPBPB2B2.10	This study
TS612	GATGATATATTTTATCTTGTGCAATG	IPCR and sequencing primer	This study
TS614	TATCGATTGTATGGGAAGCCCGA	IPCR and sequencing primer	This study
TS621	CACAGAACATACCAAATATCCA	Sequencing primer for pTS1559-9	This study

Table 3

Phenotypic and Genotypic Analysis of Trf1 insertion events under nonselective conditions.

Transposed Patch	Trf1 Plasmid	Number of Colonies				G418 ^R	G418 ^S	G418 ^S /neo ⁺	% Transposition [†]	Genotype
		NSP	SP	NSP	NS*					
YTS6716	pTS1559-9	250	7	243	2				2.8	Diploid
YTS6932	pHL449-1	250	6	244	7				2.4	Haploid
YTS7084	pTS1559-9	250	4	246	0				1.6	Haploid
YTS7133	pTS1559-9	250	7	243	1				2.8	Haploid

NSP = Nonselective Phase
SP = Selective Phase
NS* = neo⁺ by southern blot hybridization using an 851bp DIG-labeled neo probe.
[†] = Transposition frequency of each patch was determined by calculating the total number of 5-FOA^R colonies that were also G418^R.

Table 4

Analysis of Tfl Intergenic Insertions.

Strain	Chr	5' ORF	IR (bp)	3' ORF	5' and 3' ORF orientation	Insertion distance from closest ORF (bp)	Genotype
YTS6932-14	3	SPCC1223.09	2533	SPCC1223.10 (<i>eqf1</i>)	← ←	866	G418 ^S
YTS6932-38	3	SPCC1919.03	2103	SPCC1919.04	← →	114	G418 ^S
YTS6932-45	2	SPBC776.12 (<i>hsk1</i>)	1162	SPBC776.13 (<i>end1</i>)	← →	174	G418 ^S
YTS6932-121	1	SPAC4A8.03 (<i>pic4</i>)	2911	SPAC4A8.04 (<i>isp6</i>)	← →	801	G418 ^S
YTS6932-203	1	SPAC57A7.05	4263	SPAC57A7.04 (<i>pabp</i>)	← →	1375	G418 ^S
YTS6932-215	1	SPAC13A11.02 (<i>erg11</i>)	1898	SPAC13A11.03 (<i>mcp7</i>)	← →	153	G418 ^S
YTS6932-230	1	SPAC3H5.04 (<i>aar2</i>)	871	SPAC3H5.03 (<i>pk11</i>)	← →	159	G418 ^S
YTS6716-2	3	SPCC74.03 (<i>ssp2</i>)	4972	SPCC74.04	← →	215	G418 ^R
YTS6716-68	2	SPBC23G7.09 (<i>mat1-Mc</i>)	3239	SPBC23G7.10	→ ←	443	G418 ^S
YTS6716-102	2	SPBC23G7.09 (<i>mat1-Mc</i>)	3239	SPBC23G7.10	→ ←	443	G418 ^S
YTS7084-4	2	SPBC119.03	1039	SPBC119.04 (<i>mai3</i>)	→ →	580	G418 ^R
pTS1559-9	2	SPBPB2B2.09	2406	SPBPB2B2.10			Donor Plasmid

IR = intergenic region. Number represents distance in base pairs between the adjacent 5' and 3' open reading frame (ORF) from the target sequence; Chr = chromosome; bp = base pair

← → Divergent orientation
 → ← Convergent orientation
 → → Tandem orientation
 ← ← Tandem orientation

Table 5

Insertion Clone/Plasmid	Phenotype	Adjacent genes	Protein function
Gene expression (+/-)			
YTS6932-14			
-	G418 ^S	5'-SPCC1223.09	uricase *
+		3'-SPCC1223.10 (<i>eaf1</i>)	RNA pol II transcription elongation factor
YTS6932-38			
-	G418 ^S	5'-SPCC1919.03 (<i>amk2</i>)	AMP-activated protein kinase beta subunit *
-		3'-SPCC1919.04	Uncharacterized membrane protein
YTS6932-45			
-	G418 ^S	5'-SPBC776.12 (<i>hsk1</i>)	Protein kinase
-		3'-SPBC776.13 (<i>cnd1</i>)	Condensin complex subunit 1
YTS6932-121			
-	G418 ^S	5'-SPAC4A8.03 (<i>ptc4</i>)	protein phosphatase
-		3'-SPAC4A8.04 (<i>isp6</i>)	vacuolar serine protease
YTS6932-203			
-	G418 ^S	5'-SPAC57A7.05	conserved hypothetical protein
-		3'-SPAC57A7.04 (<i>pabp</i>)	mRNA export shuttling protein
YTS6932-215			
-	G418 ^S	5'-SPAC13A11.02 (<i>erg11</i>)	Probable cytochrome P450 protein
-		3'-SPAC13A11.03 (<i>mcp7</i>)	Required for meiotic recombination
YTS6932-230			
+	G418 ^S	5'-SPAC3H5.04 (<i>aar2</i>)	U5 snRNP-associated protein
-		3'-SPAC3H5.03 (<i>pk11</i>)	kinesin-like protein
YTS6716-2			
-	G418 ^R	5'-SPCC74.03 (<i>ssp2</i>)	serine/threonine protein kinase
-		3'-SPCC74.04	amino acid permease *
YTS6716-68/102			
-	G418 ^S	5'-SPBC23G7.09 (<i>mat1-Mc</i>)	Mating-type M-specific polypeptide
-		3'-SPBC23G7.10	NADH-dependent flavin oxidoreductase *
YTS7084-4			
+	G418 ^R	5'-SPBC119.03	conserved hypothetical protein
-		3'-SPBC119.04 (<i>mei3</i>)	meiosis inducing protein
pTS1559-9			
-	Plasmid	5'-SPBPB2B2.09	2-dehydropantoate 2-reductase
-		3'-SPBPB2B2.10	Galactose-1-phosphate uridylyltransferase

* predicted protein function