Insertional mutagenesis by a hybrid piggyBac and Sleeping Beauty transposon in the rat

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Running title: Transposon mutagenesis in the rat

Keywords: transposition, transposase, tyrosinase, transgenic rats, gene trap

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ABSTRACT

A hybrid piggyBac/Sleeping Beauty transposon-based insertional mutagenesis system that can be mobilized by simple breeding was established in the rat. These transposons were engineered to include gene trap sequences and a tyrosinase (Tyr) pigmentation reporter to rescue the albinism of the genetic background used in the mutagenesis strategy. Single copy transposon insertions were transposed into the rat genome by co-injection of plasmids carrying the transposon and RNA encoding piggyBac transposase into zygotes. The levels of transgenic Tyr expression were influenced by chromosomal context, leading to transgenic rats with different pigmentation that enabled visual genotyping. Transgenic rats designed to ubiquitously express either piggyBac or Sleeping Beauty transposase were generated by standard zygote injection also on an albino background. Bigenic rats carrying single copy transposons at known loci and transposase transgenes exhibited coat color mosaicism, indicating somatic transposition. piggyBac or Sleeping Beauty transposase bigenic rats bred with wild-type albino rats yielded offspring with pigmentation distinct from the initial transposon insertions as a consequence of germline transposition to new loci. The germline transposition frequency for Sleeping Beauty and piggyBac was ~10% or about one new
insertion per litter. ~50% of the insertions occurred in introns. Chimeric transcripts containing endogenous and gene trap sequences were identified in *Gabrb1* mutant rats. This mutagenesis system based on simple crosses and visual genotyping can be used to generate a collection of single gene mutations in the rat.
INTRODUCTION

The laboratory rat (*Rattus norvegicus*) is an important organism for modeling human biology and disease. These relatively large rodents are especially useful for physiological, neurobehavioral, transplantation, and toxicological studies. Many genomic resources for the rat have become available (Aitman et al., 2008). The genome has been sequenced, assembled and annotated (Rat Genome Sequencing Project Consortium, 2004), leading to current predictions of more than 22,000 genes (www.ensembl.org/Rattus_norvegicus). The rat model system is now primed for functional genomic studies. Unfortunately, relatively few single gene mutations in the rat have been isolated or generated compared to the other predominant laboratory rodent, the mouse. A large collection of mutations in the rat and a continuous source of new mutations would be a valuable resource to facilitate studies of gene function and model human biology and disease when mouse models are inadequate.

There are a variety of relatively new mutagenesis strategies that have been developed to generate mutations in rats. Chemical mutagenesis using N-ethyl-N-nitrosourea (ENU) was used to generate mutations in rats (Zan et al., 2003; Smits et al., 2005; 2006; Homberg et al., 2007; Olivier et al., 2008; van Boxtel et al., 2008; Homberg et al., 2009; van Boxtel et al., 2010).
These studies have used reverse genetics approaches to identify point mutations in specific genes of interest. In addition, an ENU-mutagenized cryopreserved rat sperm library was generated carrying random point mutations (Mashimo et al., 2008). Somatic tissues from the sperm donors used to generate these libraries can be screened by molecular methods to identify point mutations in any gene. If the point mutation is of interest, then the sperm can be used for in vitro fertilization to recover the mutation for study. Spermatogonial stem cells (SCC) are present in postnatal testes that can self-renew or differentiate into spermatozoa (Oatley and Brinster, 2008). SCCs can also be used to generate random mutations in rats (Izsvák et al., 2010). These cells are isolated from testes, expanded and genetically modified in vitro, and then injected directly into the seminiferous tubules of recipients to complete spermatogenesis. Sperm carrying the mutations can then be used to create mutant rats (Izsvák et al., 2010). Zinc-finger nucleases (ZFNs) are bi-functional DNA-binding proteins designed to recognize and cleave specific gene sequences. They can be expressed in rat zygotes by pronuclear injection to generate targeted mutations (Geurts et al., 2009; Mashimo et al., 2010; Cui et al., 2011). Recently, germline competent rat embryonic stem (ES) cell lines have been isolated (Buehr et al., 2008; Li et al., 2008; Kawamata and Ochiya, 2010; Blair et al., 2011). In addition, the first demonstration of a
targeted germline mutation created using rat ES cells was reported (Tong et al., 2010). All of these mutagenesis methods are relatively new in the rat model. It is currently unclear which of these approaches will be the most productive for generating single gene mutations in the rat (Dolgin, 2010).

Transposon-mediated insertional mutagenesis is another approach that has been established in rodents, including the mouse and rat (Carlson et al., 2003; Horie et al., 2003; Kitada et al., 2007; Lu et al., 2007; Izsvák et al., 2010). Engineered transposons contain terminal inverted repeats (TIRs) at both ends that are recognized by their corresponding transposase (Curcio and Derbyshire, 2003). These TIRs can flank cargo sequences for delivery into the genome by transposition. Most DNA transposons are mobilized through a “cut-and-paste” mechanism (Reznikoff et al., 1999). Transposon transposition into the genome or mobilization within the genome can result in insertional mutation, providing a molecular tag of the genetic lesion. Sleeping Beauty transposase inserts Sleeping Beauty transposons into highly abundant TA sequences in the genome (Ivics et al., 1997). PiggyBac transposase inserts piggyBac transposons in TTAA sequences and has been shown to have a higher activity for transposon mobilization than Sleeping Beauty, Tol2 and Mos1 in mammalian cells (Wu et al., 2006). However,
Grabundzija et al. (2010) showed that the transpositional efficiencies in human HeLa and hematopoietic stem cells is Sleeping Beauty > piggyBac > Tol2. Sleeping Beauty transposon excision is not precise, frequently leaving a 5 base pair footprint, whereas piggyBac leaves no molecular footprint (Ivics et al., 1997; Ivics et al., 2004; Lu et al., 2007; Woltjen et al., 2009). Sleeping Beauty has a higher frequency of local hops (within 4 Mb), whereas piggyBac tends to mobilize and reinsert on other chromosomes (Liang et al., 2009). In the mouse and rat, transposons are typically introduced into the germline by standard pronuclear injection of purified DNA fragments that results in multi-copy tandem arrays usually integrated randomly at a single locus (Dupuy et al., 2002). Transposons can be mobilized in the germline by transposase expression from a second transgene (Dupuy et al., 2002; Carlson et al., 2003; Horie et al., 2003). One disadvantage of mobilizing multiple copies of transposons in tandem arrays is chromosomal damage (Geurts et al., 2006). Single copy transposon mobilization for mutagenesis has yet to be reported in the rat.

We have developed and validated a dual transposon mutagenesis system in the rat that can be mobilized by either Sleeping Beauty or piggyBac transposase. A gene trap module for insertional mutagenesis is combined with a pigmentation reporter gene (*tyrosinase*, *Tyr*) for
visual genotyping. In this system, single copy transposons integrated at known loci are mobilized to new chromosomal locations by crosses with transgenic rats designed to ubiquitously express Sleeping Beauty or piggyBac transposase. Expression of the Tyr reporter is influenced by chromosomal context. Thus, transposition to a new site in the genome is usually accompanied by a visible change in pigmentation, greatly simplifying the identification of transposition events. In addition, rats hemizygous for a transposon insertion can be distinguished from homozygotes by coat color intensity. This system using either Sleeping Beauty or piggyBac transposase results in ~10% frequency of germline transposition events. Truncated endogenous transcripts containing vector sequences demonstrate the activity of the gene trap modules. This simple mutagenesis system can facilitate the generation of a collection of new mutant rat strains to model human biology and disease.
RESULTS

Generation of hybrid piggyBac and Sleeping Beauty transposons:

Two types of transposon vectors, Bhr2 and Bhr7, were constructed (Fig. 1A, B). Both vectors contain a mouse Tyr minigene expression cassette flanked by the inverted repeat/direct repeat (IR/DR) sequences recognized by Sleeping Beauty transposase and the terminal inverted repeat (TIR) sequences recognized by piggyBac transposase, i.e. a Sleeping Beauty transposon within a piggyBac transposon. The Tyr pigmentation gene was used as a visual reporter for transposon integration on a genetic background that is albino, e.g. Sprague-Dawley, Wistar, or F344 rats (Overbeek et al., 1991; Overbeek et al., 2002; Blaszczyk et al., 2005; Lu et al., 2007).

The Bhr2 transposon (Fig. 1A) contains two copies of a gene trap cassette that consists of splice acceptor (SA) and polyadenylation (pA) signals in opposite orientations flanking the Tyr minigene. Thus, integration of Bhr2 into an intron should trap endogenous transcripts independent of orientation. attP and attB sites that are recognized by PhiC31 integrase were placed between the piggyBac TIRs and Sleeping Beauty IR/DRs for potential DNA fragment exchange but were not exploited in the current study.
The Bhr7 transposon (Fig. 1B) contains a gene trap cassette that consists of a SA-internal ribosomal entry site (IRES)-reverse tetracycline-controlled transactivator (rtTA)-pA module placed 5’ of the Tyr minigene. The gene trap function of Bhr7 requires integration in one specific orientation and should result in the expression of rtTA in the pattern of the trapped gene. In contrast to Bhr2, the Tyr minigene expression cassette in Bhr7 does not contain a pA sequence but rather an IRES splice donor (SD) sequence. This was designed so that Tyr expression would depend on integration within an intron and splicing into a downstream endogenous exon to provide a pA signal, increasing the mutagenesis potential of Bhr7. However, there appear to be many cryptic pA signals within the genome not necessarily residing in exons (Jang and Behringer, unpublished observations). Thus, in practice, the IRES-SD strategy was not very specific for identifying insertions within introns of genes.

Transposition of single copy transposons into the rat genome:

To eliminate the problems associated with transposons in multicopy tandem arrays created by the injection of purified DNA fragments, we mobilized Bhr2 and Bhr7 into the rat genome as single
copy integrants using piggyBac transposase. The Bhr2 and Bhr7 transposons were introduced into the genome of Sprague Dawley outbred albino rats by injection of plasmids carrying the transposons and in vitro synthesized RNA encoding piggyBac transposase into the pronuclei of zygotes (Jang and Behringer, 2007). Fourteen (14/22 born, 64%) Bhr2 and four (4/11 born, 36%) Bhr7 founder rats were generated. All of the resulting transposon positive founders identified by PCR had darkly pigmented heads and dorsum (Fig. 2A). Thus, the Tyr reporter expressed from both types of transposons rescued the albinism and revealed the hooded mutant coat color phenotype present in the genetic background of the albino strain. If integration of the transposons was by transposition, then the transposons should be present as single copy insertions but could integrate on more than one chromosome. Therefore, founders were bred with Sprague-Dawley albino mates to generate progeny. Interestingly, 100% of the progeny from all of the founders were pigmented and pigmentation varied between individual progeny (Fig. 2B). These results suggest that each founder contained transposon insertions at multiple loci and that each insertion contributes to the total levels of Tyr expression. Southern blot analysis of the founders and progeny demonstrated that the founders had 5 to 8 transposon copies and that all transposons were present at one copy per locus (Fig. 2C and data not shown).
To facilitate our mutagenesis strategy, we bred the founders with wild-type albino rats and segregated the transposon insertions assessed by Southern blot (data not shown) to generate pedigrees with single transposon insertions (Fig. 3A). This was initiated using the Kyoto Wistar (WKY/NCrl) partially inbred rat but subsequently we used the Kyoto Wistar (WKY/NHsd) inbred strain. Each pedigree had a unique coat color, including light ash grey, medium grey, dark grey, tan or brown (Fig. 3B). In addition, two pedigrees with Bhr7 insertions were albino, indicating that either the transposons were physically damaged or epigenetically silenced perhaps by chromosomal position effects (Fig. 3B). The unique level of pigmentation associated with each transposon insertion site remained stable through subsequent generations. These results suggest that the level of expression of the Tyr pigmentation reporter from each transposon insertion site varied depending upon influences at the locus of integration.

The genomic locations of some of the single copy transposon insertions generated from the original pronuclear injections were identified by inverse PCR (iPCR), DNA sequencing, and verified by PCR genotyping (Table 1, Fig. 8). One of the Bhr2 transposon insertions was located in intron 4 of the Gamma-aminobutyric acid A receptor, subunit beta 1 (Gabrb1) locus (see below). Hemizygotes (Tn/+) had tan pigmentation and intercrosses between hemizygotes yielded
albino (+/+, tan (Tn/+), and brown (putative Tn/Tn) progeny (Fig. 3C). The brown progeny were viable with no obvious abnormal phenotypes. These genotypes were verified by PCR using specific primers for the wild-type and mutant alleles (Fig. 3E). A second example of the correlation of coat color with genotype is shown for a Bhr7 insertion in the Zbt20 locus (Fig. 3D).

Thus, the Tyr pigmentation reporter can be used for visual genotyping of the transposon insertion site without the need for molecular analysis (e.g. PCR). The two pedigrees with the Bhr7 transposon insertions that remained albino had insertions upstream of the first methionine-encoding exon of LOC685774 and ~212 bp upstream of Phex (Fig. 3B).

Interestingly, both LOC685774 and Phex are located on the X chromosome. LOC685774Bhr7 and PhexBhr7 males appeared normal and were fertile. The rats with known positions for Bhr2 in the Gabrb1 locus and Bhr7 in LOC685774 served as genetic reagents to develop our mutagenesis strategy.

**Generation and characterization of piggyBac and Sleeping Beauty transposase transgenic rats:**

To mobilize the single copy Bhr2 and Bhr7 transposon insertions to create new insertional
mutations by simple crosses, we generated transgenic rats to ubiquitously express either Sleeping
Beauty or piggyBac transposase by standard pronuclear injection of albino zygotes. The human
UBIQUITIN-C (UBC) promoter was used to express the transposase genes (Fig. 4A) (Lois et al.,
2002). For Sleeping Beauty transposase, an improved variant designated HSB3 was used (Yant et
al., 2004). The transposase gene constructs were flanked by pairs of transcriptional insulators
from the chicken beta-globin locus to reduce the influence of the site of chromosomal integration.
Founders were identified by PCR using primers to UBC and transposase cDNA (Fig. 4A). One
(1/15, 7%) UBC-SleepingBeauty (UBC-SB) and three (3/8, 38%) UBC-piggyBac (UBC-PB)
founders were generated.

The UBC-SB and -PB transgenic rats were screened for their ability to mobilize single
copy Bhr2 and Bhr7 transposon insertions. Gabrb1^{Bhr2} (tan) and LOC685774^{Bhr7} (albino)
hemizygous rats were bred with UBC-SB and UBC-PB transgenic rats to generate bigenic
progeny carrying both the transposon and transposase transgenes designated as “seed” rats. All of
the seed rats generated using Gabrb1^{Bhr2}, LOC685774^{Bhr7}, UBC-SB and UBC-PB showed coat
color mosaicism, suggesting somatic transposition to new loci (Fig. 4B, C). The pigmentation
observed in the seed rats generated using LOC685774^{Bhr7} demonstrated that Bhr7 was
functionally intact and could express the Tyr pigmentation reporter when mobilized to a permissive site within the genome. These results suggest that the UBC promoter can direct transposase expression during development to mobilize and reinsert Bhr2 and Bhr7 transposons at new genomic locations that are revealed by higher or lower levels of Tyr expression relative to the site of the initial transposon insertion.

**Mobilization of single copy of transposons in the rat germline:**

The coat color mosaicism in the seed rats indicated that transposon mobilization might also occur in their germ cells. To determine if transposition events occurred in the germ cells of the seed rats, we crossed the seed rats with albino mates. If transposition events occurred in the germ cells of the seed rat, then the progeny that inherited insertions at new loci would be revealed by pigmentation that is different from that of the original transposon insertion.

Male Gabrb1\textsuperscript{Bhr2}; UBC-SB or -PB seed rats were bred with wild-type albino females. PCR genotyping for Gabrb1\textsuperscript{Bhr2} and the transposase transgene was performed on the resulting progeny. Albino progeny were either wild-type or transposase positive. Pigmentation mosaic progeny carried Gabrb1\textsuperscript{Bhr2} and the transposase transgene. Tan progeny were Gabrb1\textsuperscript{Bhr2}
hemizygotes without the transposase transgene (Fig. 3C, 5A-C). We also obtained pups with a homogenous color that was different from the original $Gabrb1^{Bhr2}$ insertion (Fig. 5B). These pigmented rats were genotyped as wild-allele for $Gabrb1^{Bhr2}$ and lacked the transposase transgene, indicating that Bhr2 had transposed to a new locus (Fig. 5C). Southern blot analysis using a $Tyr$ fragment probe fortuitously produces similar sized bands for the endogenous $Tyr$ locus and $Gabrb1^{Bhr2}$ when digested with $PvuII$ (Fig. 3A, 5D). Two of the progeny (#1 & 3) showing new coat colors had a hybridizing band distinct in size from the endogenous $Tyr$ locus and the original $Gabrb1^{Bhr2}$ transposon insertion (Fig. 5D). In one of the progeny (#2), the size of the hybridizing band was very similar to the size of the endogenous $Tyr$ locus and $Gabrb1^{Bhr2}$ even though the rat was much darker and genotyped by PCR as wild-type for $Gabrb1^{Bhr2}$ (Fig. 5B-D). These results demonstrate that new transposition events occurred in germ cells of the male seed rats and became fixed when segregated from the transposase transgene. In addition, these new transposition events corresponded with a change in coat color.

Male $LOC685774^{Bhr7}$; $UBC-SB$ or -PB seed rats with coat color mosaicism were bred with wild-type albino females. Albino progeny from this cross were either 1) wild-type, 2) transposase positive, or 3) $LOC685774^{Bhr7}$, whereas 4) coat color mosaics carried
LOC685774<sup>Bhr</sup><sup>7</sup> and the transposase transgene. Some of the progeny had homogenous pigmentation (Fig. 6B) and were genotyped as wild-allele for LOC685774<sup>Bhr</sup><sup>7</sup> and lacked the transposase transgene (Fig. 6C). Southern blot analysis using a Bhr7-specific probe showed that the transposon in the pigmented progeny had moved to a new locus (Fig. 6D). Similar to the Gabrb1<sup>Bhr</sup><sup>2</sup> results above, the LOC685774<sup>Bhr</sup><sup>7</sup> insertion can be mobilized in the germline and segregated away from the transposase by a simple cross. In the case of the LOC685774<sup>Bhr</sup><sup>7</sup> insertion, however, transposon mobilization events are more easily recognized by the presence of homogeneously pigmented progeny (i.e. albino vs pigmented) rather than a coat color change compared to the original insertion (i.e. tan vs a different color). Based on the above results, we designed two simple transposon mutagenesis strategies to generate mutant rats (Fig. 5E, 6E).

**Transposon mutagenesis and isolation of rat mutants:**

Using our coat color-based mutagenesis system we generated a set of rats with new transposon insertions. The transposition frequency in the germline using Sleeping Beauty transposase (UBC-SB) was 8.5% (12 new insertions/141 progeny screened) and using piggyBac transposase (UBC-PB) was 11.1% (9/81). The combined frequency was 9.5%. At this frequency about one
new insertion per litter is expected.

iPCR and DNA sequencing identified the location of 16 of the 21 new insertions (Table 2, Fig. 8). Twelve of these 16 (75%) new insertions occurred on a different chromosome relative to the initial transposon insertion site, whereas 4 were on the same chromosome (Table 2). Two of the new insertions mobilized from Gabrb1<sup>Bhr2</sup> by piggyBac transposase were 4 and 11 kb away from the initial transposon insertion site. 8 of the 16 (50%) transposon insertions were in introns of genes. For Sleeping Beauty transposase, one of the new insertions mobilized from Gabrb1<sup>Bhr2</sup> remained on chromosome 14 and one of the new insertions mobilized from LOC685774<sup>Bhr7</sup> remained on the X chromosome. Five of the intron insertions were generated by Bhr2 (Table 2). Three of the intron insertions were generated by Bhr7 and all of these were in the desired orientation relative to the direction of endogenous gene transcription (Table 2).

Progeny with transposon insertions at different loci were obtained from the same male seed rat, demonstrating that multiple unique transposition events can be recovered from an individual seed rat. In addition, we did not recover progeny with new transposon insertions at the same locus from an individual male seed rat. Combining the insertions generated by the initial pronuclear injections mediated by piggyBac transposase and the insertions generated by crosses, we have
generated 12 rat lines with gene-trap insertions in the following genes: Adam9, Ataxin7, Col4a3bp, Gabrb1, LOC685774, Plcb1, Prrxl1, RGD1310778, RGD1311463, Smurf2, Suclg2, and Zbtb20 (Table 1, 2, Fig. 8).

To determine if the Bhr2 transposon can trap endogenous mRNA, we analyzed rats homozygous for Gabrb1^{Bhr2}. RT-PCR was performed using total RNA from adult brain (Fig. 7). Bhr2 is located in intron 4 of Gabrb1. Primers specific to Gabrb1 exon 4 and the SA of Bhr2 amplified cDNA from the homozygous mutants, indicating that the gene trap sequences were functional (Fig. 1A, 5A, 7). In addition, primers were designed to determine if wild-type transcripts could also be generated by the mutant locus. These experiments demonstrated that some wild-type transcripts were also generated from the mutant locus, indicating that splicing was occurring around the transposon sequences (Fig. 7). Thus, the Bhr2 transposon located in intron 4 of the Gabrb1 locus could trap the mRNA transcribed by the locus but not completely, suggesting that this is a hypomorphic allele.

DISCUSSION

We have developed a hybrid transposon mutagenesis system in rats that uses simple crosses to
mobilize single copy transposons. Our transposons located in known genomic locations can be mobilized by matings to rats that express either Sleeping Beauty or piggyBac transposase. New integration sites in the genome are identified by changes in coat color. The Sleeping Beauty transposon system has been used for mutagenesis in rats but until now there have been no reports using piggyBac transposons to generate mutations (Kitada et al., 2007; Lu et al., 2007; Izsvák et al., 2010). PiggyBac/Sleeping Beauty hybrid transposons have been previously reported and shown to function in vitro (Wang et al., 2008; Liang et al., 2009). Here we show that hybrid transposons can be mobilized by both types of transposases in rat somatic and germ cells in vivo. Once rats with new insertions are identified by coat color, the precise site of integration can be determined quickly, using standard molecular techniques, i.e. PCR and DNA sequencing. Thus, using our system requires no specialized skills to generate mutant rats in contrast to the unique methods required by other rat mutagenesis strategies, e.g. in vitro fertilization, rat ES cell culture and chimera generation, spermatogonial stem cell culture and seminiferous tubule transplantation (Buehr et al., 2008; Li et al., 2008; Mashimo et al., 2008; Oatley and Brinster, 2008; Izsvák et al., 2010; Kawamata and Ochiya, 2010; Tong et al., 2010). It may be more efficient to generate transposon mutant rats by zygote pronuclear injection rather than by breeding but again this
requires special skills and resources for micromanipulation.

Our transposon vectors contain a Tyr minigene that can rescue albinism (Yokoyama, et al., 1990; Overbeek, et al., 1991). Nearly all of our transgenic rats with single copy transposon insertions had detectable pigmentation, although even the darkest pigmented pedigrees were lighter than wild type. Thus, the Tyr minigene can express at many locations within the genome (Yokoyama, et al., 1990; Overbeek, et al., 1991; Lu et al., 2007). In addition, as the level of expression of the minigene is influenced by chromosomal context, different transposon insertion sites in the genome resulted in different levels of pigmentation (Yokoyama, et al., 1990; Overbeek, et al., 1991; Lu et al., 2007). For most transposon integrations, there was a gene dosage effect. Rats that are homozygous for the transposon insertion usually were obviously darker than hemizygotes. Thus, the Tyr minigene allows visual genotyping and greatly simplifies the identification of homozygous, hemizygotes and wild-type rats (Yokoyama, et al., 1990; Overbeek, et al., 1991; Lu et al., 2007). Therefore, no molecular analyses are required to genotype the rats once the pedigrees are established, greatly simplifying their maintenance and analysis.

We isolated two transposon insertion sites (LOC685774\textsuperscript{Bhr7} and Phex\textsuperscript{Bhr7}) that were
albino either because Tyr expression is repressed by chromosomal context or the Tyr minigene was damaged upon integration (Karpen, 1994; Pikaart et al., 1998). Bigenic rats carrying LOC685774\textsuperscript{Bhr7} and either the UBC-SB or UBC-PB transgenes showed mosaic pigmentation and outcrosses to albino females yielded pigmented progeny. Therefore, we conclude that Bhr7 in the LOC685774 locus is structurally intact but Tyr expression is repressed at this chromosomal location. These transposon insertions whose expression is repressed and therefore albino proved to be very useful because the mutagenesis screen becomes simply looking for any pigmented progeny in crosses between seed males and albino females rather than distinguishing one color from another.

Previous transposon mutagenesis studies have generated initial transgenic mice or rats by standard pronuclear injection to create single integrations with transposon concatemers (Dupuy et al., 2001; Fischer et al., 2001; Horie et al., 2001; Dupuy et al., 2002; Ding et al., 2005; Kitada et al., 2007; Lu et al., 2007; Urschitz et al., 2010). These have been crossed with transgenic animals that express the corresponding transposase in the germline, using male germ cell-specific regulatory sequences. One problem with mobilizing transposons in a concatemer is chromosomal damage and scattering of the transposons to multiple sites within the genome,
complicating the molecular identification of the new insertions (Geurts et al., 2006). To circumvent these problems, we introduced our transposons into the rat genome by transposition to obtain single copy insertions, albeit at multiple chromosomal locations (Jang and Behringer, 2007). It might be possible to generate founders with single transposon insertions, bypassing the subsequent breeding required to generate single transposon pedigrees by titrating the transposon-containing plasmid or transposase RNA injected into zygotes. Our founders had 5-8 transposon insertions and were very darkly pigmented but their progeny that inherited varying numbers of the transposons had lighter pigmentation. This suggests that multiple transposons are expressing \textit{Tyr}, and the pigmentation of the founder is a collation of \textit{Tyr} expression. Therefore, the generation of transgenic founder animals by piggyBac transposition may be useful in certain species when expression is desired and germline transmission is not required or difficult (e.g. non-human primates) (Chan \textit{et al}., 2001). This can also be achieved using lentiviral vectors (Lois \textit{et al}., 2002; Sasaki \textit{et al}., 2009).

Our initial attempts to generate transgenic rats that express Sleeping Beauty or piggyBac transposase specifically in the germline using \textit{Oct4} sequences were not successful (Yeom \textit{et al}., 1996). We therefore, decided to express these transposases ubiquitously using the
human $UBC$ regulatory sequences. This proved profitable because the seed males could be visually identified very easily because they developed coat color mosaicism, indicating transposon mobilization in melanocytes or melanocyte precursors. Crosses of the seed males containing single copy transposons with albino females demonstrated germline transposition for both Sleeping Beauty and piggyBac transposases.

The frequency of new insertions by germline mobilization of single copy transposons in our system using $UBC-PB$ or $UBC-SB$ was about 10%. Considering the average litter size of the rat strains we used, this would translate to about one new insertion per litter. There have been a few reports in mice, demonstrating the remobilization and insertion of single copy Sleeping Beauty transposons by crosses with Sleeping Beauty transposase transgenic lines. In one report, one remobilization/insertion event was obtained in approximately 100 progeny screened, using a CAGGS-SB10 transgene (Geurts et al., 2006). In another study, remobilization/insertion was much more efficient ranging from 17 to 50%, using a $Prm1-HSB16$ transgene (Ruf et al., 2011). The variation in the frequency of remobilization/insertion of single copy piggyBac or Sleeping Beauty transposons may be due to the different transposase-expressing transgenic lines used. Until now there has been no report of mobilizing single copy transposons by simple crosses in
rats. We obtained transposition from the $Gabrb1^{Bhr2}$ and $LOC685774^{Bhr7}$ loci to sites on the same and different chromosomes. Although the numbers are small, it appears that both Sleeping Beauty and piggyBac transposition onto the same chromosome occur at similar frequencies in our system. For the transpositions onto the same chromosome we generated two additional alleles for $Gabrb1$. These were located 4 and 11 kb from the original intron 4 insertion site into the same intron, respectively. Therefore, perhaps for larger genes, our system could be used to generate an allele series for a particular gene of interest.

In this initial screen, we generated insertions in 12 genes in the rat genome. 6 of these genes have corresponding mouse mutants, including $Adam9$, $Col4a3bp$, $Plcb1$, $Prrxl1$, $Smurf2$, and $Zbtb20$. Thus, at this point in time about half of our rat mutants do not have an equivalent mouse mutant. $Prrxl1$ knockout mice have skin and pain sensitivity abnormalities and die at about 3 weeks of age (Chen et al., 2001). Our $Prrxl1$ homozygous mutant rats appear normal. It is not clear if this is because our allele is a hypomorph or that the rat genetic background is not permissive for expression of the skin or postnatal viability phenotype.

For the initial $Bhr2$ insertion into the $Gabrb1$ locus, molecular analysis demonstrated that the gene trap sequences were functional, however wild-type transcripts could also be
detected in the homozygotes most likely due to splicing around the gene trap sequences (McClive et al., 1998; Voss et al., 1998). The Gabrb1 homozygous mutant rats appeared normal and were fertile. There is currently no mouse knockout for this gene, however other GABA receptor mutants have been reported to be overtly normal perhaps because of the complexity of the GABA receptor system (Homanics et al., 1997; Sur et al., 2001). Molecularly, this Gabrb1 mutation is a hypomorphic allele. Hypomorphic alleles for certain types of genes could be very useful in cases when a null allele causes lethality at early embryonic stages or before adulthood. Perhaps Bhr2 and Bhr7 could be modified to generate more efficient gene traps, e.g. different splice acceptor sequences. However, our current system has been used by others to create a protein null allele in the rat Bobby Sox homolog, Bbx (Chun-Ming Chen, National Yang Ming University, personal communication).

Our initial results indicate that germline transposition can produce new integrations sites that are dispersed throughout the genome, implying that mutations can be created at many different loci. This is also true for chemical mutagenesis strategies (Zan et al., 2003; Smits et al., 2005; 2006; Homberg et al., 2007; Olivier et al., 2008; van Boxtel et al., 2008; Mashimo et al., 2008; Homberg et al., 2009; van Boxtel et al., 2010) and transposon mutagenesis in
spermatogonial stem cells (Izsvák et al., 2010). Current methods to generate targeted mutations in specific genes include gene targeting in rat ES cells, zinc finger nucleases, and TALE nucleases (Geurts et al., 2009; Mashimo et al., 2010; Tesson et al., 2011; Tong et al., 2012).

Many of these systems required very specialized technical skills not available in most laboratories. We are cryopreserving our transgenic rat strains, including Gabrb1<sup>Bhr2</sup>, LOC685774<sup>Bhr7</sup>, UBC-SB, and UBC-PB. Because our system requires breeding rats, visual genotyping, and standard molecular techniques most biomedical laboratories should be able to perform a mutagenesis screen using these rat strains. This system will lead to the generation of a large collection of single gene mutations for new rat models of human biology and disease.

**ACKNOWLEDGEMENTS**

We thank Allan Bradley for the mPB plasmid and Thom Saunders for helpful advice generating transgenic rats. Supported by National Institutes of Health (NIH) grant RR022904, the Ben F. Love Endowment, and Kleberg Foundation to R.R.B. DNA sequencing and veterinary resources were supported by the NIH Cancer Center Support Grant CA16672.
MATERIALS AND METHODS

Rats:

Sprague-Dawley outbred and Wistar Kyoto (WKY/NCrl) partially inbred rats were purchased from Charles Rivers. WKY/NHsd inbred rats were purchased from Harlan Laboratories.

Transposon-carrying rats were generated using Sprague-Dawley zygotes and then backcrossed to WKY/NCrl and subsequently WKY/NHsd. UBC-SB and UBC-PB transgenic rats were generated using WKY/NHsd zygotes and maintained on the same genetic background. All animal manipulations were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals and approved by the University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Construction of the Bhr2 and Bhr7 transposons and transposase gene constructs:

Bhr2 is shown in Fig. 1A. Bhr2 was constructed by inserting two copies of a gene-trap cassette which consists of an adenovirus splice acceptor (Ad2) and SV40 poly(A) signal (SA-pA) in opposite orientations flanking a mouse Tyr minigene in pXL-BacII piggyBac (Li et al., 2001).
The SA-pA fragment was subcloned by PCR from pT2/Bart3 (Lu et al., 2007). The Tyr minigene was isolated from pT-TyBS (P. Overbeek, unpublished), and modified by PCR-cloning a 290 bp minimal promoter, and ligating it to the Pmel site. The Sleeping Beauty transposon 5’ arm was PCR-cloned from pT3 (Yant et al., 2004), to flank the SA-pA cassettes and Tyr minigene.

*AttPlattB* sites were inserted in between piggyBac and Sleeping Beauty arms using oligonucleotides.

Bhr7 is shown in **Fig. 1B**. Bhr7 was constructed by inserting two copies of the Sleeping Beauty 5’ arm, PCR-cloned from pT3, into pXL-BacII to flank a gene-trap module and Tyr minigene. rtTA2S-M2 coding sequence led by an IRES sequence from the hXIAP 5’ UTR (Holcik et al., 1999) was inserted into the SA-pA cassette mentioned above to create the gene-trap module. The Tyr minigene coding sequence was followed by an ECMV IRES sequence (Borovjagin et al., 1991) and the splice donor site from rabbit beta-globin intron (van Ooyen et al., 1979). The Tyr minigene is similar to the one in Bhr2 except that it lacks a pA.

*UBC-SB* and *UBC-PB* transposase gene constructs are shown in **Fig. 4A**. The human UBC promoter is used to direct Sleeping Beauty or piggyBac transposase expression. The constructs are flanked by chicken transcriptional insulators (cHS4) (two copies on each side).
The *UBC* promoter was isolated from the UI4\_GFP\_SIBR plasmid (provided by Dr. David Turner, University of Michigan). The Sleeping Beauty transposase coding sequence was isolated from pCMV-HSB3 (*Yant et al.*, 2004). The piggyBac transposase coding sequence was isolated from pcDNA3-mPB (*Cadinanos and Bradley* 2007). A rabbit beta-globin intron and poly(A) signal (*van Ooyen et al.*, 1979) was subcloned downstream of the transposase coding sequences. The cHS4 insulator sequence was isolated from pNI-CD (*Bell et al.*, 1999).

**Generation of transgenic rats:**

Transgenic rats carrying transposons were generated by co-injecting circular plasmids with the transposons and piggyBac transposase RNA synthesized from pcDNA3-mPB, into zygotes (*Jang and Behringer* 2007). Transgenic rats expressing transposases were generated using standard methods (*Filipiak and Saunders*, 2006).

**Southern blot analysis:**

Toe DNA was used for Southern blot analysis as described (*Sambrook* 1989). Genomic DNA was cut with *PvuII* for Bhr2 or *ApaLI/BamHI* for Bhr7, separated by electrophoresis through a
1% agarose gel, and transferred to Hybond-XL (GE Healthcare). A 909 bp \textit{Hind}\textsubscript{III}/\textit{Sca}\textsubscript{I} fragment of mouse \textit{Tyr} was isolated from Bhr2 and used as a probe for detecting Bhr2. A 487 bp \textit{Apa}\textsubscript{I}/\textit{Pac}\textsubscript{I} fragment containing IRES and SD sequences from Bhr7 was used as a probe for detecting Bhr7. Probes were labeled with \textsuperscript{32}P-dCTP using the Rediprime II Random Prime Labeling System (GE Healthcare).

**Inverse polymerase chain reaction:**

To identify the genomic locations of the transposon integration events in the rat genome, we used an iPCR strategy (Chen \textit{et al.}, 2010). The locations of primers within the transposon and their sequences used for iPCR are shown (Fig. 1A, B, Table 3). We used I1/I2 and N1/N2 primer sets to identify transposon locations after initial pronuclear injections or after mobilization by piggyBac transposase by crosses. The primer sets of I1/IS2 and N1/NS2 were used to identify transposon locations after mobilization by Sleeping Beauty transposase. 1.5 mg of genomic DNA was digested by \textit{ApaI}, \textit{BamHI}, \textit{EcoRI} or \textit{XbaI} and heated at 70°C for 10 min to inactivate the enzyme. The digested genomic DNA was self-ligated using T4 DNA ligase (Roche) and purified by ethanol precipitation. The precipitate was dissolved in 25 \mu l of TE and 2 \mu l of the self-ligation
mixture was used as template for a first round PCR reaction. The first round PCR was performed using the following conditions: 94 °C for 1 min followed by 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C 3 min. A second round of PCR was performed using 2 µl of the PCR products from the first round of amplification as the template using an annealing temperature of 58 °C. Amplified DNA fragments were fractionated and purified for direct sequencing using primers N2 or NS2. The chromosomal location of the transposon was analyzed using the BLAST Search program provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ensembl (http://www.ensembl.org/Multi/blastview).

**PCR genotyping:**

Transposon detecting primers were Tyr-F and Tyr-R that recognized the Tyr minigene. The UBC-SB or UBC-PB transposase rats were identified by primers UBC-F1 and SB-R or UBC-F1 and PB-R, respectively. After the transposon locus was identified by iPCR, the transposon rats were confirmed by locus-specific primers. Gabrb1Bhr2 rats were genotyped using wild-type allele primers Gab-F and Gab-R and transposon allele primers Gab-F and B2-3, LOC685774Bhr7 rats were genotyped using wild-type allele primers LOC-F and LOC-R and transposon allele primers.
LOC-F and PB-IR5.2. The sizes of the amplification products and primer sequences are listed in Table 3.

**Gabrb1<sup>Bhr2</sup> RT-PCR:**

One microgram of total RNA isolated using the TRIzol Reagent (Invitrogen) from adult brain was subjected to RT-PCR analysis, using the SuperScript II first-strand synthesis system (Invitrogen), according to the protocol provided by the manufacturer. The purity and quantity of cDNAs were analyzed with rat beta-actin specific primers B-act F and B-act R. Primers used to detect the gene transcripts are: Gab-RT5F, Gab-RT5R, Gab-RTIF, Gab-RTIR, B2-RT, Gab-RT3F, Gab-RT3R. The locations for each primer are indicated in Fig. 5A. The sequence and size of the DNA fragments amplified by the primers are listed in Table 3.
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Improved generation of rat gene knockouts by target-selected mutagenesis in mismatch


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**FIGURE LEGENDS**

**Fig. 1. Transposons for transgenic rat production.** A, Bhr2 and B, Bhr7 transposon constructs.

Bhr2 and Bhr7 contain terminal inverted repeat sequences recognized by piggyBac transposase (light gray arrowheads) and the inverted/direct repeat sequences recognized by Sleeping Beauty transposase (dark gray arrowheads). Pink and green circles are *attB* and *attP* sequences, respectively, not exploited in this study. The primers for initial iPCR are I1, I2 and IS2 (black arrowheads) and for nested iPCR are N1, N2 and NS2 (blue arrowheads). The primer for RT-PCR for Bhr2 is B2-RT (red arrowhead). The primers, Tyr-F and Tyr-R (green arrowheads), are used for detecting the transposon in the rat genome. A portion of the *Tyr* reporter gene in Bhr2 and the splice donor and a subregion of the 3’ IRES sequence in Bhr7 were used as probes (Probe) for Southern blot analysis. Primer sequences for iPCR, genotyping and RT-PCR are described in the Table 3. SA, Ad2 splice acceptor; pA in Bhr2, SV40 polyadenylation signal; pA in Bhr7, human growth hormone polyadenylation signal; Pro, *Tyr* promoter; pA after *Tyr*, *Tyr* polyadenylation signal; IRES, internal ribosomal entry site; rtTA, reverse tetracycline transactivator; SD, splice donor of rabbit beta-globin intron; A, *Apa*I; AL, *Apa*LI; B, *Bam*HI; E, *Eco*RI; P, *Pvu*II; X, *Xba*I.
**Fig. 2. Generation of transgenic rats by piggyBac transposition.** A, Litter generated from piggyBac transposon Bhr2 injected to albino Sprague-Dawley rat zygotes. Bhr2 piggyBac transposon founder transgenic rats are pigmented. Sprague-Dawley rats are homozygous for the hooded mutation that restricts pigmentation to the head and dorsum. B, Offspring from a piggyBac transposon founder male crossed with a Sprague-Dawley female. All progeny were pigmented and each individual had a different coat color. C. Southern blot analysis using a Bhr7-specific probe, showing multiple transposon integrations in a founder (F) and their segregation in progeny (1 to 8) generated by crossing with a Sprague-Dawley female.

**Fig. 3. Segregation and analysis of initial piggyBac transposon insertions.** A, Southern blot analysis, showing the isolation of single Bhr7 transposon gene insertions in separate pedigrees. L, LOC685774; P, Phex; +, wild type. Bhr2 transposon insertion in the Gabrb1 locus (G). The Tyr probe used for Bhr2 Southern analysis recognizes a single band of approximately 7.4 kb for the endogenous Tyr locus. The Bhr2 transposon insertion in the Gabrb1 locus shows a single band of approximately 7.6 kb and therefore appears the same as wild type. B, Coat color differences in
rats with different single transposon insertions. The rats with insertions in LOC685774 or Phex loci remained albino. C, Rats with the Bhr2 transposon in the Gabrb1 locus. Tn/Tn rats are darker than Tn/+ rats. D, Rats with the Bhr7 transposon in the Zbtb20 locus. Tn/Tn rats are darker than Tn/+ rats. E, PCR genotyping of progeny from a cross between Gabrb1\textsuperscript{Bhr2} hemizygotes.

**Fig. 4. Generation and functional analysis of piggyBac and Sleeping Beauty transposase-expressing transgenic rat lines.** A, Diagram of UBC-SB and UBC-PB gene constructs. The Sleeping Beauty or piggyBac transposase expression is driven by the human UBC promoter and flanked by chicken hypersensitive site 4 transcriptional insulators (cHS4) to reduce the influence of chromosomal integration sites. Transposase transgenic rats were identified by PCR using UBC-F and SB-R or PB-R primers (black arrowheads). Primer sequences for genotyping are described in the Table 3. pA; rabbit beta-globin intron and polyadenylation signal. B, Male containing the Bhr2 transposon in the Gabrb1 locus (left) and seed male containing the Bhr2 transposon in the Gabrb1 locus and Sleeping Beauty (middle) or piggyBac (right) transposase transgenes. All seed rats exhibited coat color mosaicism. C, Male
containing the Bhr7 transposon in \textit{LOC685774} locus (left) and seed male containing the Bhr7 transposon in the \textit{LOC685774} locus and Sleeping Beauty transposase (middle) or piggyBac transposase (right) transgene. All seed rats were pigmented.

\textbf{Fig. 5. Structure of the Bhr2 transposon insertion in the \textit{Gabrb1} locus and germline mobilization.} A, Diagram of the Bhr2 transposon insertion site in the \textit{Gabrb1} locus. Brown arrow shows the direction of \textit{Tyr} transcription. Primers for genotyping, arrowheads. Gab-F and Gab-R can detect the wild-type allele and Gab-F and B2-3 can detect the transposon allele. Primers for RT-PCR analysis, red arrowheads; Ex., exon. B, Coat colors of the progeny with new transposon insertions originating from the \textit{Gabrb1} Tn locus generated by crosses of seed males and transposase-expressing rats. 1-3 have different pigmentation compared to the original Bhr2 transposon \textit{Gabrb1} rat. G, \textit{Gabrb1} Tn/+ rat. C, PCR analysis of genomic DNA. The Sleeping Beauty transposase transgene is no longer present in the progeny (1-3) carrying the transposon at a new position (upper panel). The rat with the Bhr2 transposon in the \textit{Gabrb1} locus has a wild-type (450 bp) and transposon (628 bp) allele but the rats with the new transposition events only have the wild-type band, demonstrating that the Bhr2 transposon has transposed away from
the Gabrb1 locus (lower panel). 1-3 and G are the same as in B. PC, positive control; Wt, wild type. D, Transposition events revealed by Southern blot analysis of offspring from seed males using Tyr probe. The Bhr2 transposon in the Gabrb1 locus and the endogenous Tyr locus digested with PvuII resulted in 7.6 kb (G) and 7.4 kb (Wt) bands, respectively. Progeny generated from seed male with different coat colors compared to Gabrb1Bhr rats have an additional band (*). Progeny #2 likely has a new insertion that yields a band of similar size to the Gabrb1 insertion. 1 to 3, and G are the same as in C. E, Mutagenesis strategy using Bhr2 transposon insertion in the Gabrb1 locus. The seed male carrying both the transposon and transposase transgene that has coat color mosaicism is bred with an albino female. Progeny carrying the transposon at a different locus should have a different coat color compared to the original Bhr2 Gabrb1 insertion. Primer sequences for genotyping and RT-PCR are described in the Table 3.

Fig. 6. Structure of the Bhr7 transposon insertion in the LOC685774 locus and germline remobilization. A, Diagram of Bhr7 transposon insertion site in the LOC685774 locus. Brown arrow shows the direction of Tyr transcription. Primers for genotyping, arrowheads. LOC-F and
LOC-R can detect the wild-type allele and LOC-F and PB-IR-5.2 can detect the transposon allele.

Ex., exon. B, Coat color of progeny (1 and 2) with new transposition insertion sites. C, PCR analysis of genomic DNA for the piggyBac transposase transgene (upper panel) and transposon insertion in LOC685774 (lower panel). The upper panel shows that the piggyBac transposase transgene is not present in the rats carrying the Bhr7 transposon at a new position (1 and 2). The lower panel shows the transposon in the LOC685774 locus is not present in the pigmented progeny (1 and 2). 1 and 2 are the same as in B. L, Bhr7 transposon in the LOC685774 locus of a Tn/+ female; Wt, wild type, S; seed rat. D, Transposition events revealed by Southern blot analysis of offspring from seed males using a Bhr7-specific probe. The seed male (S) has multiple bands because of multiple transposition events in somatic tissues. The progeny that are pigmented have single bands of different size compared to the Bhr7 transposon in the LOC685774 (1 and 2). 1, 2, L, Wt and S are the same as in C. E, Mutagenesis strategy using Bhr7 transposon insertion in the LOC685774 locus. Rats carrying the Bhr7 transposon in the X chromosome-linked LOC685774 locus are albino. The seed male rat carrying both the transposon and transposase transgene that shows coat color mosaicism is bred with an albino female. Progeny carrying the transposon at a different locus can become pigmented. Primer sequences
for genotyping are described in the Table 3.

**Fig. 7. Expression analysis in Gabrb1 mutant rats.** Adult brain mRNA of wild-type (left) and Gabrb1 (Tn/Tn) (right) rats. Primers for RT-PCR (red arrowheads) are shown in Figs. 1A and 5A. Primers G-RT5F/G-RT5R, G-RTIF/G-RTIR and G-RT3F/G-RT3R amplify mRNA before (5), between (I) and after (3) the transposon insertion site, respectively. Primers G-RTIF/B2-RT amplify mRNA trapped by the transposon in the presence of reverse transcriptase (+RT). All primer sets do not amplify DNA in the absence of RT (–RT). The Tn/Tn rat shows trapped mRNA (Tn) and wild-type mRNA (I and 3). Primers b-act F/b-act R were used to detect beta-actin expression (C). Primer sequences for RT-PCR are described in the Table 3. m, 100 bp ladder DNA marker.

**Fig. 8. Diagram of transposon insertions in the rat genome.** Yellow circles, initial transposon insertions; blue circles, insertions mobilized from Gabrb1^Bhr2 Bhr7 by piggyBac transposase; red circles, insertions mobilized from Gabrb1^Bhr2 Bhr7 by Sleeping Beauty transposase.
### Table 1. Summary of initial transposon insertions in founders

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<tr>
<th>Tn</th>
<th>Locus</th>
<th>Chr</th>
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<tbody>
<tr>
<td>Bhr2</td>
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<td>Bhr2</td>
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<td>Bhr7</td>
<td>Ataxn7</td>
<td>15</td>
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</tr>
<tr>
<td>Bhr7</td>
<td>Mug *</td>
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<tr>
<td>Bhr7</td>
<td>Phex **</td>
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Chr, chromosome; IR, intergenic region; Tn, transposon.

*iPCR sequence data for this single copy Tn insertion matches 4 genes (LOC297568, Mug1, Mug2 and F1M4L8_RAT) linked within a ~500 kb region. F1M4L8_RAT is Cpamd8 which is Mug2 in the mouse. The precise location of the Tn is currently ambiguous because the genes have high similarity with each other. Only in the case of F1M4L8_RAT is the Tn in the correct orientation for gene trapping.

**Tn insertion located in promoter region in reverse orientation relative to the direction of transcription.
Table 2. Summary of transposon insertions after transposition from the initial transposon insertions.

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<th>New locus</th>
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<th>Transposase</th>
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<td>Gabrb1 *</td>
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<td>PB</td>
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<td>Gabrb1 (14)</td>
<td>Gabrb1 **</td>
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<td>PB</td>
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<td>1, 1, 9, X</td>
<td>SB</td>
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</table>

Chr, chromosome; IR, intergenic region; PB, piggyBac; SB, Sleeping Beauty; Tn, transposon. * and ** are independent transposition events within the same intron 3' of the original Tn insertion.
**Table 3. Primer sequences for iPCR, genotyping transgenic and Tn rats and RT-PCR.**

### iPCR

<table>
<thead>
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<td>I1</td>
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### Genotyping

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<tr>
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<td>(Tyr-R) 5'-GTGGTCCCTCAGGTGTTCCATCG-3'</td>
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<tr>
<td></td>
<td>(SB-R) 5'-GTACAGATGAACGTGGTACCGCAG-3'</td>
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<tr>
<td>T'ase (PB)</td>
<td>(UbC-F) 5'-CTGAAGCTCCGGTTTTGAACATATGC-3'</td>
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<tr>
<td>LOC685774 (Tn)</td>
<td>(LOC-F) 5'-GCTCACAATCTACCATTCTCTTGATGCGCC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(PBIR-5.2) 5'-TCCTAAATGCACAGCGACGGATTC-3'</td>
<td>319</td>
</tr>
</tbody>
</table>

### RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-actin</td>
<td>(b-act F) 5'-CAGTCCGCTGATGAGCATCATG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b-act R) 5'-CCATCACAATGCGAGGATCGGAC-3'</td>
<td>471</td>
</tr>
<tr>
<td>Gene</td>
<td>RTF</td>
<td>RT5F</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Gabrb1 (5')</td>
<td>(G-RT5F) 5'-CCATGGTTTGTTGTGCACACAGCTCC-3'</td>
<td>(G-RT5R) 5'-GTGGTTCAGCTACCCCTGTGGTCAAGG-3'</td>
</tr>
<tr>
<td>Gabrb1 (Tn (-))</td>
<td>(G-RTIF) 5'-ACAACAGGGTAGCTGACCAACTCTGG-3'</td>
<td>(G-RTIR) 5'-ACTGATGGTTGTCATGGTCAGCACC-3'</td>
</tr>
<tr>
<td>Gabrb1 (3')</td>
<td>(G-RT3F) 5'-TGTGTTCGTGTTCTCCTGCTACTGGG-3'</td>
<td>(G-RT3R) 5'-CGCTGTCGTAGTACATAGTGCC-3'</td>
</tr>
<tr>
<td>Gabrb1 (Tn (+))</td>
<td>(G-RTIF) 5'-ACAACAGGGTAGCTGACCAACTCTGG-3'</td>
<td>(B2-RT) 5'-CACTGCATTCTAGTTGGTGGTCCAAAACCATC-3'</td>
</tr>
</tbody>
</table>

WT, wild type; Tn, transposon; T'ase, transposase
Fig. 1

A Bhr2

Terminal Inverted Repeat (PB) ▲ Inverted/Direct Repeat (SB)

B Bhr7

Terminal Inverted Repeat (PB) ▲ Inverted/Direct Repeat (SB)
**Fig. 4**

A. Diagram showing a transposase insertion into the UBC Pro domain.

B. Bhr2 (Gabrb1 locus)

C. Bhr7 (LOC685774 locus)
Fig. 5

A. **Gabrb1 locus**

B. Image of wild-type and mutant alleles.

C. Diagram showing wild-type and mutant alleles with genetic markers.

D. Gel electrophoresis results showing bands at 10kb, 8kb, 7kb, and 5kb.

E. Cartoon illustration of mouse breeding and genetic inheritance.
Fig. 6

A

LOC685774 locus

LOC-F  LOC-R  Ex.1

Wild-type allele

LOC-F  PB-IR-5.2  LOC-R  Ex.1

Mutant allele

Ex.2

B

1  2

C

PB

LOC

D

1  2  L  Wt  S

10kb  8kb  6kb  5kb  4kb  3kb  2kb  1kb

E

Tn  T'ase

Seed male  Wild-type

new insertion
Fig. 7

**Wild type**

- **m**
- **5**
- **I**
- **3**
- **Tn**
- **C**

**Tn/Tn**

- **m**
- **5**
- **I**
- **3**
- **Tn**
- **C**

**RT**

**-RT**