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Manipulation of the Mouse Genome by Transposon and Recombinase Techniques

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Chapter 1

Abstract

The importance of understanding mammalian gene function has become more and more relevant since the increased insights on the sequence level. Especially in the context of human genetic diseases, the knowledge of gene function is one major focus of current research. Techniques like the generation of gene knock-outs in model organisms, provide concrete understanding of loss-of-function mutations. But this technology requires a certain knowledge about the target gene and a connection to the disease, and hence is based on reverse engineering.
A different strategy is random mutagenesis, followed by phenotypical screens, like gene trap or ENU technology. Both techniques are highly efficient and lead to mutations that might show a link to certain genetic diseases in human. Another random method has proven its usefulness by combining advantages of both screens, which is transposon-based insertional mutagenesis.
Transposon systems can efficiently cause insertional mutations in vivo and in vitro in nearly all species. The transposase enzyme manages the excision and reintegration of a transposon sequence in a genomic context. The cargo sequence can be chosen unrestricted, as long as it is flanked by the transposase binding sites. Therefore transposon systems can efficiently cause mutations that are easy to localize, by using the transposon sequence as a tag. They also provide the advantage of in vivo activity, which makes phenotypic screens in model organisms a lot faster.
In this work, several different transposon systems (subforms of *Sleeping Beauty* and *piggyBac*) were established by generating mouse lines which express the respective transposase, and others that harbor transposon constructs, which make in vivo detection of transposition events possible. By generating double transgenic mouse lines and backcrossing them with wild type mice, offspring then shows new insertions of the transposon sequence (potential mutations) and can be screened phenotypically.
Transposon systems show different characteristics, some reinsert totally random, some show preference of local reintegration with respect to the initial integration site. Therefore another technique was used in combi-

nation, Flp recombinase mediated cassette exchange of transposon constructs with FlEx gene trap clones. This technique works highly efficient and allows the single-copy exchange of any desired sequence with gene trap insertions. In context of a transposon-based mutagenic screen, this method offers the possibility of an allelic series of insertional mutagenesis, starting from a known and verified gene trap locus. Especially in human diseases which are caused by multiple mutations in a critical region (like the Williams-Beuren Syndrome), the generation of allelic series of mutations are reasonable. RMCE additionally represents an efficient approach to generate knock-in alleles. The described technique can be of great advantage to gain more knowledge and understanding of genetic diseases in model organisms.
The benefit of both techniques established in this work, the transposon-based mutagenesis and the recombinase mediated cassette exchange is, that they offer the possibility to combine already established mutagenic screens, to generate loss-of-function mutations in either a random, a local or a "directed-random" manner.

Chapter 2

Introduction

2.1 Forward Genetics - Random Mutagenic Screens

Whole genomes of numerous different species are completely known; the technologies of high throughput sequencing have opened up a lot of insights on transcriptomes and individual sequence variation. But one major goal was not achieved yet: the understanding of the function of every single gene in the mammalian genome. The mouse model is perfectly suited for functional genomics: 1. mice and man show 93% overall genetic similarity; 2. several lines of mouse ES cells are available for genetic manipulation of the mouse germline, and 3. with a generation time of 9 weeks, mice are easily maintained and handled compared to other mammals.
The motivation to understand gene function is primarily the identification of genes or gene networks which underlie human genetic disease therefore, a phenotype-driven mutagenic approach seems favorable. Forward genetics phenotype-driven screens generate random mutations in vivo, produce animals with a phenotype related to the human disease of interest, followed by the determination of the responsible gene.
The first well established mutagenic forward genetic screen is ENU (N-ethyl-N-nitrosourea) chemical mutagenesis. Treatment with ENU of adult male mice leads to highly efficient mutagenesis (mainly point mutations) of the male germ cells, which then result after breeding to wild type female mice in offspring carrying the mutated allele.
ENU mutagenic efficiency is about two times higher than insertional mutagenesis (Justice et al., 1999), and has been mostly used to generate mouse models for dominant genetic diseases, but the localization of the mutated gene underlying the phenotype is often very time and work consuming, since the point mutation needs to be positionally cloned (de Angelis et al., 1998; Acevedo-Arorena et al., 2008).
An alternative way to efficiently manipulate the mouse genome is insertional mutagenesis like gene trap technology (Friedrich and Soriano, 1991;

Gossler et al, 1989), which generates random loss-of-function mutations of active genes in ES cells and reports level and location of expression of the disrupted gene by integrating a reporter gene that is driven by the endogenous promoter. Advantages of gene trap mutagenesis are not only the high efficiency of integration of the mutagenic vector, also the fast and easy localization of the insertion site by using Splinkerette PCR or RACE protocols (Horn et al., 2007; Wiles et al., 2000; Glaser et al., 2005; Stanford et al., 2001).
Disadvantages are that with commonly used promoter trap vectors, only genes that are active in ES cells can be trapped successfully, so genes with a later onset get lost. After trapping a new locus, the generation of a mutant mouse by blastocyst injection is time consuming and predictions about resulting phenotypes are difficult to be made in advance. A striking potential in the mouse is clearly the possibility to generate conditional mutations, either in certain tissue, or at a given timepoint, by using FLEx gene trap vectors (Schnütgen et al., 2005; Floss and Schnütgen, 2008) and breeding gene trap mice with various Cre mouselines.
One way to combine all advantages of both mutagenic screens described above is to establish a transposon-based insertional mutagenic in vivo screen. Different transposon systems are functional in all species, and a number was also shown to be efficiently active in the mouse germline. Like in gene trap technology, the transposon sequence itself can be utilized as a tag for rapid identification of the insertion site, and like in ENU, by creating an appropriate transposon vector, which contains detection markers, phenotype-driven in vivo mutagenesis could be established. Fig. 2.1 schematically shows ENU and gene trap technology used for mutagenic screens and points out the advantages that can be combined in a transposon based screen.

2.2 Transposon Mutagenesis

Until Barbara McClintock discovered transposons, "jumping genes" (McClintock, 1950), it had been believed that genes were fixed at their location on the chromosome. Such mobile genetic elements not only occur in maize, they are found in a variety of organisms. The transposase (TS) enzyme is able to bind to certain recognition sites, the transposase binding sites (TBS), cut the flanked sequence, the transposon (TP), out of its location and reintegrate it into another genomic locus (van Luenen et al., 1994). Some transposases reintegrate the excised sequence randomly, some only in short target sequences.
There are two main classes of transposon systems described, retrotransposons (class I), which mobilize the transposon as an RNA intermediate and thereby duplicate their sequence during transposition, and DNA transposons (class II), which either work also in a copy-and-paste, or in a

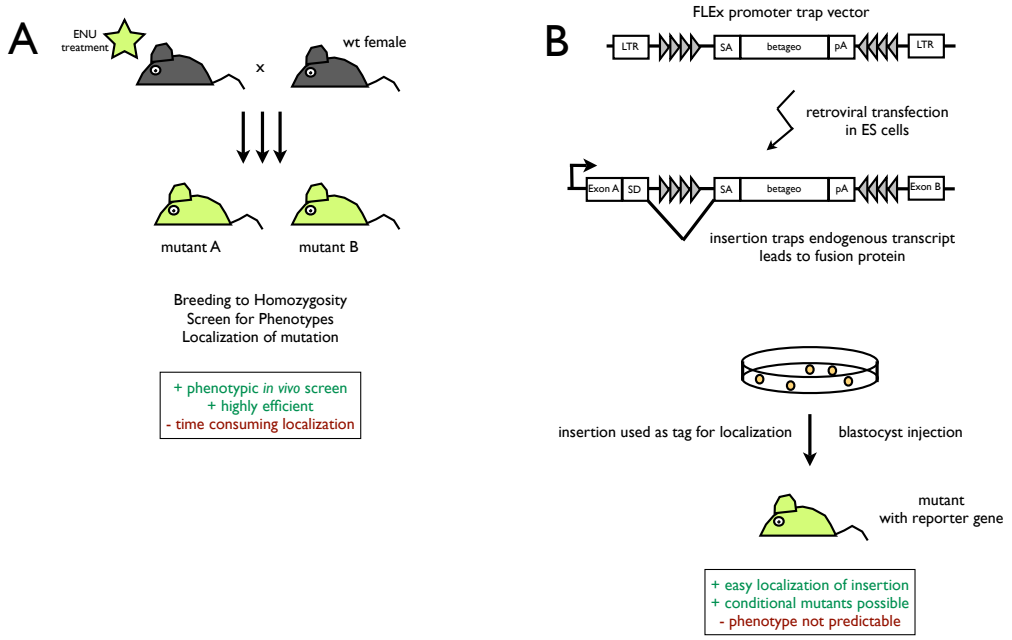


Figure 2.1: *Forward genetic random insertional mutagenic screens*
(A) Schematic outline of ENU mutagenesis. ENU treated male mice are crossed with wild type mice to give rise to mutant offspring, which then can undergo a phenotypic screen and breeded to homozygosity. (B) Insertional mutagenesis by gene trap technology in vitro. A promoter trap vector is integrated via retroviral transfection and leads to disruption of an endogenous transcript. ES cell clones with insertions can then be injected into blastocysts and mutant mice can be produced.
Advantages (green) and disadvantages (red) of each screen are pointed out in boxes below. A transposon-based in vivo screen can combine advantages of both techniques.

cut-and-paste manner. The latter does not lead to the duplication of the transposon sequence (see Fig. 2.2) and represents the major mechanism within DNA transposons (Kaufman and Rio, 1992).
The sequence flanked by the TBS is often the coding sequence of the respective transposase enzyme, but if the TS enzyme is expressed in trans, any cargo can be mobilized as long as it contains the required sequences for recognition (Craig et al., 2002), so transposons reveal an effective system to deliver any genetic feature into the genome (gain-of-function) or work as insertional mutagens (loss-of-function), by inserting themselves into a gene. In model organisms like yeast, *C. elegans* or *Drosophila*, transposon systems have already been widely used to gain more insight in gene function.

2.2.1 Picking the Transposon System

The aim of the present work was a transposon-based mutagenic screen in mice. For this, the first step was to decide for the appropriate trans-

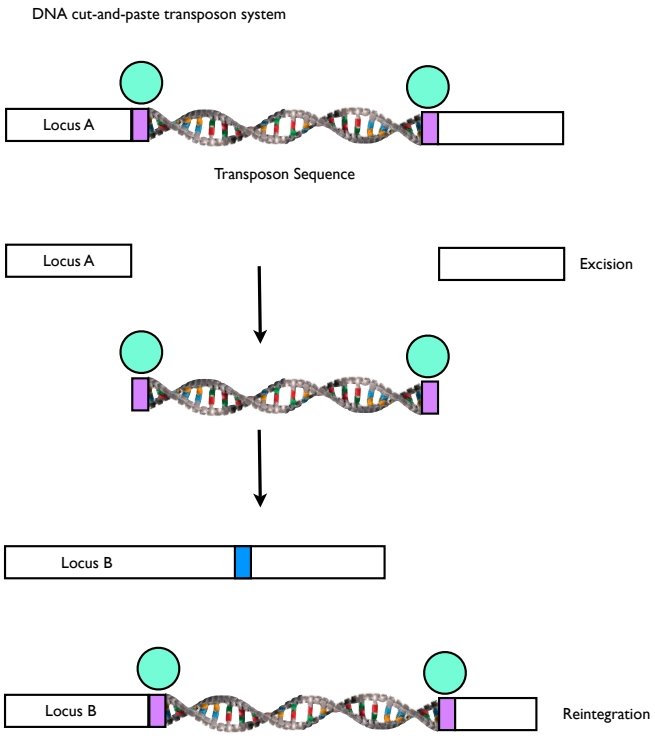


Figure 2.2: *DNA cut-and-paste transposon system*
Shown is the schematic excision and reintegration mechanism of a DNA cut-and-paste transposon system. The transposase enzyme (circle) binds to certain transposase binding sites (TBS, purple) that flank the transposon sequence, excises the sequence and reintegrates it either randomly or into a certain target sequence (blue) at a new genomic locus.

poson system which is sufficiently active in vertebrates and functional in the mouse germline. Vertebrates have genetically inactivated endogenous transposons during evolution by various point mutations. Therefore, it was reasonable that in general, high activity of transposases is not tolerated by the vertebrate genome (Ivics et al., 1997).

Other criteria for choosing an appropriate system are for example the independence of host factors of the transposase. Further, it was necessary to consider whether there are known reintegration hotspots that could limit a wide-range insertion spectrum, the ability of single copy mobilization and the magnitude of factors like cargo size and methylation for efficiency of transposition.

Based on these requirements, two different transposon systems were chosen to establish a mutagenic screen. Both represent DNA transposon systems and had been previously used in the mouse with success, however, both showed also different advantages concerning on their re-mobilization abilities.

2.2.1.1 *Sleeping Beauty*

Sleeping Beauty (SB), originally reconstructed from salmonid fish (Ivics et al., 1997), is a member of the Tc1/mariner superfamily, which is widespread in nature and represented in ciliates, plants, fungi and animals (Plasterk et al, 1999), also in several vertebrate genomes including human (Smit and Riggs, 1996). The family is named after its best studied members, Tc1, discovered in *C. elegans* (Emmons et al., 1983) and the related mariner transposon, found in *Drosophila mauritiana* (Jacobson et al., 1986). It has been shown since its reconstruction, that *Sleeping Beauty* is active in tissue culture, in living mice and in the germline of mice (Fischer et al., 2001; Dupuy et al., 2001; Horie et al., 2001).
SB transposon system has recently shown that its application as a cancer gene discovery tool and its conditional expression in certain tissues is highly effective (Starr et al., 2009; Dupuy et al., 2006; Keng et al., 2009; Collier et al., 2005). Unwanted negative effects seem to occur only if the transposon sequence is integrated multiple times as chromosomal concatemers, then transposon mobilization can also lead to genomic rearrangements involving up to tens of millions of base pairs (Geurts et al., 2006), but this "disadvantage" can also be used on purpose for a desired deletion of certain regions in the genome (Kokubu et al., 2009).
The binding sites of SB transposase are inverted and direct repeats (IRDR) of about 230 bases at each side of the transposon sequence, which contain two imperfect direct repeats of about 32 base pairs, separated by a spacer sequence (Cui et al., 2002). Each direct repeat represents a binding site for one transposase molecule hence, four molecules bind during transposition (Izsvak et al., 1995). Originally the sequence flanked by the IRDRs codes for the transposase enzyme (see Fig. 2.3) and typical Tc1/mariner elements are about 1300-2400 bp in lenght (Plasterk et al., 1999).
Sleeping Beauty transposase requires a TA dinucleotide sequence for integration, which is duplicated after transposition. If the transposon is mobilized, not only the duplication stays behind, also a three base pair footprint is left at the donor site after excision (see Fig. 2.4). These footprints are the consequence of the donor site repair by end-joining, when target sequences got duplicated after insertion, but stay behind at the donor site after excision (Luo et al., 1998; Coen et al., 1986; Weil and Kunze, 2000).
Even if the potential insertion sites seem limited by that, the approximately 200 million TA sites in the mouse genome, among these, 10% will be favored as integration site (Geurts et al., 2006), represent still a huge amount of potential mutations.
SB has shown a small but significant bias to integrate the transposon sequence into genes and their upstream regulatory sequences, but was not dependent on their transcriptional activity (Yant et al., 2005).
SB transposition requires mainly the transposase enzyme itself, but some studies have shown that host factors like HMGB1 are cofactors for trans-

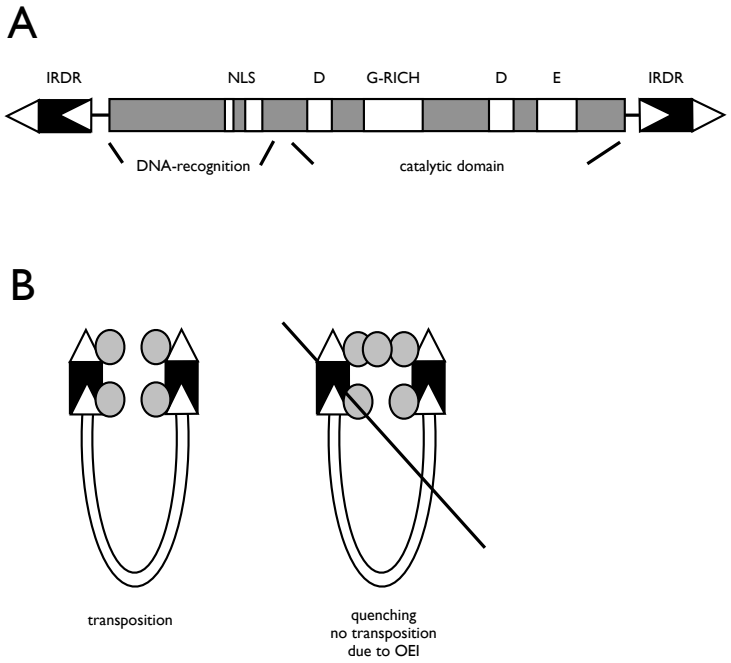


Figure 2.3: *(A) Schematic map of the original Sleeping Beauty transposon. Inverted and direct repeats (IRDR) flank the coding sequence for the transposase enzyme. The N-terminal half is the DNA binding domain and overlaps with a bipartite nuclear lokalization signal (NLS). The C-terminal half, the catalytic domain, catalyzes the transposition and contains the DDE domain and a glycine-rich motive (Ivics et al., 1997). (B) Model for overexpression inhibition of Sleeping Beauty. Four transposase molecules bind to the IRDR which flank the transposon sequence and manage the transposition event. If the expression of transposase is too high, additional molecules bind (quenching) and transposition gets inhibited (Hackett et al., 2007).*

position (Zayed et al., 2003) and the methylation status of the transposon has a positive influence on the transposition efficiency (Yusa et al., 2004; Ikeda et al., 2007).

The size of the excised cargo shows to have a direct effect on transposition efficiency; with increasing size, the efficiency drops nearly exponentially in contrast to wild type size, approximately decrease of efficiency is 30% per additional kb (Izsvak et al., 2000; Geurts et al., 2003).

This fact implies potential problems, but since several hyperactive versions of the SB transposase have been generated by our collaborator Zoltan Ivics, which all showed increased efficiencies in respect to the older versions, size limitations appear less important. SB11 shows a roughly threefold transposition efficiency increase with respect to the older version SB10 (Geurts et al., 2003), SB M3a a four-fold increase (Ivics et al., unpublished results) and the latest version SB100X even a 100-fold increase (Mates et al., 2009).

Mariner transposon systems reach efficiency limitations also beyond a certain point of increasing amount of transposase enzyme, this effect is called

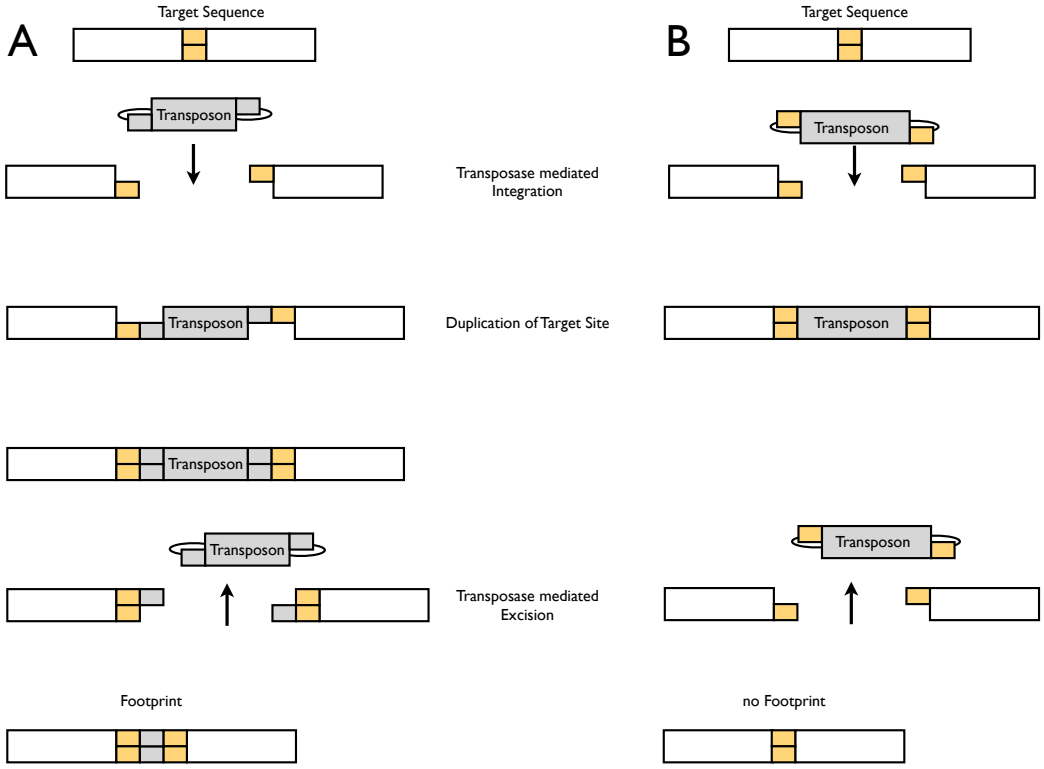


Figure 2.4: *Footprint and precise cut-and-paste transposition*
(A) Schematic outline of a footprint cut-and-paste mechanism like in case of Sleeping Beauty. The transposon integrates into a TA nucleotide and a staggered double-stranded DNA break is introduced, which leads to a duplication of the target sequence. Also excision leads to a staggered DNA cut at the ends of the transposase binding sites, which after DNA repair leaves a two base footprint behind (Luo et al., 1998). (B) PiggyBac in contrary integrates and exises the transposon sequence precisely and without a footprint and also the TTAA target sequence which is required for integration, is not duplicated during transposition (Elick et al., 1996).

overexpression inhibition (OEI) and results from quenching - transposase molecules bind to those bound to the IRDR - if the ratio of transposase to transposon exceeds 4 to 1 (Hartl et al., 1997; Hackett, 2007, see Fig. 2.3 B). In vitro studies, using different strong promoters driving the transposase, didn't show this effect in case of SB (Izsvak et al., 2000). By testing same effect within a much broader range (17:1 to 1:33 transposon to transposase amount) a dramatic inhibition of transposition efficiency with higher transposase amounts could be observed (Geurts et al., 2003).

One crucial feature of the *Sleeping Beauty* transposon system is the so called "local hopping" effect. Studies have shown that transposition events out of a chromosomal donor site do not occur randomly over the whole genome, but occur in 50% probability on the same chromosome (Keng et al., 2005). 25% of cut-and-paste events even take place within a 200 kb region (Horie et al., 2003), 9% within 100 kb of the donor site (Wang et al., 2008). Through this effect *Sleeping Beauty* is a unique tool in respect to the generation of al-

lelic series of mutations within a critical region for human disease (e.g. Williams-Beuren Syndrome, X-linked mental retardation).
For an *in vivo screen* the SB transposon system has some attractivity, despite the described limitations concerning cargo size, methylation and overexpression inhibition. By choosing a hyperactive form of the transposase enzyme, expressing the transposase in trans with an appropriate promoter and suitable transposon design, these limitations could be overcome.
In vivo, the SB system was demonstrated highly efficient: up to 90% of offspring of double transgenic mice (expressing the transposase and harboring the transposon), exhibit transposon mobilizations (Horie et al., 2001; Carlson et al., 2003). Furthermore, SB is capable to produce stable single-copy insertions to chromosomes (Izsvak et al., 2000) which could result in long-term expression. With respect to the goal of establishing mouse models for human disease, the local hopping preference of SB is its major advantage, because a number of human diseases result from large chromosomal deletions with multiple candidate genes, which could be mutated after inserting the primary insertion next to it.
In order to pursue this task, a system for targeted delivery of the transposon to defined chromosomal regions had to be established (see below). In this work the hyperactive form of *Sleeping Beauty*, SBM3a, was chosen for the in vivo screen. Although *in vivo* work was not done with SBM3a up to then, its enhanced activity appeared to be well suited to overcome possible disadvantages which had been reported from SB11.

2.2.1.2 *PiggyBac*

PiggyBac (PB, formerly IFP2) transposon system belongs to the family of TTAA-specific short repeat elements. Members of this group (class II transposons) show similar structures and movement properties and insert in TTAA target sites (Wang et al., 1989; Cary et al., 1989). *PiggyBac* itself was isolated from the cabbage looper moth *Trichoplusia ni* and has been widely used in insects, but its usefulness and high efficiency was also demonstrated in human cell lines (Wilson et al., 2007), mouse ES cells (Wang et al., 2008) and living mice (Ding et al. 2005) and recently also as tool for inducing pluripotency in mouse cells (Woltjen et al., 2009; Yusa et al., 2009).
The *piggyBac* element is 2.4 kb in size and terminates in 13 bp perfect inverted repeats with additional internal 19 bp inverted repeats that are located asymmetrically with respect to the ends (Cary et al., 1989).
In contrast to *Sleeping Beauty*, *piggyBac* shows precise cut-and-paste events, the target sequence TTAA is not duplicated after excision, therefore no footprints stay behind after transposition (Elick et al., 1996; Fraser et al., 1995; Mitra et al., 2008, see Fig. 2.4 B).
Overexpression inhibition and reduced transposition efficiency caused by larger cargo size have not been reported from *piggyBac* (Hackett, 2007), which implicates a wider use and less restrictions for random insertional

mutagenesis.
The activity of *piggyBac* transposase seems to be significantly higher than of SB11 and the transposase shows less sensitivity towards molecular modifications (Wu et al., 2006). Studies have shown that the original PB transposase is 10 times more efficient than SB100 and a mouse codon optimized version of *piggyBac* (mPB, Cadinanos and Bradley, 2007) even 13 times. These studies were performed side-by-side in vitro, with a single copy chromosomal integration of equal size for each tested transposase (Liang et al., 2009). The local hopping effect of SB was confirmed in this study, whereas PB showed clearly random reintegration sites. *PiggyBac* inserted with a slightly higher preference into genes, but both transposon systems preferred active transcribed loci and 5' integrations. The reintegration rates of both systems were about the same (roughly 50%), even if the overall activity was different.
PiggyBac has proven its wide range of applications in mutagenesis and shows attractive characteristics and less limitations than *Sleeping Beauty*. Both available subforms (iPB and mPB) of this transposon system were chosen as a backup strategy, in case SBM3a may show low efficiency due to described reasons, and may turn out unsuitable for a large scale in vivo screen.

2.2.2 Setting up the *in vivo* Mutagenic Screen

The goal was to generate 2 different mouse lines for each transposon system, one which expresses the transposase enzyme (jump starter) and one harboring a single-copy of the transposon vector (mutator) flanked by the respective binding sites. These mouse lines should then be intercrossed to generate the double transgenic "seed mice", which are positive for TS and TP.
In these mice, somatic and germ cell transposition events could occur. Therefore, by backcrossing the seed mice with wild type mice (negative for TS and TP), a part of the offspring should harbor a novel insertion of the transposon (transposition occurred in germ cell, potential mutation is generated) but should be negative for the transposase (new insertion is stable). Hence, the second step after choosing the transposon system, was to design an appropriate insertional mutagenesis transposon vector, which will not only disrupt genes, but also report transposition events *in vivo*.

2.2.3 Transposon Design

As indicated above, the transposon should ideally consist of two functional parts, one which works as "gene breaking" cassette that will lead to the disruption of the endogenous transcript and result in a fusion protein which will then also report the expression of the mutated gene. The

second part should consist of a cassette, which will report the occurence of novel transposition events into a gene, to be able to rapidly and easily identify offspring with a new insertion (cut-and-paste occured in germ cell). This part of the vector should not be dependent of the activity of the respective gene, so a polyA-trap cassette seemed reasonable (see Fig. 2.5).

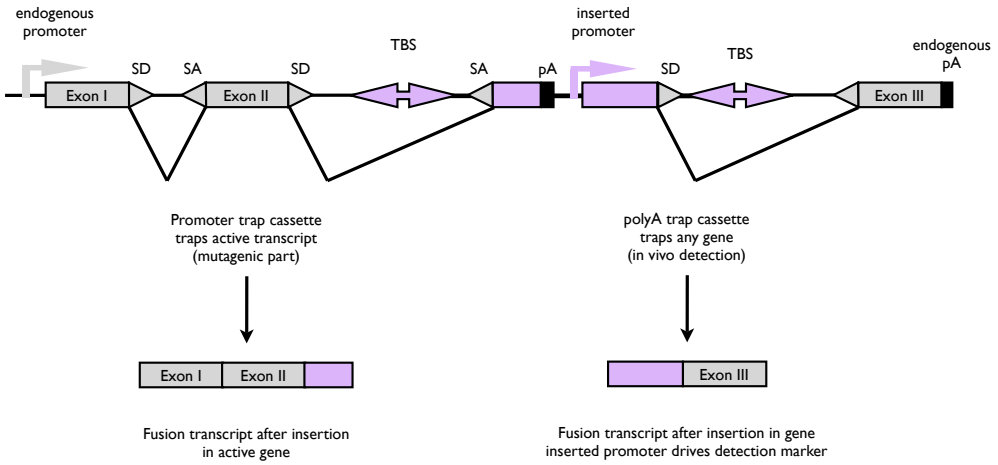


Figure 2.5: *Schematical outline of transposon insertion in an intron of an endogenous gene. A transposon construct is flanked by transposase binding sites (TBS) and contains two different functional cassettes. A promoter-trap cassette in the 5' half, which will trap an endogenous transcript and disrupt it (gene-breaking). The second part is a promoter containing polyA-trap cassette, which will lead to a fusion transcript if inserted in a gene, independent of its expression status, and therefore report new insertions into genes (in vivo detection).*

The donor insertion site can be chosen randomly for the *piggyBac* system, because of its unbiased reintegration preference, but should be directed, next to a critical region for *Sleeping Beauty*, which should then reinsert locally, according to its well described characteristics.
In order to take advantage of the transposition detection marker, the donor integration needs to be in an inactive, non mutagenic state, and should become active only after excision and reintegration in the mutagenic orientation. Reintegrations in non mutagenic orientations will be lost, but identification of transposition events will be much easier (see Fig. 2.6).

2.2.4 Generation of the Transposase Expressing Mouse Line

All transposases used (SBM3a, iPB, mPB) were driven by the ubiquitously active Caggs (chimeric promoter derived from chicken β-actin and cytomegalovirus immediate early promoter sequences; Okabe et al., 1997) promoter and integrated in the well described Rosa26 locus via recombinase mediated cassette exchange (RMCE) by C31 integrase (derived from Streptomyces phage, Branda and Dymecki, 2004). Rosa 26 locus is ubiquitously active and represents an adaequate knock-in location, since it does not result in known phenotypes when mutated. The Caggs promoter has

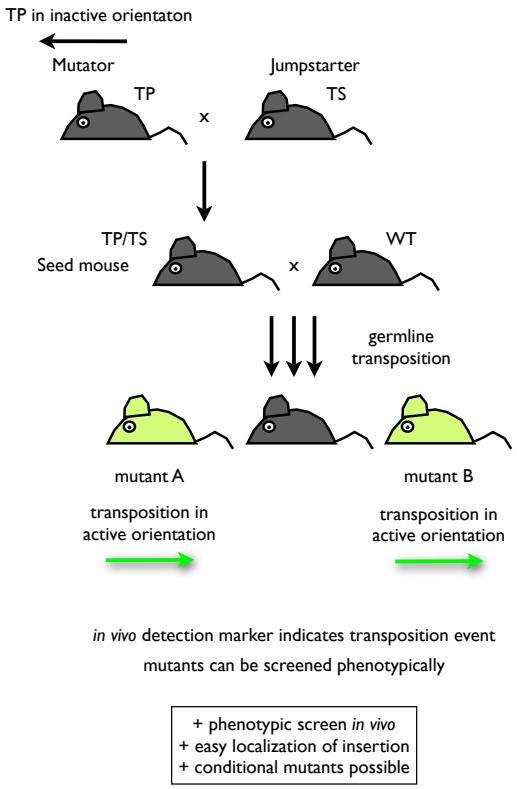


Figure 2.6: *Schematic outline of a transposon-based in vivo screen.*
A transposon harboring mouse, the mutator, which shows integration in an inactive orientation is crossed with the transposase expressing mouse line (jumpstarter) to receive a double transgenic seed mouse. Crossing back to wild type mice will then lead to germ line transpositions and to stable mutagenic insertions at a new locus in the offspring, if transposase negative. The transposon reintegrates in an active orientation and makes the detection of cut-and-paste events into genes visible. Mice can then be screened for phenotypical abnormalities.

ubiquitous activity and appeared strong enough for high expression levels of the transposase gene.

To achieve this, an already established vector/ES cell system by R. Kühn was used. The promoter driven transposase had to be transferred into an cassette exchange vector with an ES cell selection marker, all flanked by the C31 recombinase recognition sites (RRS, attB). The cell line already shows a stable integration of another vector in the Rosa26 locus, containing the respective RSS (attP), which after cotransfection will be partly replaced by the transposase plasmid and RSS will recombine to attL/R sites. Further details see Fig. 2.7.

By using this method, a single copy integration in Rosa26 could be achieved, which then leads to a stable expression of the integrated transposase gene.

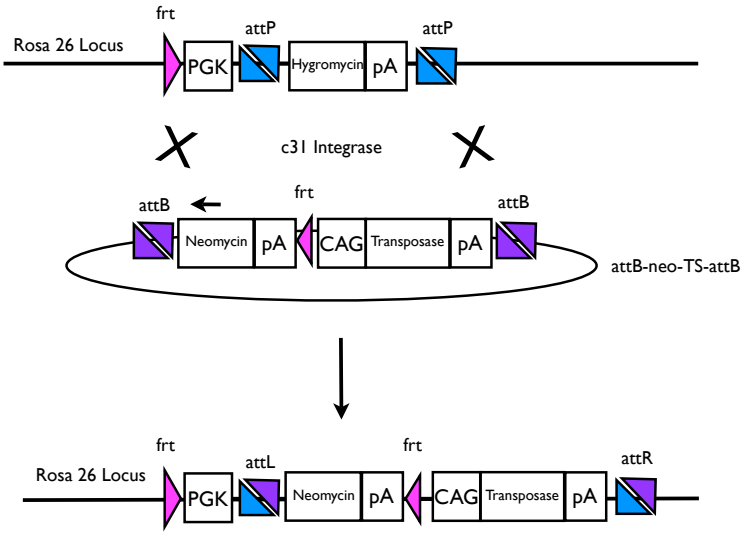


Figure 2.7: *Schematic drawing of C31 RMCE in targeted ES cells*
C31 RMCE was performed in IDG3.2pR26.10 F1 ES cell line, which has a stable integration of a PGK driven hygromycin resistance gene in the Rosa 26 locus. The hygromycin coding sequence is flanked by C31 RRS (attP), so a cotransfection of C31 expression plasmid and the attB-neo-transposase-attB plasmid, flanked sequences will be exchanged by the integrase. After exchange, the inserted promoterless neomycin resistance gene will be driven by the PGK promoter and enable selection on positive exchange.

2.2.5 Generation of the Transposon Harboring Mouse Line

The transposon construct needed to be integrated in a single copy manner and a directed integration was necessary, especially for the use of the SBM3a transposon, since the screen should take advantage of the local hopping phenomenon. To achieve this, another RMCE system was chosen, the Flp recombinase (flippase, named after its ability to flip DNA segments in *Saccharomyces cerevisiae*, Branda and Dymecki, 2004), which is capable to exchange of the transposon vector with any given conditional gene trap clone. By choosing Flp RMCE into gene trap clones, thousands of potential donor sites next to any region of interest (critical for human disease) are provided.

As described, gene trap insertions contain a adSA-betageo-pA cassette which is flanked by wild type and mutant recombinase recognition sites (RRS) in FlEx configuration (Flp excision, Schnütgen et al., 2003, see Fig. 2.1 B) for CRE ("causes recombination" in the phage P1 genome) and Flp recombinases. By flanking the transposon construct with the respective sites and adding a selection marker for ES cell culture, the construct can be integrated into a known locus next to the region of interest for the mutagenic screen.

2.3 Flp RMCE Efficiency Studies

A small number of recent studies already showed the advantages of recombinase mediated cassette exchange (RMCE) to generate knock-in alleles or "humanized mice". All these studies mainly followed the one-gene-at-a-time approach which also led to elaborate vector design and targeting efforts (Cesari et al., 2004; Liu et al, 2006; Jaegle et al., 2007, Bateman and Wu, 2008; Sato et al., 2008).
First starting Flp-mediated RMCE as a side project to manage the single copy integration of the transposon sequence into gene trap insertion sites in mouse ES cells, systematical RMCE of gene trap clones was performed to gain more insights in optimal conditions, efficiency and consequences of reading frame and location of insertion.
Since the GGTC library contains several thousand conditional insertions (56.296 known insertions in over 6000 independent genes), it represents a powerful target for exchanging any given cDNA which then will be under control of the endogenous promoter. A great advantage of this technique is the possibility of secondary modifications of previously inserted cassettes (Seibler and Bode, 1997; Seibler et al., 1998).
Gene trap cassettes are introduced randomly by retroviral transfection into ES cells and are flanked by RRS for Cre and Flp recombinases. At each side of the vector, the mutated (substitutions within the spacer or the repeats) and the wild type RSS for Cre and Flp is present (loxP and FRT: wildtype, lox5171 and F3: mutated).
RRS usually consist of two palindromic sequences separated by an asymmetric spacer, which determines their orientation. Depending on the orientation of two homotypic (either two wild type or two mutated) RSS towards each other (head-to-tail or head-to-head) the flanked sequence is excised or inverted respectively (Branda and Dymecki, 2004), whereas heterotypic RSS do not reccombine in general, although exceptions from this rule have been described (see Fig. 2.8 A B).
By cotransfecting Flp recombinase along with the exchange vector (exchange sequence flanked by head-to-head oriented F3 and FRT sites) into any conditional gene trap clone, the recombinase will technically manage the excision between the homotypic sites located in the insertion and in the vector, which leads to the exchange of the flanked sequences (see Fig. 2.8 C).
The goal of this side project was to test various aspects of Flp RMCE on gene trap insertions. First the exchange efficiency per se, next the importance of the in frame exchange reaction (exchange vector and gene trap vector in same frame) and finally any possible influence due to the insertion localization (5′ or 3′ of a gene) on exchange efficiency. Further studies were done concerning the expression of the inserted vector by the endogenous promoter after Cre deletion and the SB100X mediated transposition of the vector into surrounding genomic locations and the ability to gener-

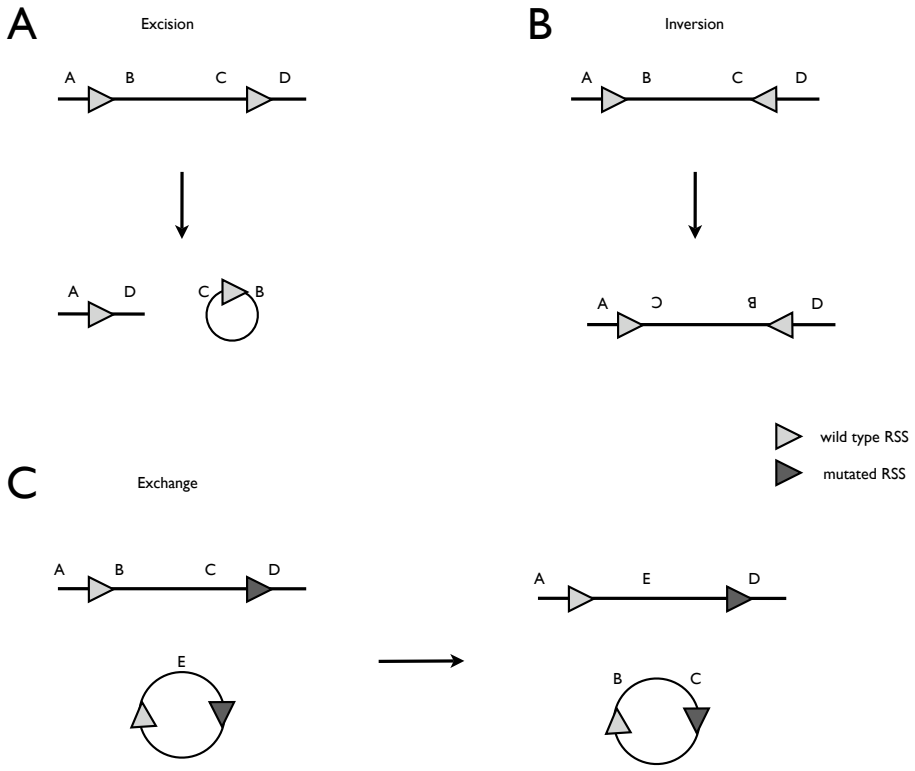


Figure 2.8: *Outline of possible events mediated by Cre or Flp recombinase.*
Three possibilities can occur between wild type (bright grey triangles) or mutated (dark grey triangles) recombinase recognition sites (RSS) for each recombinase. Depending on their orientation towards each other, the recombinase can either excise (A), invert (B) or exchange (C) the flanked parts.

ate mutant mouse lines from exchanged clones.
This technique is a powerful tool since gene trap insertions are not only available in high quantity, insertions also are determined and verified and they provide conditionality, which opens the way to a tissue-specific expression of any cDNA of choice.

Chapter 3

Materials

3.1 Instruments

autoclave	Aigner, type 667-1ST
balances	Sartorius
bottles for hybridization	ThermoHybaid
cassettes for autoradiography	Amersham, Hypercassette
centrifuges	Sorvall, Evolution RC;
centrifuges	Eppendorf, 5415D, 5417R;
centrifuges	Varifuge 3.0R
centrifuges	Multifuge 3L-R
chambers for electrophoresis (DNA)	MWG Biotech; Peqlab
developing machine	Agfa, Curix 60
digital camera	Zeiss, AxioCam MRc
homogenizer	IKA, Ultra-Turrax T25
freezer (-20°C)	Liebherr
freezer (-80°C)	Heraeus HFU 686
fridges (4°C)	Liebherr
gel documentation system	Herolab, E.A.S.Y.
gel-/blottingsystem	Invitrogen
Genesis RSP 200 robot	TECAN
glass homogenizer (tissue grinder, 2 ml)	KIMBLE / KONTES
glass pipettes	Hirschmann
glassware	Schott
ice machine	Scotsman, AF 30
imaging analyzer	Fuji, FLA-3000
incubators (for bacteria)	NBSC, innova 4230
incubators (for ES cells)	Heraeus
laminar flow	Nunc Microflow 2

light source for microscopy	Leica KL 1500
liquid scintillation counter	Hidex, Triathler
luminometer	Berthold, Orion I
magnetic stirrer / heater	Heidolph, MR3001
microscope	Zeiss Axioplan 2
microwave oven	Sharp R-937 IN
Neubauer counting chamber	Brand
oven for hybridization	Memmert, UM 400
oven for hybridization	MWG-Biotech, Mini 10
oven for hybridization	ThermoElectron, Shake'n'Stack
PCR machine	Eppendorf, MasterCycler Gradient
pH-meter	InoLab, pH Level 1
photometer	Eppendorf, Biophotometer 6131
pipetteboy	Eppendorf
pipettes	Gilson; Eppendorf
power supplies for electrophoresis	Consort, E443;
radiation monitor	Berthold, LB122
rotating rod apparatus	Bioseb, Letica LE 8200
shaker	Heidolph, Promax 2020
sonifier	Branson sonifier, cell disrupter B15
stereomicroscope	Zeiss, Stemi SV6
thermomixer	Eppendorf, comfort
UV-DNA/RNA-crosslinker	Stratagene, UV-Stratalinker 1800
UV-lamp	Benda, N-36
vortex	Scientific Industries, Vortex Genie 2
water bath	Lauda, ecoline RE 112;
water conditioning system	Millipore, Milli-Q biocel

3.2 Chemicals

α-P-dCTP	Amersham
3,3'-diaminobenzidine (DAB)	Sigma
4-NBT (Nitro blue tetrazolium)	Roche
acetic acid	Merck
acetic anhydride	Sigma
agarose	Gibco Life Technologies, Biozym
ammonium acetate	Merck
ampicillin	Sigma
Ampuwa	Fresenius
β-Mercaptoethanol	Sigma, Gibco
bacto agar	Difco
bacto peptone	BD Biosciences

BCIP (5-bromo-4-chloro-3-indolyl phosphate)	Roche
bicine	Fluka
bis-tris	Sigma
Blocking reagent	Roche
Blocking reagent	Perkin Elmer
boric acid	Merck
bovine serum albumin (BSA, 20 mg/ml)	NEB, Sigma
bromphenol blue	Sigma
calcium chloride ($CaCl_2$)	Sigma
carrier DNA	Sigma
chicken serum	Perbio
chloral hydrate	Sigma
chlorobutanol	Sigma
citric acid	Sigma
Complete Mini (protease inhibitors)	Roche
cresyl violet acetate	Sigma
dextran sulphate	Sigma
dithiotreitol (DTT)	Roche
DMEM	Gibco
DMSO	Sigma
dNTP (100 mM dATP, dTTP, dCTP, dGTP)	MBI
EDTA	Sigma
EGTA	Sigma
Eosin Y	Sigma
ethanol absolute	Merck
ethidiumbromide	Fluka
ethylene glycol	Sigma
fetal calf serum (FCS)	PAN, Hybond
Ficoll 400	Sigma
formaldehyde	Sigma
formamide	Sigma
freezing medium Tissue	Tek, OCT compound
gelatin	Sigma
Glasgow MEM	Gibco
glucose	Sigma
glycerol	Sigma
hematoxylin	Fluka
Hepes	Gibco
human chorion gonadotropin (hCG/Ovogest)	Intervet
Hyb-mix	Ambion

hydrochloric acid (HCl)	Merck
hydrogen peroxide (30%)	Sigma
hygromycin	Sigma
iodoacetamide	Sigma
isopropanol	Merck
kanamycin	Sigma
levamisol	Sigma
lithium carbonate	Fluka
luxol fast blue	MBS CHROMA
M2 medium	Sigma
magnesium chloride ($MgCl_2 \cdot 4H_2O$)	Merck
maleic acid	Sigma
MEM nonessential aminoacids	Gibco
MES hydrate	Sigma
methanol	Merck
mineral oil	Sigma
MOPS	Sigma
NeutrAvidine biotin-binding protein	Pierce
Nonidet P40 (NP-40)	Fluka
orange G	Sigma
paraformaldehyde	Sigma
PBS (for cell culture)	Gibco
Pertex mounting medium	HDScientific
PIPES	Sigma
polyvinylpyrrolidone 40 (PVP 40)	Sigma
potassium chloride (KCl)	Merck
potassium ferricyanid ($K_3Fe(CN)_6$)	Sigma
potassium ferrocyanid ($K4Fe(CN)_6 \cdot 3H_2O$)	Sigma
potassium hydroxid (KOH)	Sigma
potassium phosphate ($KH_2PO_4 \cdot H_2O, K_2HPO_4$)	Roth
pregnant mare's serum gonadotropin (PMSG)	Intervet
RapidHyb buffer	Amersham
RNaseZAP	Sigma
Roti-HistoKit II	Roth
Roti-Histol	Roth
salmon sperm DNA	Fluka
sheep serum	Gibco
skim milk powder	BD Biosciences
sodium acetate (NaOAc)	Merck, Sigma
sodium chloride (NaCl)	Merck

sodium citrate	Sigma
sodium desoxycholate	Sigma
sodium dodecylsulfate (SDS)	Merck
sodium hydroxide (NaOH)	Roth
sodium iodate	Sigma
sodium phosphate ($NaH_2PO_4 \cdot H_2O, Na_2HPO_4$)	Sigma
spermidin	Sigma
sucrose	Sigma
triethanolamine	Merck
TriReagent	Sigma
Tris (Trizma-Base)	Sigma
Triton-X 100	Biorad
Trizol	Invitrogen
tRNA	Roche
trypsin	Gibco
tryptone BD	Biosciences
Tween 20	Sigma
xylol	Fluka
yeast extract	Difco

3.3 Consumables and Others

1kb+ DNA Ladder	Invitrogen
cell culture dishes	Nunc
centrifuge tubes (15 ml, 50 ml)	Falcon, Sarstedt
cuvettes for electroporation (0.4 cm)	Biorad
films for autoradiography	Kodak: Biomax MS
filter paper	Whatman 3MM
filter tips 10 μl, 20 μl, 200 μl, 1 ml	Art, Starlab
gloves	Kimberley-Clark
Hybond N Plus (nylon membrane)	Amersham
Hyperfilm (chemiluminescence detection)	Amersham
MicroSpin S-300	Amersham
NuPAGE Novex Bis-Tris gels, 10% (protein)	Invitrogen
one-way needles	Terumo, Neolus 20G, 27G
Pasteur pipettes	Brand
PCR reaction tubes (0,2 ml), lids	Biozym
Phase Lock Gel, heavy	Eppendorf
pipette tips	Gilson
plastic pipettes (1 ml, 5 ml, 10 ml, 25 ml)	Greiner
PVDF membrane (protein)	Pall Biosciences
reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf

SeeBlue Plus2 Prestained protein ladder	Invitrogen
SmartLadder DNA marker	Eurogentec

3.4 Kits

BCA protein assay kit	Pierce
DNA Highspeed Maxi Prep Kit	Qiagen
DNA Mini Prep Kit	Qiagen
ECL Detection Kit	Amersham
PCR Purification Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
RediPrime II DNA Labeling Kit	Amersham
SuperScript First-Strand Synthesis System for RT-PCR	Invitrogen
TOPO TA Cloning Kit	Invitrogen
Wizard Genomic DNA Purification Kit	Promega
Zero Blunt TOPO PCR Cloning Kit	Invitrogen

3.5 E. coli Strains

DH5α	Gibco Life Technologies
DB3.1	Invitrogen
TOP10	Invitrogen

3.6 Enzymes

DNase I (RNase-free)	Roche
Klenow fragment of DNA Polymerase I	NEB
PCR-Mastermix 5x 5	PRIME
polynucleotide kinase (PNK)	NEB
proteinase K	Roche
restriction enzymes	Roche, MBI, NEB
RNA polymerases (T7, SP6)	Roche
RNase A	Serva
RNasin RNAse inhibitor	Roche
Shrimp alkaline phosphatase (SAP)	Roche
SuperScriptII	Invitrogen
T4 DNA ligase	NEB

Chapter 4

Methods

4.1 Molecular Analysis

4.1.1 Standard Methods

All techniques on DNA, RNA or Protein level were performed according to standard protocols (Sambrook et al., Molecular Cloning: A Laboratory Manual).

4.1.2 Individual Methods

4.1.2.1 Southern Probes

All Southern probes used in this work were plasmid fragments, which had to be extracted. Used plasmid names, restriction enzymes and resulting probe fragments are listed in Table 4.1.

Probe	Plasmid	Enzymes	Fragment	Source
Neomycin	pKJ1-Neo	PstI	600 bp	T. Floss
Hygromycin	pvbb-Hygro	NotI/NcoI	365 bp	R. Kühn
DsRed	SA-dsRed-pA	SacI/HindIII	691 bp	P. Chang
T2onc	TopoT2onc	EcoRI	296 bp	D. Largaespada
Rosa	pCRII-Rosa5'	EcoRI	466 bp	R. Kühn

Table 4.1: *Overview of Southern Blot Probes*

4.1.2.2 General PCR techniques

All primers used for genotyping of genomic DNA of either mice or ES cell clones, primers for amplifications, RT-PCR or sequencing are listed in Fig. 4.1.

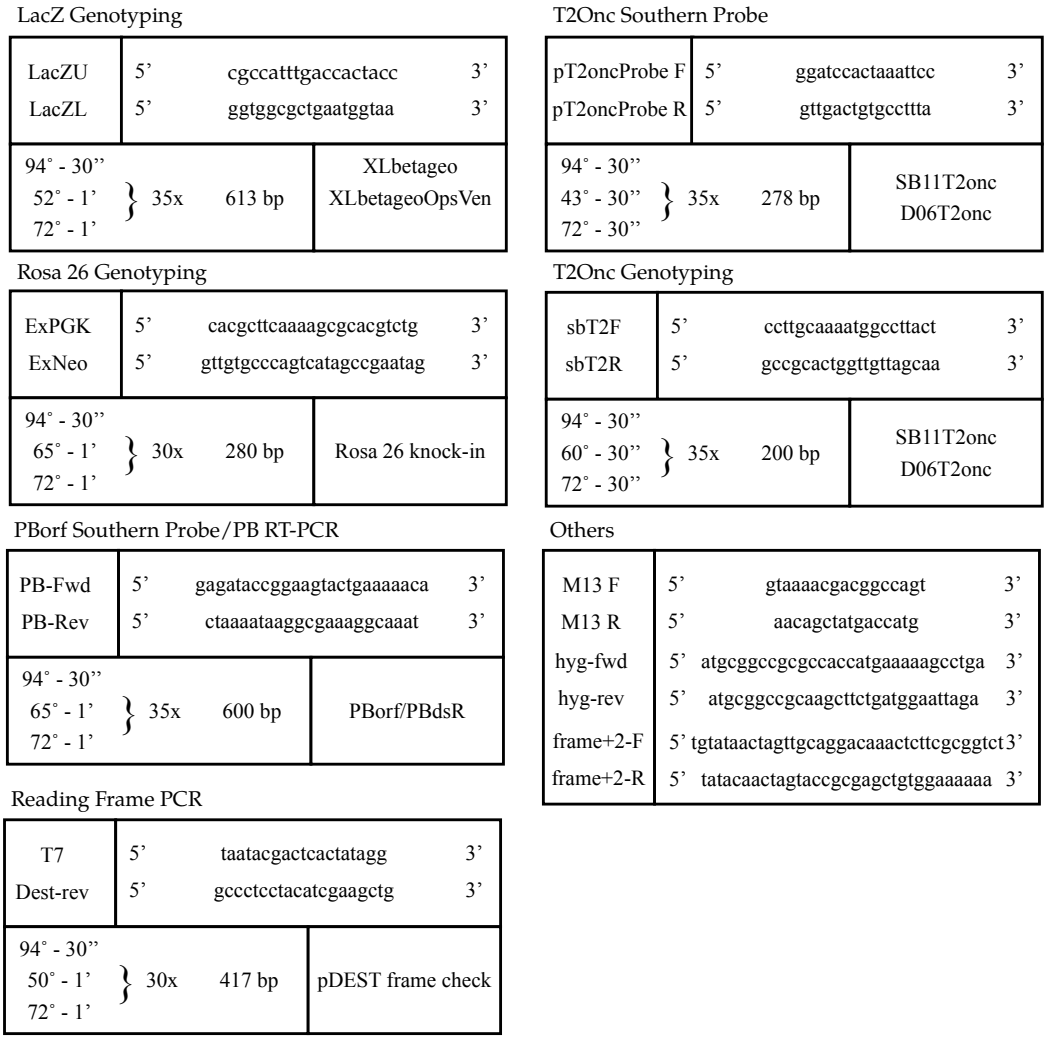


Figure 4.1: *PCR Conditions*
Indicated are primer names, primer sequences, PCR cycle conditions and expected band size for each reaction. Indicated is also which program was used for Genotyping of which mouse or ES cell strain. All other primers were used for fragment amplification or sequencing.

4.1.2.3 PCR Analysis prior to RMCE

Selected FlEx gene trap clones for RMCE transfections had to be screened with several PCR techniques prior to transfection. The presence of the 5' frt site, which is crucial for efficient exchange reactions had to be determined. Further the Splk PCR results for insertion localization had to be verified by PCR, using primers located within the gene trap vector, combined with external primers which had to be designed according to Splk PCR sequences. Further the orientation of the gene trap vector, also in comparison with the exchange vector, had to be checked by PCR. All primers and programs are listed in Fig. 4.2.

PCR for presence of 5′ frt site in gene trap clones

B034	5’	tgtaaaacgacgggatccgcc	3’
SR2	5’	gccaaacctacaggtggggtcttt	3’

94° - 30”	650 bp	5’ frt present
61° - 1’ } 35x		
72° - 1’	550 bp	5’ frt lost

PCR to determine insertion orientation

B045	5’	ctccgcctcctcttcctccat	3’
B048	5’	cctcccccgtgccttccttgac	3’
B050	5’	tttgaggggacgacgacagtat	3’

95° - 15”		
53° - 15” } 10x	630 bp	original
72° - 1’		
-1° each cycle	800 bp	turned
95° - 15”		
53° - 15” } 25x		
72° - 1’	839 bp	pEX-Flp Exchange

Primers to verify insertion site (internal)

1. splirev1	5’	gctagcttgccaaacctacaggtgg	3’
2. splirev2	5’	gccaaacctacaggtggggtcttt	3’
3. iPCR4	5’	tacccgtgtatccaataaaccc	3’

94° - 30”		
T° - 1’ } 35x	X bp	combine one primer with an external primer according to SPLK result
72° - 1’		

Primers to verify insertion site (external)

E311D09	5’	gccggaagagatgctgagtc	3’	3
E068C09	5’	aaactggttttcattggggatca	3’	3
E326E05	5’	tcccccatgtttctgtaattgg	3’	2
E287F07	5’	no product	3’	X
E079H11	5’	atcgaatgtagcccaggatg	3’	2
E326C04	5’	catcttgaacctcaagtttgcctttt	3’	3
E284H06	5’	ctggcttcctcccacttgtgtt	3’	3
E224H09	5’	agaggtgaccctgctggaaatg	3’	3
E288B02	5’	ccaaggtgggaaagatgaggtg	3’	2
E326E12	5’	cacatggtgaccttcagagcag	3’	2
D045A10	5’	acaggctaccgtatttcgtaaccaa	3’	1

Figure 4.2: *PCR analysis of FlEx gene trap clones*
Shown are PCR protocols (primers and conditions) and the possible band sizes and their meaning for gene trap clone analysis. Clones used in this work and the respective external primers for insertion verification are shown on the right, also the internal primer (1, 2, or 3) are indicated. The protocol to determine the orientationj of the insertion was also used after RMCE, resulting band size is indicated.

4.1.2.4 PCR Analysis after RMCE

After RMCE transfection and selection of gene trap clones, resistant colonies had to be screened for successful exchange of pEX-Flp using primers TP and SR2.
After transient transfection with either SB100X or Cre expression plasmids, exchange clones had to be screened for the respective deletion or mobilization of vector parts.
All PCR programs and primer sequences are shown in Fig. 4.3.

4.1.2.5 Splinkerette PCR of T2onc Insertion Sites

This protocol is adapted from Dupuy et al., 2001.

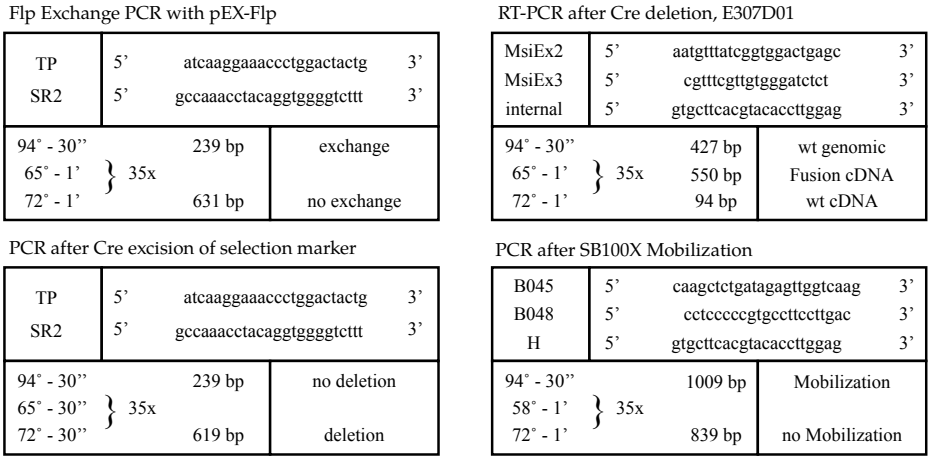


Figure 4.3: *PCR Analysis of RMCE clones*
Primers and PCR techniques for analysis of successful exchanged RMCE clones are shown. Also PCR conditions for analysis of further modification (Cre deletion and SB100 excision) are shown.

Genomic tail clip received DNA of mice from double transgenic mice intercrossed with wild type mice (either SB11-T2Onc or SBM3a-T2Onc) is digested with BfaI (to analyse the integration starting from the left IRDR) and in a separate reaction with NlaIII (to analyse the integration starting from the left IRDR) in 50 μl Vol. Purify after digest. Prior to ligation, linkers have to be prepared by heating the mixtures containing the respective linkers (50 μl of 100 μM Linker) to 95°C for 5 min and let them slowly cool down afterwards. Respective linker mixtures for either left or right side are then ligated with the respective purified digest in 20 μl Vol. over night at 16°C. Purify after reaction. A second digestion is performed with BamHI for the left side reactions and XhoI for the right side reactions for 6-18 hrs at 37°C. Purify after reaction. 3μl of each reaction is then used as template for the primary PCR with the respective Spl IRDR 1 1 primer and Spl Link 1 1 primer. Diluted PCR product is used as template for secondary PCR with respective Spl IRDR 2 1 primer and Spl Link Nest 1 primer. For primer sequences and PCR programs see Fig. 4.4.

4.2 Cell Culture

4.2.1 General

All *in vitro* procedures were done according to established standard methods (Hogan et al., 1994). ES cells were grown at 37°C and 5% CO_2. E14 Tg2A.4 cells were cultured in medium containing 10 % FCS (Hybond), 1mM Sodium Pyruvat, 1x MEM nonessential AA, 0.1 mM β-Mercaptoethanol, 1000 U/ml LIF in Glasgow MEM. F1 and TBV2 cells were cultured in medium containing 15 % FCS (PAN), 20 mM Hepes, 1x MEM nonessen-

Adapter and Primer

BfaI Linker	5'	GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC	3'
BfaI Linker	5'	Phos-TAGTCCCTTAAGCGGAG-NH2 C7	3'
NlaIII Linker	5'	GTAATACGACTCACTATAGGGCTCCGCTTAAGGGACCATG	3'
NlaIII Linker	5'	Phos-GTCCCTTAAGCGGAGCC -NH2 C7	3'
Spl IRDR R1 1	5'	GCTTGTGGAAGGCTACTCGAAATGTTTGACCC	3'
Spl IRDR L1 1	5'	CTGGAATTTTCCAAGCTGTTTAAAGGCACAGTCAAC	3'
Spl Link1 1	5'	GTAATACGACTCACTATAGGGC	3'
Spl IRDR R2 1	5'	CCACTGGGAATGTGATGAAAGAAATAAAAGC	3'
Spl IRDR L2 1	5'	GACTTGTGTCATGCACAAAGTAGATG	3'
Spl Link Nest 1	5'	AGGGCTCCGCTTAAGGGAC	3'

Primary PCR		Secondary PCR	
Spl IRDR R1 1 Spl IRDR L1 1 Spl Link1 1	Template: 3µl of digest Vol.: 50µl Use R1 1 OR L1 1	Spl IRDR R2 1 Spl IRDR L2 1 Spl Link Nest 1	Template: 6µl of 1st PCR Vol.: 100µl Use R2 1 OR L2 1
94° - 30'' 60° - 30'' } 30x 72° - 90''	Dilute 1:75 in H_2O	94° - 30'' 60° - 30'' } 30x 72° - 90''	Use for sequencing TOPO cloning and Gel electrophoresis

Figure 4.4: *Splk PCR primer and programs*
Indicated are all primers, adaptors and PCR conditions used for Splk PCR analysis of reintegrated T2Onc transposons of mouse strains SB11-T2Onc and SBM3aT2Onc. For more details see, Dupuy et al., 2001.

tial AA, 0.1 mM β-Mercaptoethanol, 1500 U/ml LIF in DMEM. All electroporations were done with an EPI 2500 Elektroporations Impulsgenerator 0-2500 V (Fischer) in 700μl PBS (phosphate-buffered saline). Cells were pulser for 2 ms at 300V and plated after at least letting them stand for 10 min at RT. TBV2 cells (Wiles et al., 2000) and IDG3.2pR26.10 F1 ES cells were plated on culture dishes coated with a layer of mouse embryonic fibroblasts (MEF). E14 Tg2A.4 cells (a gift from Kent Lloyd to T. Floss) were plated on 1% gelatine solution coated culture dishes. To derive mouse lines of positive ES cell clones, expanded cells were injected into C57Bl/6 host blastocysts after superovulation.

4.2.2 Transfections

4.2.2.1 C31 Mediated RMCE in Targeted Rosa26 Locus

10^7 IDG3.2pR26.10 F1 ES cells were electroporated with 25 μg of C31 integrase expression plasmid and 25 μg of pbs-attB-neo-attB vector containing the Caggs driven transposase gene. Cells were plated on 10x10 cm culture dishes. After 48 hrs cells were selected for neomycin resistance for 9 days (150 μg/ml, Gibco). Resistant clones were transferred to 96-well dishes and expanded to 2 replicates of 48-wells. One plate was frozen as stock and one was used to isolate genomic DNA for further analysis (Southern

blotting and PCR).

4.2.2.2 *PiggyBac* Mediated Random Insertions

10^7 TBV2 cells were electroporated with different amounts (see Fig. 5.22) of Cag-mPB expression plasmid and XLbetageo or XLbetageoOpsVen plasmid. Each electroporation was plated on 5x10 cm culture dishes and selected for neomycin resistance after 48 hrs (150 μg/ml, Gibco). Resistant colonies were transferred to 96-well plates and expanded on 2 48-well plates. One plate was frozen as stock, one was used to isolate genomic DNA for further analysis (Splk PCR, done by AG Ruiz, Charite Berlin).

4.2.2.3 Transposon Random Integration

10 μg of linearized TPOPS vector (SspI) were transfected in $1x10^7$ TBV2 cells and plated on 5x100 mm culture dishes. Hygromycin selection (150 μg/ml, Sigma-Aldrich) was started after 24 hrs. Resistant colonies were transferred after 9 days of selection on 96-well plates and expanded on 2 48-well plates. One plate was frozen and one was used to isolate genomic DNA for further analysis (PCR and Southern blotting).

4.2.2.4 *PiggyBac in vitro* Excision Assay

10^6 mPB-20 cells (stable knock-in clone of Caggs-mPB in Rosa 26 locus) and IDG3.2pR26.10 F1 ES cells were electroporated with 5 μg supercoiled XLbetageo vector each. Each electroporation was plated on 1x100 mm culture dish and after 48 hrs LacZ stain (Uez et al., 2008) was performed.

4.2.2.5 FlpO RMCE

All electroporated clones except D045A10 (TBV2) were derived from E14 Tg2A.4 cell gene trap lines. Cells of each clone were electroporated with 30 μg supercoiled plasmid DNA of pEX-Flp and 70 μg of FlpO. 10^7 cells were electroporated per experiment and plated afterwards on 2x100 mm culture dishes. Selection on hygromycin resistance began after 48 hrs (150 μg/ml, Sigma-Aldrich). Resistant colonies were transferred to 96-well dishes and expanded to 3 replicates of 48-wells. One plate was frozen as stock, one was used to determine β-gal activity and one to isolate genomic DNA for further analysis (Southern blot and PCR).

4.2.2.6 Cre Transfection

Clone A03 (E307D01 derivate of RMCE transfection) was transfected with 50 μg of supercoiled Caggs-Cre-IRES-Puro plasmid. 50 % of electroporated cells were plated on 5x100 mm dishes and cultured for 48 hrs without selection. Pooled genomic DNA served as template for PCR analysis.

The remaining cells of the electroporation were plated on a 1x100 mm dish and selected for puromycin resistance for 5 days (1 μg/ml, Sigma). After expansion total RNA was extracted (Trizol) and subsequent RT-PCR was performed.

4.2.2.7 SB100X Transfection

Clone A03 (E307D01 derivate of RMCE transfection) was transfected with different amounts of supercoiled SB100X expression plasmid (0, 10 and 70 μg). Electroporations were plated each on 1x100 mm culture dishes and genomic DNA for PCR analysis was extracted after 48 hrs without selection.

4.3 Animal Housing

Mice at the HelmholtzZentrum München are bred in accordance with national and institutional guidelines. Mice were group housed with maximal five animals per cage, and were kept in open cages at a light/dark cycle of 12 hours. Food and water were provided *ad libitum*. For breeding one male was paired with one or two females, pups were weaned at 21 days of age. Mice were then seperated according to their gender and received earmarks for identification. Genomic DNA was extracted from tail clips.

Chapter 5

Results

5.1 Vectors and Cloning

5.1.1 Gateway Two-Component System for RMCE

5.1.1.1 Destination Vector

Since two different approaches were pursued by using our Gateway compatible exchange vector, either the integration of a *Sleeping Beauty* transposon in a single copy manner in a critical locus of interest, or the investigation of the efficiency of FlpO mediated cassette exchange in general, two different names were chosen. For the general efficiency studies, the two component vector system is called pEX-Flp and pENTR-EX, for the integration of the mutagenic transposon in the mouse genome, vectors are called pDEST and pENTR-T2B, although pEX-Flp and pDEST are identical.
The backbone of the exchange vector was pvbb-Hygro, which consists of a NotI-linkered PCR-amplified hygromycin resistance cassette, which was cloned into the synthesized vector pvbb[1], which harboured the Flp and CRE specific recombinase recognition sites (RRS), the adenoviral splice acceptor and unique NotI, XhoI, SalI and PmeI restriction sites.
The 5′ FRT and the 3′ F3 sites in a head-to-head configuration flank all other features and are required for the recombinase mediated cassette exchange (RMCE) by FlpO. The head-to-tail oriented loxP sites flank the XhoI, SalI and NotI restriction sites, which were integrated to create all three reading frames and to insert the NotI linkered hygromycin resistance. The loxP sites were integrated for CRE mediated removal of the selection marker hygromycin, if required.
The PmeI restriction site, located between the 3′ loxP site and the F3 site was designed for integrating the Gateway cassette (chloramphenicol resistance and ccdB gene, flanked by attR1/2 sites) to enable later insertion

[1] http://www.geneart.com/

of any sequence of interest by a one step gateway clonase reaction (Invitrogen). The hygromycin resistance gene followed by the PGK pA signal was amplified by PCR using NotI linkered primers (template: pPGK-Hygromycin-pA from R. Kühn), cloned into pCRII-Topo vector, and integrated in pvbb-Hygro by NotI digestion (PCR and ligation done by A. Ettinger).
The last step to obtain pDEST in frame +1 was to ligate the blunt Gateway cassette A (Invitrogen) in the PmeI digested pvbb-Hygro vector. Critical features and cloning steps are indicated in Fig. 5.1. After insertion of the Gateway cassette, E. coli DB3.1 cells (Invitrogen) had to be used for transfection, since other bacteria do not tolerate the ccdB gene.

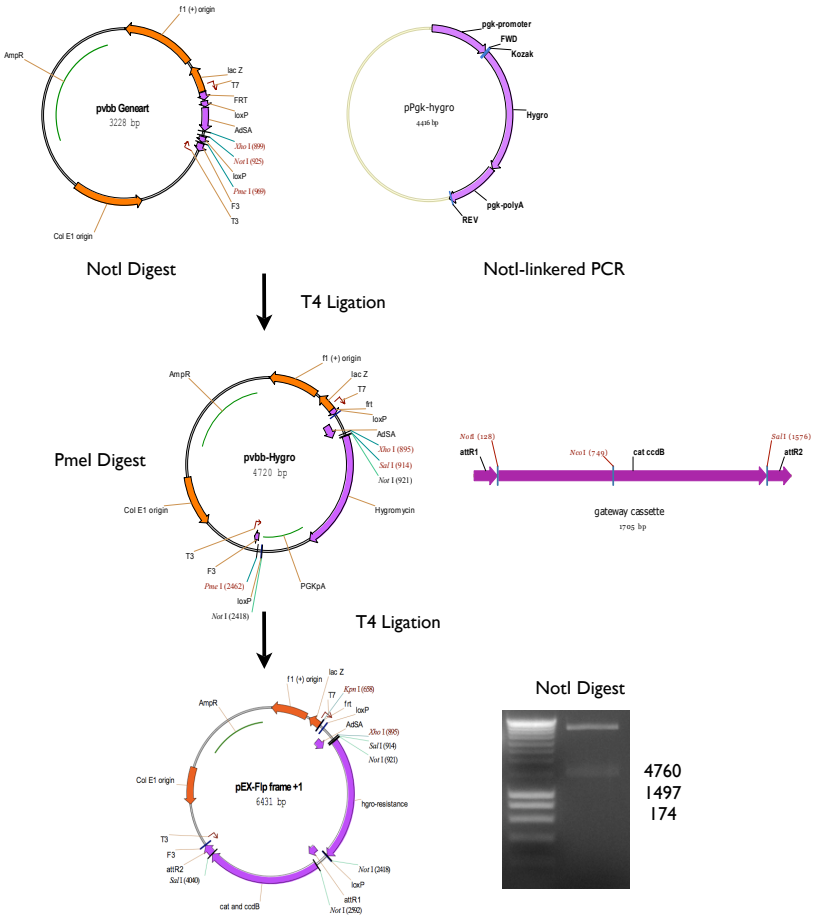


Figure 5.1: *Cloning strategy of pEX-Flp/pDEST*
NotI linearized pvbb vector was ligated with PCR amplified hygromycin-polyA fragment (primers FWD and REV indicated) to receive pvbb-Hygro (A. Ettinger). A blunt ended Gateway cassette A (Invitrogen) was then ligated with PmeI linearized pvbb-Hygro to result in the destination vector for Gateway reaction pEX-Flp/pDEST in reading frame +1 (plasmid digestion using NotI is shown, 174 bp fragment weakly visible).

In order to generate all reading frames to be able to perform FlpO RMCE with all existing conditional gene trap clones, pDEST (frame +1) was di-

gested with XhoI and SalI and religated, which resulted in the loss of 19 bases (frame +0) and both involved restriction sites. Frame +2 was created by amplifying pDEST+1 with overhang primers (pDest-sense, pDest-antisense), which harboured one SpeI overhang and a stop codon mutation (PCR and transformation done by C. Wolf). The resulting vector was enlarged by 11 bases and showed one additional SpeI site.

The three resulting exchange vectors (pDEST/pEX-Flp) all harboured the Gateway cassette A, consisting of a chloramphenicol resistance and the ccdB gene, flanked by attR1/2 sites, necessary for later Gateway reaction, the adSA-hygromycin-pA cassette for selection in ES cells and all required RRS in reading frames +0 (6412 bp), +1 (6431 bp) and +2 (6442 bp). All three vectors and different frame sequences are indicated in Fig. 5.2 and were sequence verified with primers T7 and Dest-rev.

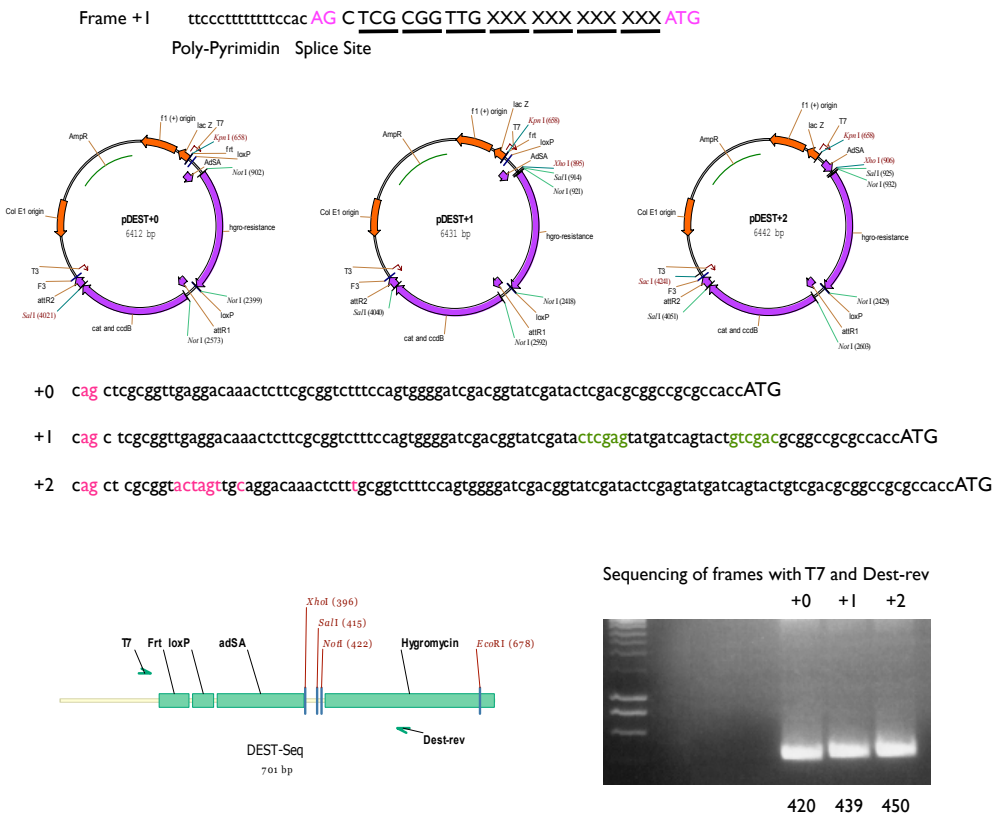


Figure 5.2: *Reading frames of pEX-Flp/pDEST*
Reading frame is defined on top, bases between the splice site in the splice acceptor sequence and the ATG start codon of the coding sequence are counted in triplet codons, no remaining base equals frame +0, one or two remaining bases equal frames +1 and +2 respectively. All generated vectors are indicated schematically, and critical sequences are shown. Frame +1 was digested with XhoI/SalI (restriction sites indicated in green) and religated to generate frame +0. Frame +2 was generated by PCR amplification, added bases are indicated in red. All frames were sequence verified using primers T7 and Dest-rev.

5.1.1.2 Entry Vector

To receive the final exchange vector, a custom made ENTRY vector, containing the gene(s) of interest (GOI) flanked by attL1/2 sites had to be established, to perform the Gateway reaction and end up with the GOI 3' of the hygromycin selection marker (see Fig. 5.3).

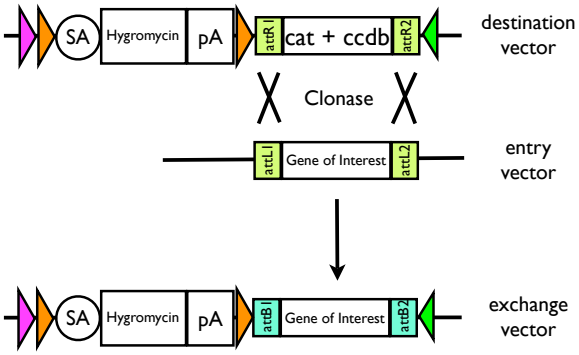


Figure 5.3: *Gateway Reaction*
The clonase enzyme catalyzes in an overnight reaction the exchange of attL1/2 flanked gene of interest sequence in the entry vector with attR1/2 flanked chloramphenicol resistance and ccdB gene (cat+ccdB) in the destination vector. After reaction, the gene of interest has recombined into the destination vector, attR1/2 have changed to attB1/2 sites and previous insert of ccdB cassette enables low background bacterial transformation by switching from DB3.1 to DH5α E. coli strain.

Since a transposon-based mutagenic screen in vivo was the goal, the integrated sequence had to be flanked by transposase binding sites, inverted and direct repeats (IRDR) for *Sleeping Beauty* (Tc1/mariner DNA cut-and-paste transposon system). The idea was to design this transposon as a mutagenic element, which should act as a gene trap vector, capable to indicate cut-and-paste events in vivo. As mutagenic ("gene-breaking") part, an adenoviral splice acceptor dsRed fluorescence protein-pA cassette was chosen (promoter-trap). After insertion into an intron of an active gene, this cassette would lead to the disruption of the endogenous transcript and result in a fusion protein. Expression of the dsRed gene would display the activity status (time and location) of the trapped gene.
Since an in-vivo-detection of transposition events was desireable, a second cassette was chosen, but in this case a polyA-trap cassette. This cassette consists of a promoter driven (mouse opsin promoter) Venus gene, followed by an IRES sequence and a splice donor. Insertion of the cassette into an intron of a gene would also lead to a fusion protein, but would be independent of the activity status, since the cassette harbours its own promoter. The opsin promoter should lead to green fluorescent retinae of those mice, where the transposon jumped out of its original inactive location and reintegrated into an intron in an active orientation. For this reason, the transposon entry vector was planned in an inverted orientation

with respect to the selection marker hygromycin, to end up with an originally inactive Venus cassette. This mouse would serve as "seed mouse", offspring could be screened for fluorescence. The third feature of the vector are lox5171 sites flanking the promoter containing part (polyA trap), in case it should be removed in vivo by CRE deletion.

As a backbone for pENTR-EX and pENTR-T2B, pENTR4 (Invitrogen) was used, which contains the attL1/2 sites flanking a ccdB cassette required for the Gateway reaction. As donor for the SB binding sites (IRDR, inverted and direct repeats), pT2B-neo (PGK-Neomycin-pA flanked by SB IRDR, a gift of Z. Ivics) was used. Both plasmids were digested with EcoRI and SalI and the transposon insert was ligated with the pENTR4 backbone to receive pENTR-T2B-Neo (done by A. Ettinger). Next step was the removal of the NotI and SalI restriction sites, to enable further cloning steps. This was achieved by digesting with the respective enzyme followed by blunting and religating the linearized plasmid. In case of NotI, overhangs were blunted by Mung Bean Nuclease (NEB), SalI overhangs were blunted by Klenow (see Fig. 5.4).

Vector plox5171 (Geneart) harboured the head-to-tail oriented lox5171 sites, flanking an AscI site to insert the Venus cassette, in addition a SalI site 5' of the lox5171 sites, to insert the dsRed cassette. All functional parts were flanked by HindIII sites, to be able to isolate and religate these into pENTR-T2B-Neo, after digesting with HindIII, to remove the PGK driven neomycin-pA cassette within the IRDRs and replace it with the plox5171 insert, to end up with pENTR-T2B-lox5171 (with the SalI site closer to attL2).

Next the two functional cassettes could be inserted. AdSA-dsRed-pA was extracted by XhoI/SalI digest of SAdsRedpA and ligated with the SalI linearized pENTR-T2B-lox5171 plasmid. Finally, Venus cassette was extracted by AscI digest from puc57-Opsin-Venus-IRES-SD plasmid (gift of Z. Ivics) and ligated with the AscI digested pENTRT2B-SAdsRed-lox5171 plasmid in the same orientation as the dsRed cassette, beginning closer to the attL2 sites, to end up with an inverted (inactive) orientation with respect of the selection marker of pDEST (see Fig. 5.5). The final pENTR-T2B-OpsVenus was used for Gateway reaction with pDEST in all three frames.

The vector pENTR-EX, for the efficiency studies, was cloned identically, with the difference that the dsRed cassette and the lox5171 fragment were inserted in the opposite orientation (active), to end up with a pEX-FLP vector where selection marker hygromycin and dsRed cassette pointed in the same direction. No Venus fragment was inserted in pENTR-EX (see Fig. 5.6).

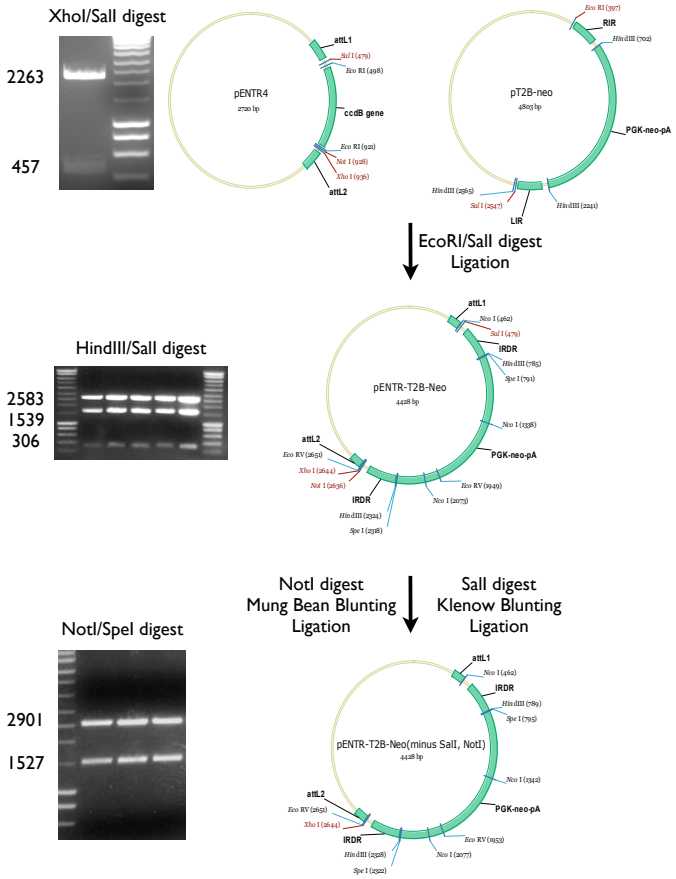


Figure 5.4: *Cloning strategy of pENTR-T2B-Neo*
To integrate the Sleeping Beauty binding sites in the entry vector, EcoRI/SalI fragment of pT2B-neo was ligated in EcoRI/SalI digested backbone of pENTR4 (Invitrogen). Then two restriction sites had to be removed (NotI and SalI) to allow subsequent cloning by digesting with the respective enzyme, blunting NotI with Mung Bean Nuclease and SalI with Klenow polymerase in seperate reactions and religating. Testdigestions are shown on the left.

5.1.1.3 Gateway Reaction for Final Exchange Vector

After cloning the respective entry and destination vectors, Gateway reaction could be performed. The clonase enzyme catalyzed the cassette exchange between the attL1/2 sites of the entry vector and the attR1/2 sites in the destination vector to achieve the final exchange vector for Flp RMCE.
The att sites in the destination vector recombine after Gateway reaction to attB1/2 sites, which prevents further recombination by the clonase. Since after Gateway cassette exchange, the reaction was transformed in DH5$alpha$E. coli competent cells, clones which harboured the Destination vector prior to exchange formed no colonies, due to toxicity of ccdB gene. Further the resistance gene in the destination vector was ampicillin, in the entry vector kanamycin, therefore ampicillin selection reduced the background significantly.

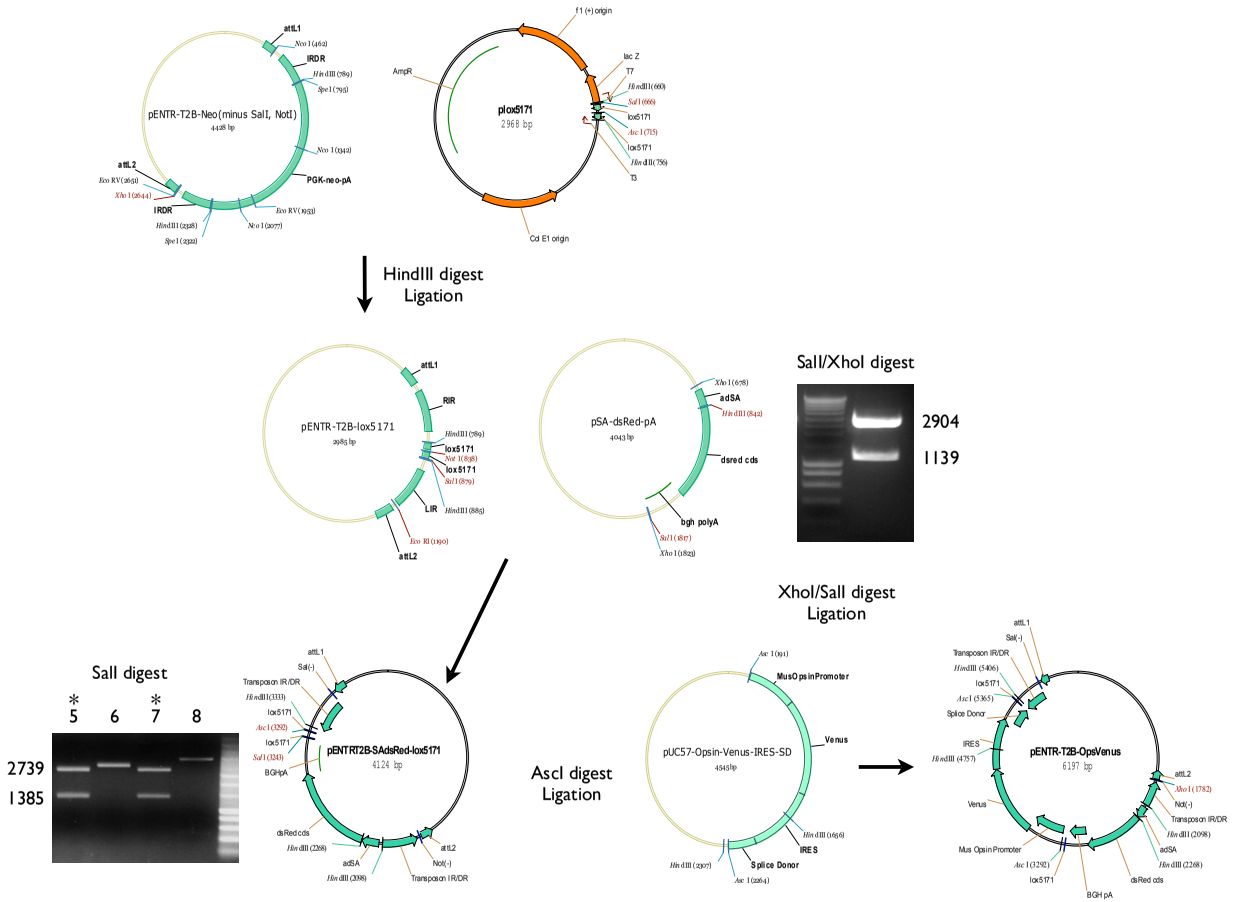


Figure 5.5: *Cloning strategy of pENTR-T2B-OPSVenus for in vivo mutagenesis*
PGK-neo-pA Fragment in pENTR-T2B-Neo was cut out by HindIII digest and replaced by the HindIII fragment of plox5171 (Geneart), which harboured designated lox5171 sites and several unique cloning sites to insert further cassettes (SalI, AscI); orientation: SalI site closer to attL2. In resulting pENTR-T2B-lox5171 vector, SA-dsRed-pA cassette (XhoI/SalI fragment) was inserted after SalI linearization (SA sequence of fragment "closer" to attL2 to recieve inactive orientation after Gateway reaction, SalI testdigest of correct clones 5 and 7). AscI digested pUC57-Opsin-Venus-IRES-SD cassette was inserted after AscI digest of pENTR-T2B-SAdsRed-lox5171 to recieve the entry vector pENTR-T2B-OPS-Venus used for the mutagenic screen.

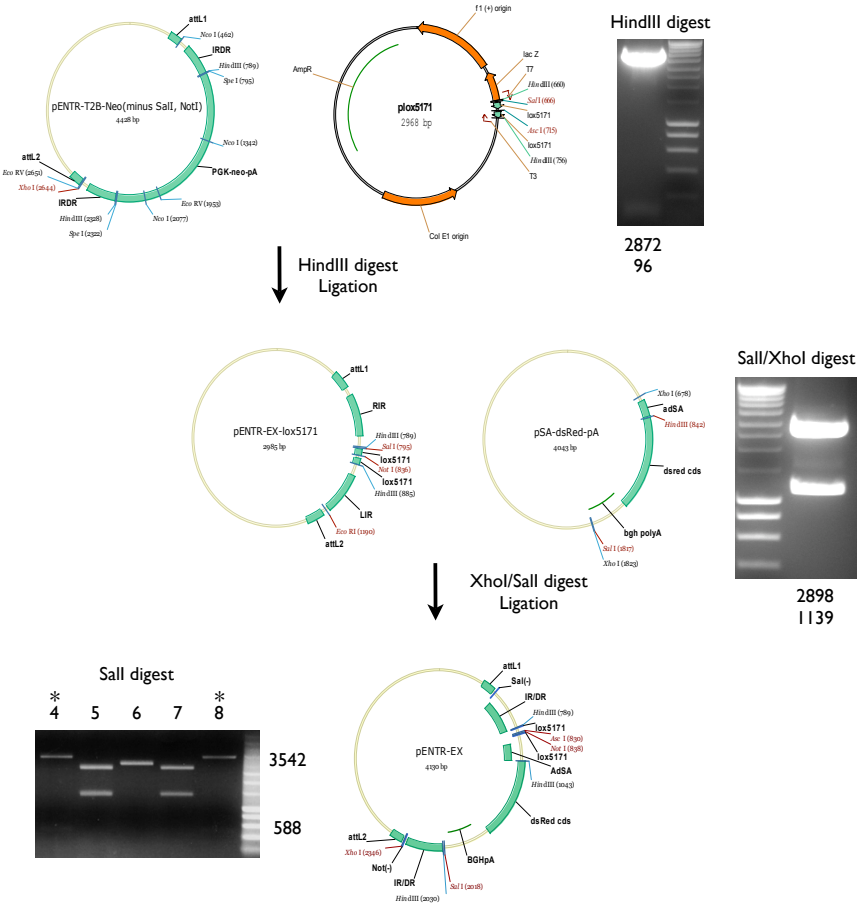


Figure 5.6: *Cloning strategy of pENTR-EX for RMCE efficiency studies*
Cloning steps are similar as in Fig. 5.5, only the orientation of the HindIII and AscI fragments into pENTR-T2B-Neo are inverted. Differences are only in inverted inserted lox5171 and dsRed cassette, with SalI site of lox5171 fragment and SA of dsRed cassette closer to attL1 site (active orientation, SalI testdigest of correct clones 4 and 8). Venus cassette was not inserted.

Gateway reactions were performed with all three reading frames of pDEST and pEX-Flp and the corresponding entry vector. Vectors and final plasmids for Flp RMCE with test-digestions are depicted in Fig. 5.7 and 5.8.

5.1.2 Rosa26 RMCE Vectors

All transposases used in this work, were integrated in the Rosa26 locus, which was first described first by P. Soriano and co-workers (Friedrich and Soriano, 1991) and shows the advantage that Rosa 26 knock-out mice display no phenotype, but the endogenous promoter is ubiquitously active in ES cells and mice.
A *piggyBac* transposase expressing plasmid (gift of M. Frasier[2]) was used as backbone for the vector exchanged into Rosa26 locus by C31 integrase.

[2]http://piggybac.bio.nd.edu/plasmid.htm

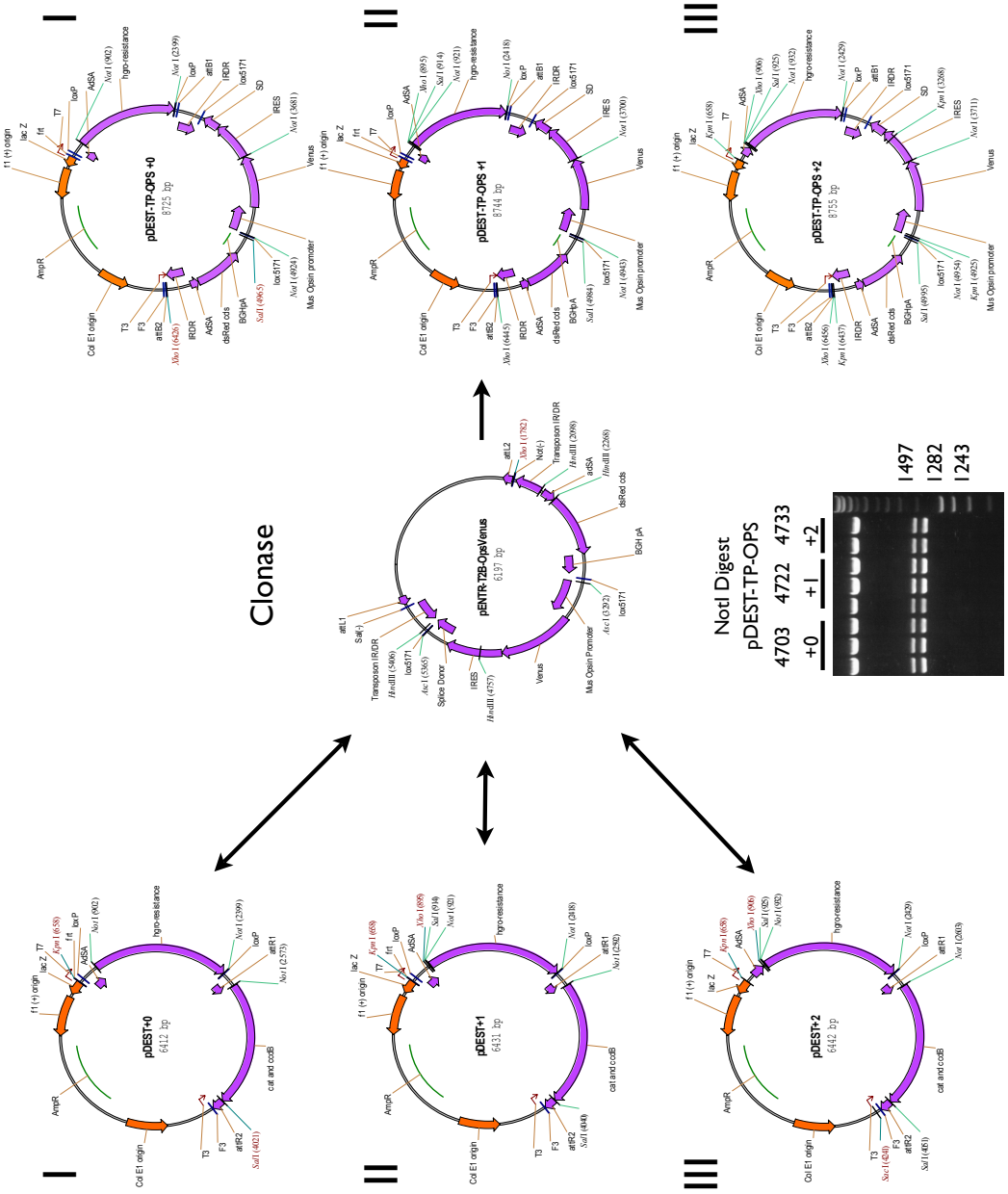


Figure 5.7: *Gateway reaction of pDEST and pEX-Flp*
Gateway reaction of pENTR-T2B-OPS-Venus and pDEST in all three reading frames is schematically shown. Test digestions with NotI of the resulting exchange vector are shown for each Gateway reaction.

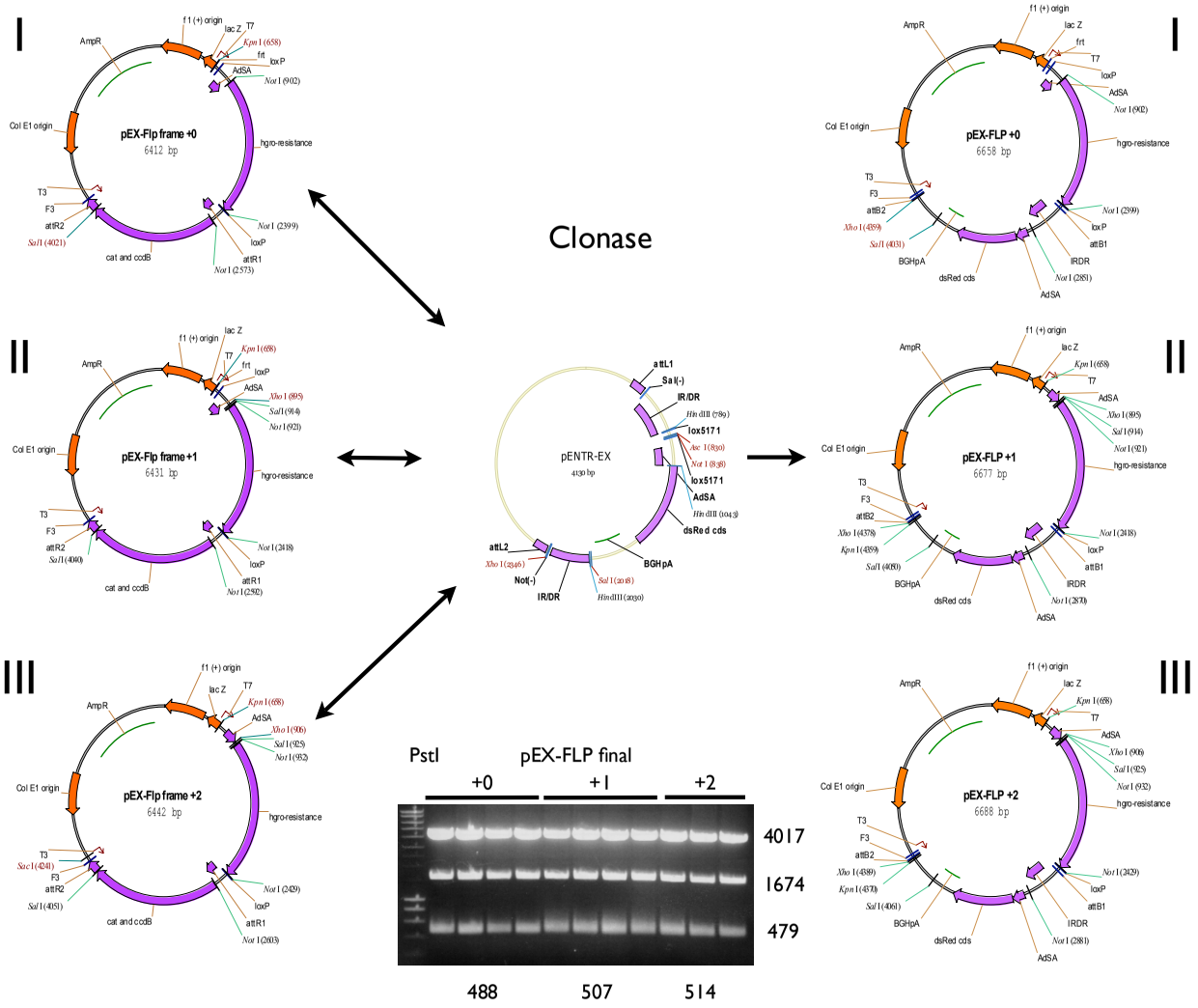


Figure 5.8: *Gateway reaction of pENTR-EX and pEX-FLP in all three reading frames is schematically shown. Test digestions with PstI of the resulting exchange vector are shown for each Gateway reaction.*

The plasmid harboured the CMV driven iPB, followed by an 3xP3 promoter driven dsRed gene. Two different transposase expressing vectors were planned, one with and one without the dsRed expressing parts. To achieve this, p3xP3-DsRed-orf was digested in two different approaches.

To generate a plasmid with the Caggs driven iPBorf, p3xP3-DsRed-orf was digested with NheI and XhoI and blunted with Klenow polymerase to end up with the iPBorf coding sequence (2032 bp). PCAG-SBM3a-pA was digested with PmeI and NotI, followed by Klenow polymerase blunting, to remove SBM3a coding sequence driven by a Caggs promoter. Then Caggs containing backbone was ligated with the iPBorf fragment, to generate pCAG-PBorf, a vector with Caggs driven *piggyBac* transposase.

To generate the Caggs driven iPBorf plasmid which also harbored the p3xP3 driven dsRed gene (pCAG-PBorf-3xP3-dsRed), p3xP3-DsRed-orf was digested with NheI, blunted with Klenow, digested with NotI and also ligated with the Caggs promoter containing backbone described above (see Fig. 5.9).

Then both fragments (Caggs driven iPBorf, one with 3xP3 driven dsRed) were cloned in the RMCE vector for the Rosa 26 targeted ES cell line. PCAG-PBorf was digested with XhoI, blunted with Klenow and the 3799 bp fragment was ligated with the SbfI digested and Mung Bean nuclease blunted pbs-attB-neo-attB vector to receive pbs-attB-neo-CAG-PBorf-attB. PCAG PBorf-3xP3-dsRed was digested with AscI and NotI, blunted with Klenow polymerase and ligated with the same pbs-attB-neo-attB backbone as described above to receive pbs-attB-neo-Cag-PB-dsRed-attB (see Fig. 5.10).

The Caggs driven SB100X and the optimized *piggyBac* transposases were also cloned into the pbs-attB-neo-attB exchange vector, by cutting the respective segments out with NotI/SspI, blunting them with Klenow polymerase and ligating them in SbfI digested and Klenow blunted pBS-attB-neo-attB vector (see Fig. 5.11).

5.1.3 *PiggyBac* Venus Transposon

pXLbetageo vector was modified to generate a second *piggyBac* transposon to be able to detect transposition in vivo. The vector was digested with NotI, dephosphorylated by CIP (calf intestinal phosphatase) and ligated with the AscI fragment of pUC57-Opsin-Venus-IRES-SD, which was blunted by Mung Bean nuclease and NotI-linkered, to receive a transposon containing the βgeo gene trap cassette, followed by the Venus polyA trap cassette, XLbetageoOpsVen (see Fig. 5.12)

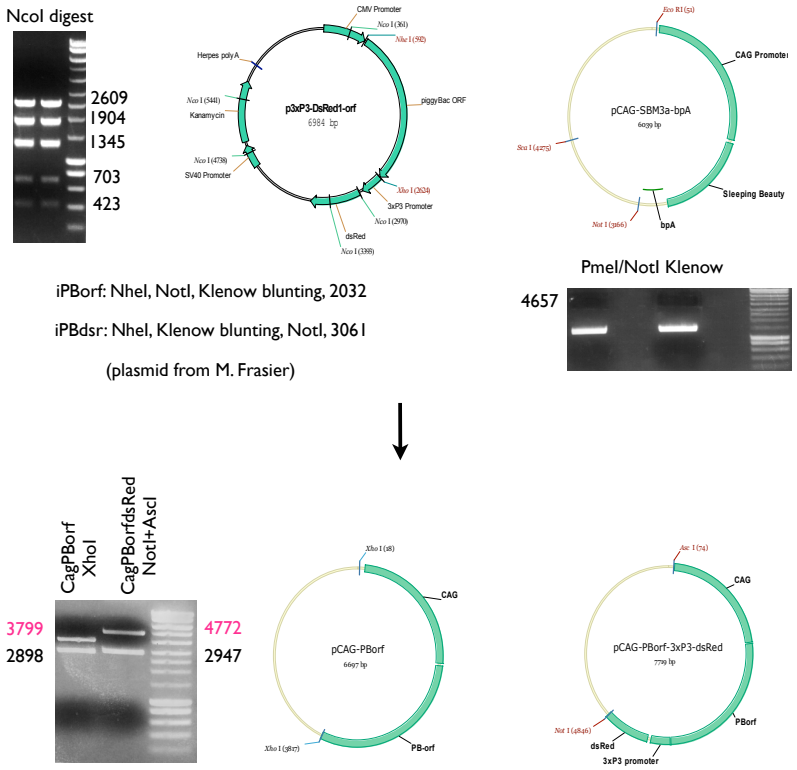


Figure 5.9: *Cloning of Caggs promoter driven piggyBac vectors*
Sleeping Beauty M3a coding sequence of vector pCAG-SBM3a was replaced by piggyBac coding sequence alone or followed by 3xP3 driven dsRed gene, by digesting pCAG-SBM3A with PmeI/NotI followed by Klenow polymerase blunting and religating the backbone with Klenow blunted NheI/NotI fragments of p3xP3-DsRed1-orf fragments. Plasmid digestions of p3xP3-DsRed1-orf with NcoI and the resulting pCAG-PBorf and pCAGP-BorfdsRed vectors are shown on the left, extracted Caggs promoter containing backbone after gel extraction is shown on the right.

5.1.4 External Vectors

5.1.4.1 Plasmids for C31 RMCE

The following plasmids were used to generate the exchange vectors, to manage the RMCE reaction into ES cells and to analyse resistant clones by Southern blot.

- pCAG-C31Int(NLS)-bpA
  Caggs driven C31 integrase (6576 bp) which manages cassette exchange in ES cells between attB and attP sites.

- pbs-attB-neo-attB
  Exchange vector for C31 mediated RMCE in Rosa26 targeted IDG 3.2pR26.10 F1 ES cells. The plasmid starts with a Frt site, followed by a promoterless neomycin resistance gene polyA signal followed

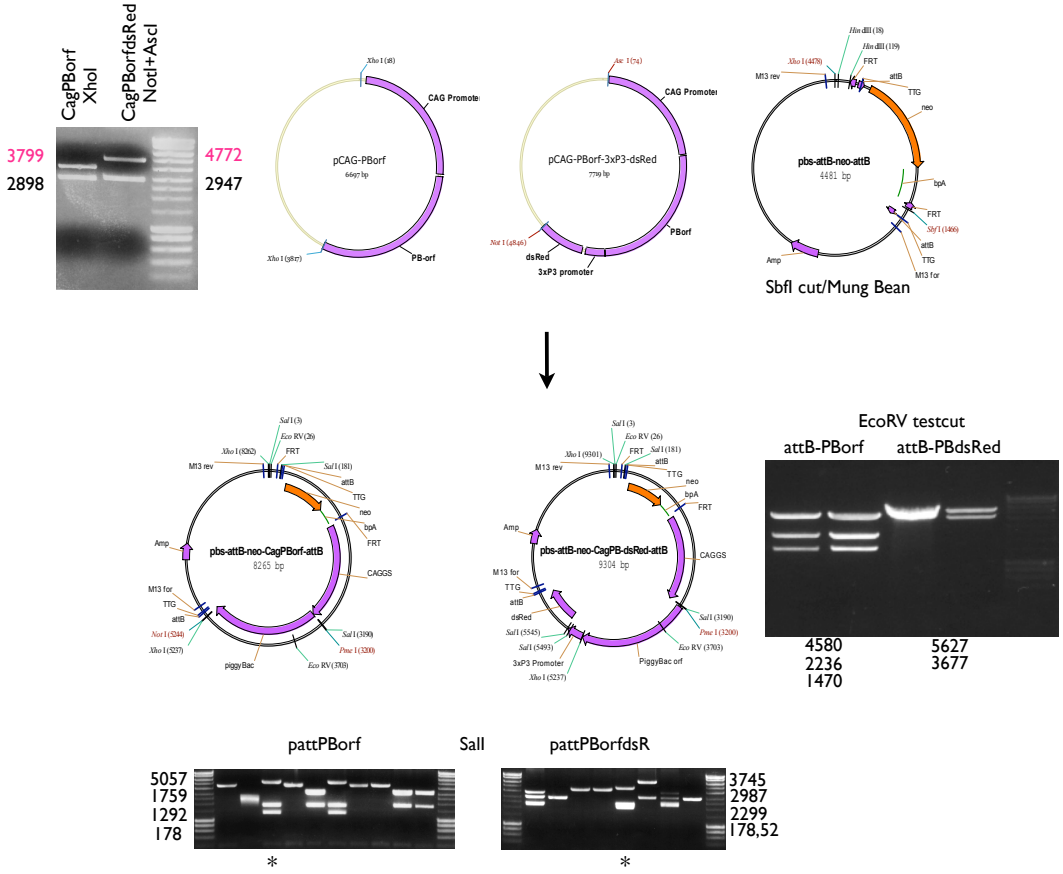


Figure 5.10: *Cloning of C31 exchange vectors harbouring PiggyBac transposase*
The two Caggs driven piggyBac transposase cassettes (with or without following 3xP3-dsRed sequence) were cut with either XhoI or AscI/NotI, blunted and each fragment ligated with SbfI digested and Mung Bean nuclease blunted pBS-attB-neo-attB vector (R. Kühn). Final vectors were used for C31 mediated RMCE in Rosa26 locus of ES cells. Two correct SalI digestions of both vectors are marked with asterisks and EcoRV digest is shown on the right.

by a Frt site, all flanked by attB sites. *Sleeping Beauty* and *piggyBac* transposases were inserted to this vector.

- pCR-II-Rosa5'probe (3-5)
  PCRII-TOPO vector containing the PCR amplified 5' probe (digest with EcoRI) to screen for successful C31 mediated cassette exchange in IDG 3.2pR26.10 F1 ES cells by Southern blot analysis.

All vector sheets are shown in Fig. 5.13.

5.1.4.2 Plasmids for Flp RMCE and CRE Excision

- pPGK-FlpO-pA
  PGK promoter driven optimized Flp recombinase for RMCE of destination vectors and conditional gene trap insertions.

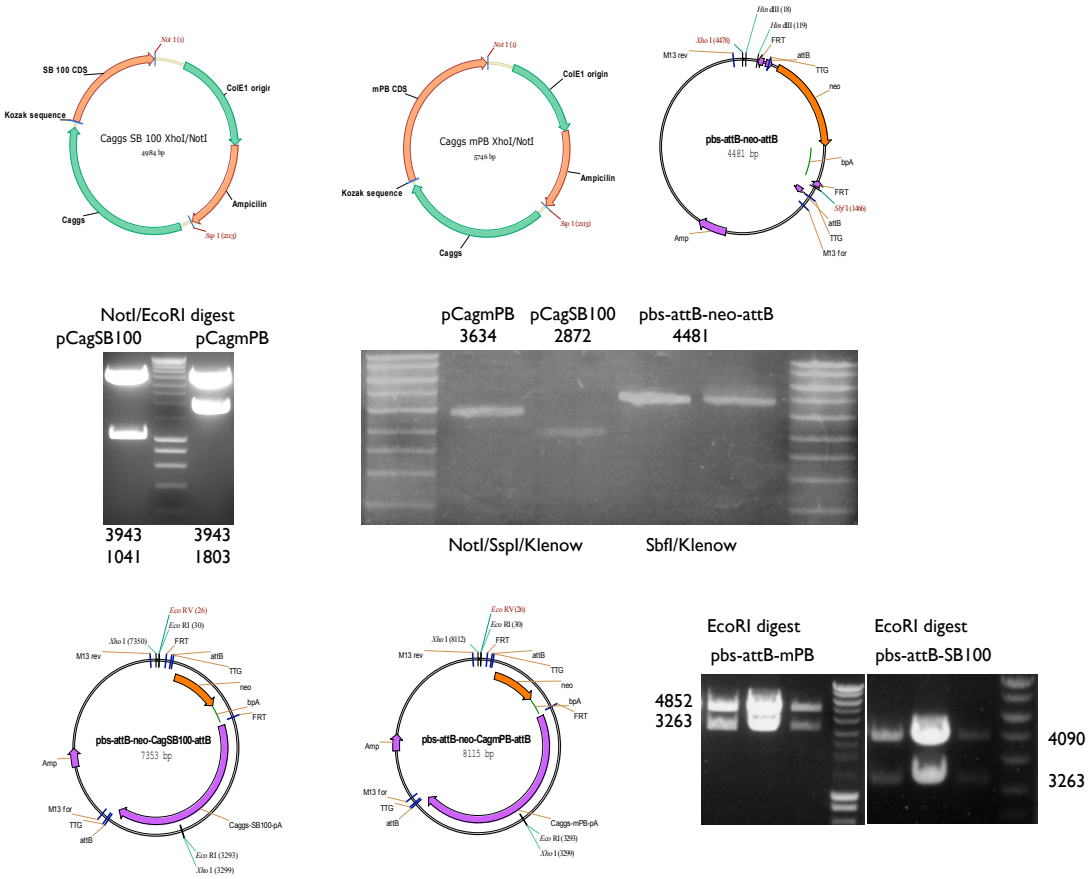


Figure 5.11: *Cloning of C31 exchange vectors harbouring SB100X and optimized PiggyBac Caggs promoter driven expression plasmids for SB100X and mPB (I. Grabundzija) were digested with NotI/SspI and blunted with Klenow DNA polymerase, to ligate each of them in SbfI digested and Klenow blunted pBS-attB-neo-attB exchange vector. Control digests of plasmids, extracted fragments and digested resulting vectors are shown in gel pictures.*

- rsFRosabetageo(CRE) gene trap vector
  All gene trap clones used in this work harboured an insertion of this vector in different reading frames[3]. Prior to Flp RMCE, gene trap clones had to be analysed by PCR concerning the presence of the 5′ frt site by using primers B034 and SR, which led to different sized bands with or without the frt site. Additionally, the annotated insertion site (based on splk results) had to be verified by PCR after designing external (genomic) primers for each used clone, and combining them with either primers Splirev2 or iPCRu4.

- pCAG-CRE-IRES-Puro
  Caggs driven CRE recombinase followed by an IRES and puromycin resistance, to be able to select for transient expression of CRE recombinase in ES cells.

[3]http://www.genetrap.de/

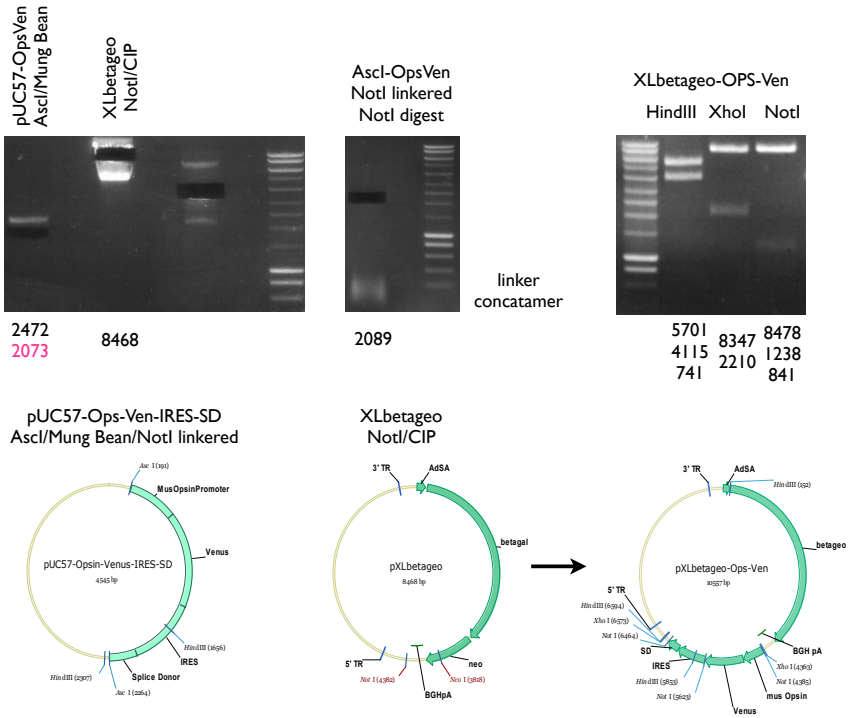


Figure 5.12: *Integration of Venus cassette in PiggyBac transposon*
Opsin promoter driven Venus fluorescence gene followed by IRES SD was digested with AscI, blunted with Mung Bean nuclease and ligated with 10-mer NotI linker. After NotI digest, resulting fragment was ligated in pXLbetageo vector linearized with NotI, to receive a transposon for piggyBac transposase also harbouring the in vivo cut-and-paste detection cassette. Gelextractions and test digestions are shown above.

Vector sheets, test digestions, exemplaric 5′ frt PCR and insertion verification PCR are shown in Fig. 5.14. CAG-CRE-IRES-Puro is shown in Fig. 5.31.

5.1.4.3 Plasmids for Further Cloning

- pattB-neo-SB M3a-attB
  The hyperactive form of *Sleeping Beauty* transposase M3a driven by the Caggs-promoter, was extracted by digesting the plasmid pCAG-SBM3a-bpA (a gift of Z. Ivics) with EcoRI, NotI and ScaI (3115 bp) and blunted with Klenow DNA-polymerase. The pBS-attB-neo-attB vector (a gift of R. Kühn) was cut with SbfI and FseI (located between neomycin selection marker and 3′ attB site), blunted with T4 polymerase and dephosphorylated with shrimp alkaline phosphatase. Last step was ligation of the linearized vector with the transposase fragment to achieve pbs-attB-neo-SB M3a-attB (done by A. Ettinger). Vector sheet shown in Fig. 5.9.

- Caggs-SB100X-pA

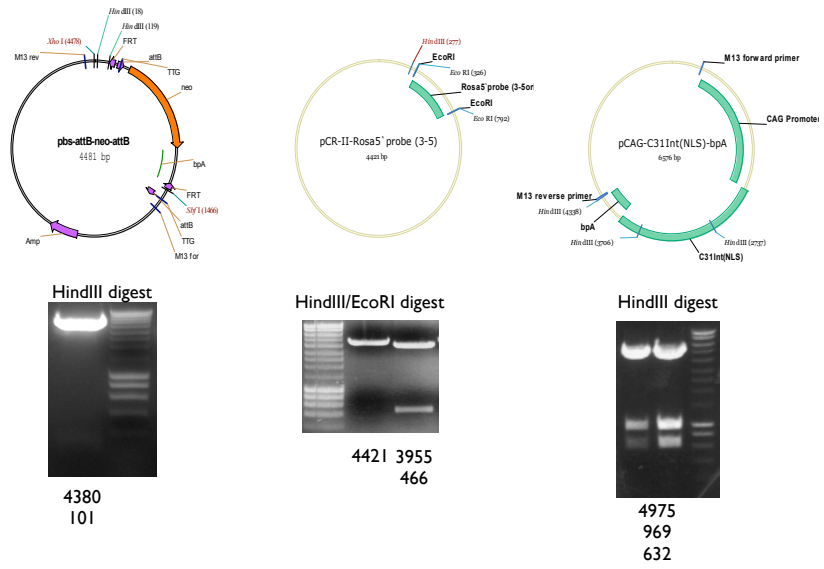


Figure 5.13: *Plasmids for C31 RMCE*
Shown are the unmodified plasmids for the C31 RMCE, the exchange vector pbs-attB-neo-attB, the vector harbouring the Rosa 5′ probe for Southern analysis of ES cell clones and the C31 expression plasmid, which was cotransfected with the exchange vector.

The Caggs promoter driven SB100X coding sequence, another mutated hyperactive form of the *Sleeping Beauty* transposase (gift of Z. Ivics, Mates et al., 2009).

- pSAdsRedpA
  The coding sequence of the red fluorescent protein (dsRed, from *Discosoma sp.*) was amplified from DsRed2-N1 (a gift of Peter Chang) using the oligonucleotides:
  5′-ATAAGCTTACCATGGCCTCCTCCGAGGAC-3′ (fwd)
  5′-ATGAGCTCCTACAGGAACAGGTGGTGGCG-3′ (rev)
  and cloned into pCRIITopo. The Topo-dsRed HindIII/SacI fragment was then ligated with the HindIII/SalI and the SacI/SalI fragments of the βgeo gene trap vector (adenoviral splice acceptor, β-galactosidase neomycin-phosphotransferase fusion gene, bovine growth hormone polyadenylation sequence, a gift of Phil Soriano to T. F.) to yield an adSA-dsRed-BGHpA harbouring vector (done bei A. Ettinger). Vector sheet shown in Fig. 5.5.

- Caggs-mPB-pA
  A plasmid which expresses the mouse codon optimized *piggyBac* transposase (Cadinanos and Bradley, 2007) driven by the Caggs promoter (plasmid from Z. Ivics/I. Grabundzija).

- XLbetageo
  βgeo gene trap cassette flanked by *piggyBac* binding sites (cloned by

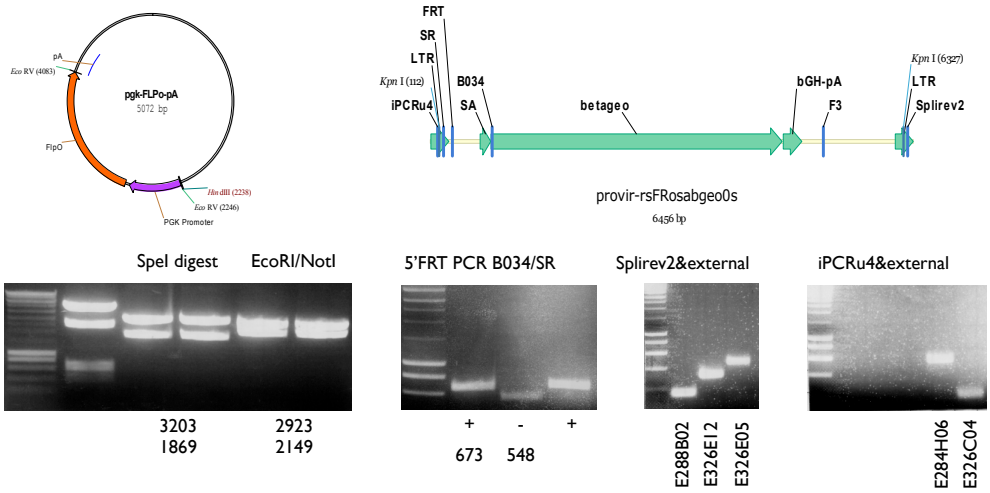


Figure 5.14: *Plasmids and PCR screens for Flp RMCE*
The optimized Flp (FlpO) under the control of the PGK promoter was cotransfected into gene trap ES cell clones with the exchange vector. All gene trap clones used in this work harboured a rsFRosabetageo insertion, which is indicated schematically. Before cotransfection existence of required 5′ frt site and annotated insertion site of each clone had to be verified by PCR methods. Complete 5′ frt site led to a bigger band (673 bp), whereas lack of this site led to a 548 bp band. Splirev2 and iPCRu4 are located either in upstream or downstream direction within the LTRs and led to a band according to the design of the external genomic primer.

I. Grabundzija). Used for cotransfection experiments in vitro, to generate single copy random insertions mediated by the transposase, to generate PB transposon harbouring mouselines. Vector sheet shown in Fig. 5.12.

- SBbetageo
  Betageo gene trap cassette flanked by *Sleeping Beauty* binding sites (cloned by I. Grabundzija). Used for cotransfection experiments in vitro.

Transposase expressing vectors are shown in Fig. 5.11.

5.2 Transposase Expressing Cells and Mice

5.2.1 C31 Mediated RMCE

5.2.1.1 Integration of *piggyBac* and *Sleeping Beauty*

Two different transposase systems were integrated in the Rosa26 locus by c31 mediated RMCE, *Sleeping Beauty* and *piggyBac*. As receiver IDG 3.2pR26.10 F1 ES cell line was used (R. Kühn). This cellline harbours an integration in the Rosa26 locus, a PGK promoter driven hygromycin resistance, where the hygromycin polyA part is flanked by attP sites (RRS for

C31 integrase). Additionally, an frt site is in front of the PGK promoter. This cellline was established by homologous recombination by R. Kühn. Also the integrase expressing plasmid and the donor plasmid for RMCE were cloned by R. Kühn.
The donor plasmid is described above and after inserting a sequence of choice (here different Caggs driven transposases), supercoiled cotransfection of donor vector and C31 expressing plasmid were performed (each 25 μg). C31 integrase managed the exchange of the attB flanked donor sequence and the attP flanked resistance gene. Successfully exchanged clones showed a neomycin resistance after 9 days selection. After exchange, genomic attP sites recombined with plasmid attB sites and changed to attL/R sites.
Screening of resistant clones was done by Southern blot analysis, using a 5' Rosa probe and by PCR analysis with a PGK and a neomycin primer (ExPGK, ExNeo). The Southern blot analysis led to up to 4 different bands, the Rosa wt band, the attP-band, the imperfect 5' exchange band and the correct exchange band with different sizes. PCR analysis resulted in a bandsize of 280 bp. Schematic RMCE is shown in Fig. 5.15.

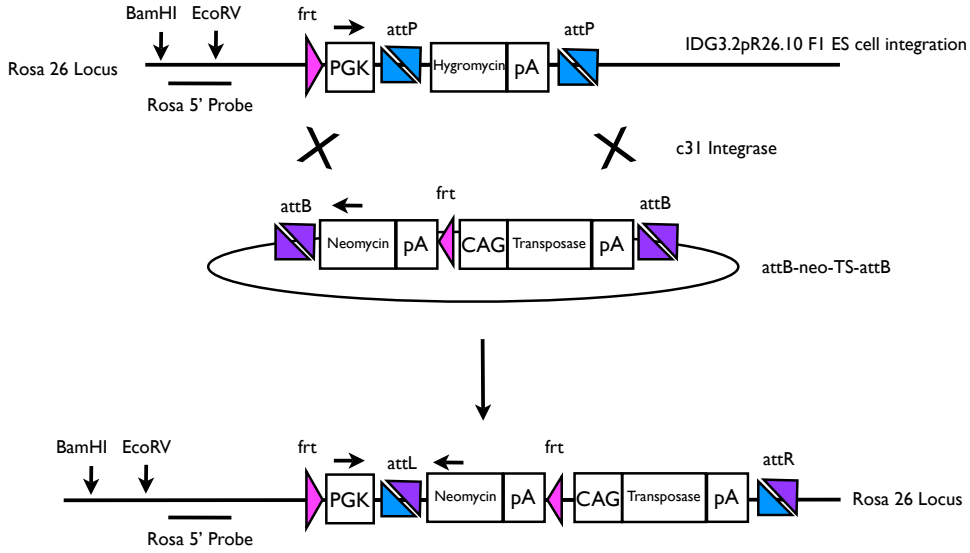


Figure 5.15: *Schematic drawing of C31 RMCE in targeted ES cells*
C31 RMCE was performed in IDG3.2pR26.10 F1 ES cell line, which has a stable integration of a PGK driven hygromycin resistance gene in the Rosa 26 locus. The hygromycin coding sequence is flanked by C31 RRS (attP), so a cotransfection of C31 expression plasmid and the attB-neo-transposase-attB plasmid (harboring the corresponding attB sites), flanked sequences will be exchanged by the integrase. After exchange, genomic attP sites recombine to attR/L sites, and no further recombination can take place. The inserted promoterless neomycin resistance gene will now be driven by the PGK promoter and enable selection on positive exchange. All transposases described in this paper were integrated in ES cells using this method. Cell line and helper plasmids from R. Kühn. Primers, restriction enzymes and Southern probe for screening are indicated.

Fig. 5.16 shows the Southern blot result of the RMCE of pattB-neo-SB M3a-

attB. Genomic DNA was digested with EcoRV and gave rise to a wild type band of 11,5 kb, an attP-band of 4.5 kb, a 5' exchange band of 8 kb and the successful exchange band of 16,8 kb.

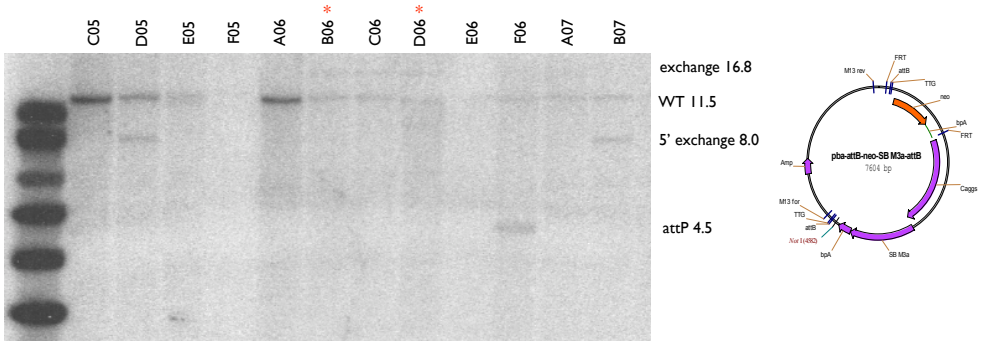


Figure 5.16: *Southern blot screen of C31 RMCE of SBM3a*
Neomycin resistant clones were screened by Southern blot analysis with a Rosa 5' probe (R. Kühn). Four different band sizes were visible, the wild type Rosa 26 band, the attP band (PGK-hygro integration still present), the 5' exchange band (imperfect exchange with only one attB/attP recombination) and the correct exchange band. Injected positive clones which gave rise to knock-in mouse lines expressing SBM3a are indicated by asterisks.

Fig. 5.17shows Southern blot screen of c31 RMCE clones with the two iPB exchange vectors, the mPB and the SB100 exchange vectors. Pbs-attB-neo-CagPB-dsRed-attB led after digesting genomic DNA with EcoRV to a 11,5 kb wild type band, a 4,5 attP-band and a 9.2 exchange band. Pbs-attB-neo-CagPBorf-attB cotransfection led after BamHI digest of genomic DNA to a 5,8 kb wild type band, a 8,3 attP-band and a 12,1 kb exchange band. Pbs-attB-neo-Cag-mPB-attB and pbs-attB-neo-Cag-SB100-attB led to a 4,5 kb attP band, a 11,5 wild type band, a 8,0 5' exchange band or the successful exchange band of 16,0 kb.
Positive clones (marked with *) were injected into Blastocysts and germline offspring was genotyped by PCR with primers ExPGK/ExNeo. Transgenic SB100 mice died shortly after birth, so no further breedings could be performed. Optimized *piggyBac* knock-in mice were received in April 2009, so no further results are accessible by now.

5.2.1.2 Analysis of Transposases *in vivo* and *in vitro*

Tissue of SB M3a knock-in mice (lines D06 and B06, received from different blastocyst injections), wild type mice and SB11 expressing mice (received from D. Largaespada) was screened by Western blot analysis with a polyclonal SB antibody to analyse in vivo expression. The polyclonal AB (rabbit anti SB) was used in a 1:5000 dilution, second AB was goat-anti-rabbit in a 1:5000 dilution. Tissue of spleen and testis was used and only the knock-in lines and the SB11 positive control showed the expected 40 kDa band (see Fig. 5.18).

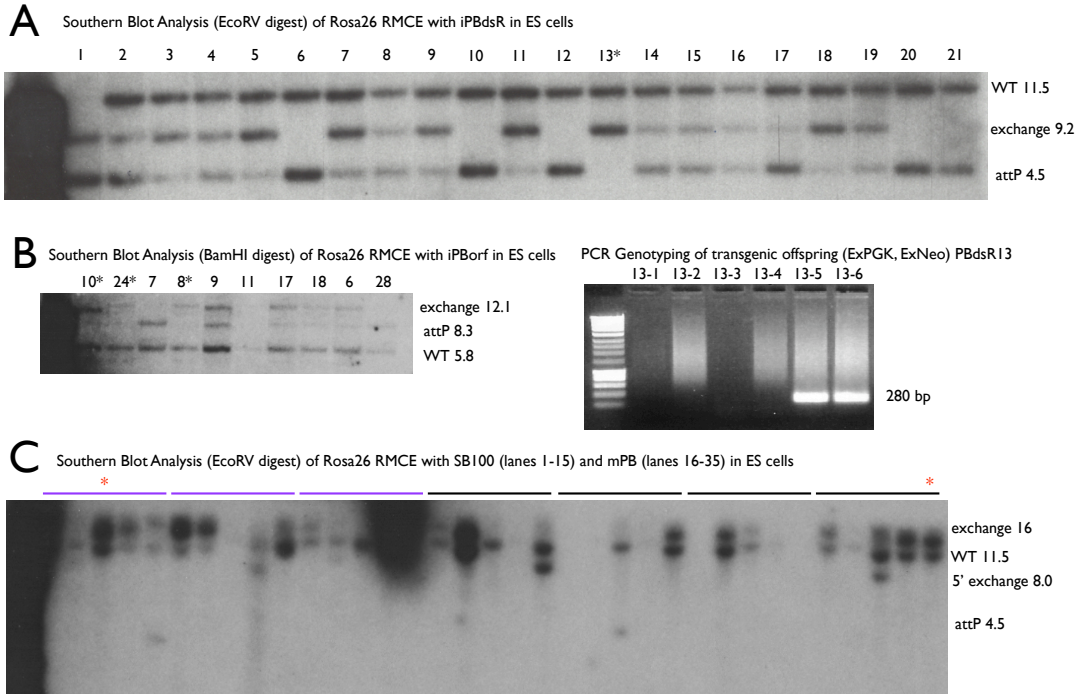


Figure 5.17: *Southern blot screen of C31 RMCE of iPB, mPB and SB100*
Shown are the Rosa 5' Southern blot screens of further four RMCE generated transposase expressing cell lines mediated by C31 integrase. Two different constructs of the iPB, one followed by a dsRed gene (A, B), the mouse codon optimized mPB and another form of SB (D), all driven by the Caggs promoter were integrated by C31 RMCE in the targeted Rosa 26 locus. Possible band sizes are indicated. Blastocyst injected clones are marked with asterisks. Germline offspring was screened by PCR using primers ExPGK and ExNeo, as an example a PCR gel of 6 PBdsR13 offspring is shown (C). Knock-in mice show the 280 bp Rosa exchange band by using primers ExPGK/Neo.

Since no *piggyBac* specific antibody was available, knock-in mice of iPB were analysed by RT-PCR on expression of the transposase. Total RNA of brain and testis of PBorf, PBdsR and wild type mice was extracted, cDNA was made and analysed with *piggyBac* internal primers. The gel of the PCR was further analysed by Southern blot analysis with a *piggyBac* internal probe overlapping the PCR product. All knock-in mice showed expected bands, negative controls (wild type tissue, DNase digested samples without reverse transciptase) showed no bands in the PCR and in Southern blot (see Fig. 5.19).

As further experiment, iPB expressing cells were transiently transfected with supercoiled XLbetageo vector and after 48 hours lacZ staining was performed. As negative control IDG3.2pR26.10 F1 ES cells were used. Only iPB cells showed blue parts, which stongly indicates that the transposase is expressed and functional in vitro (see Fig. 5.20).

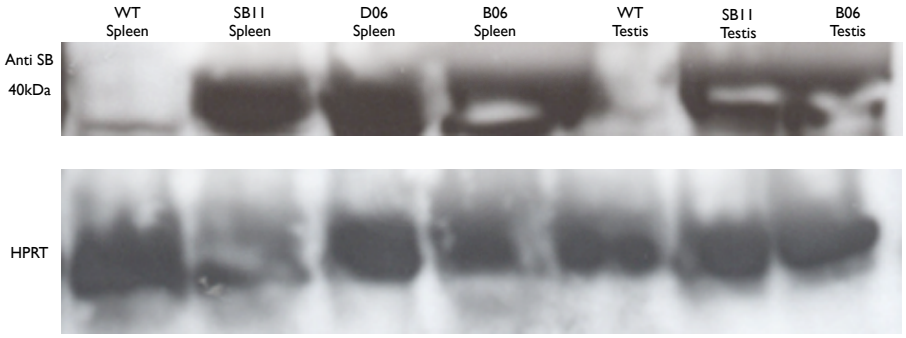


Figure 5.18: *Western Blot analysis of SBM3a expressing mouse tissue*
SBM3a knock-in mice (D06, B06) were tested by Western blot analysis using a polyclonal Anti-Sleeping Beauty antibody. Protein was extracted from spleen and testis, as negative control wild type (WT) tissue was used. As positive control SB11 transgenic mice (Dupuy et al., 2005) were used. All transgenic lines showed a strong band of 40 kDa, whereas the wild type mice showed no band. As loading control HPRT antibody was used.

5.3 Transposon Harbouring Cells and Mice

5.3.1 Random Insertion

Since the Flp mediated RMCE did not lead to positive exchanged clones in the beginning, a transfection of 10 μg linearized pDEST-TPOPS +1 was performed (SspI digest). After 24 hours electroporated cells were selected for 9 days with hygromycin and resistant clones were analysed by Southern blot with a hygromycin and a dsRed probe.
Additionally genomic DNA was screened by PCR, using primers located at the 3' parts of the functional vector cassettes, to ensure the complete insertion of the linearized vector. One positive clone, which showed both bands (dsRed and hygro) in a Southern blot and gave rise to the expected PCR band of 849 bp, was injected into blastocysts and agouti offspring of chimeras was screened by Southern blot using a dsRed probe. Transposon harbouring mice showed no fluorescence in their retinae, which was not surprising since the orientation of the cassette was in inactive configuration (see Fig. 5.24). Southern blots, probes and PCRs are shown in Fig. 5.21.

5.3.1.1 Cotransfection of Transposon and Helper Plasmid

In case of *piggyBac*, a supercoiled cotransfection of mPB expressing plasmid and XLbetageo or XLbetageoOpsVen was performed, to select for transposon harbouring cell clones, in which the transiently expressed transposase managed the transposon insertion, to ensure the complete integration of all crucial vector parts.
Different amounts of both vectors were coelectroporated into TBV2 cells,

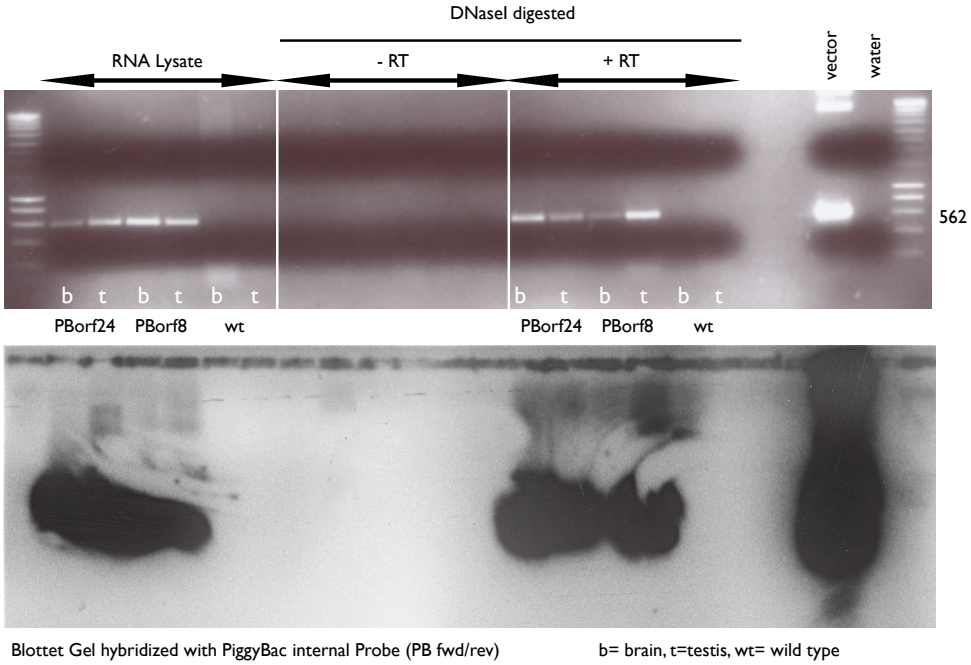


Figure 5.19: *RT-PCR of iPB expressing mouse tissue*
RT-PCR of PBorf8 and PBorf24 mouse lines was performed. Total RNA was extracted from brain (b) and testis (t) tissue. Wild type (wt) mouse tissue was used as negative control, iPB expression vector as positive control. Only transgenic lines showed the expected band size (562 bp) in subsequent PCR using PB internal primers, also negative control of DNase I digested RNA without reverse transcriptase (-RT) showed no bands. Additionally the gel was Southern blotted and hybridized with a PB probe amplified with the same internal primers.

after 48 hours the plated cells were selected with neomycin for 9 days and resistant clones were screened by Southern blot analysis (HindIII digest, neomycin probe) for multiple insertions. Further, Splk PCR was performed (AG Ruiz, Charite Berlin) to localize insertion sites. In the first batch of electroporations, the ratio of transposase to transposon were 2 and 10, 10 and 2, 10 and 10, 2 and 2, 0 and 2 μg. Clones which showed only one neo band in Southern blot analysis and matching 3′ and 5′ Splk results were injected into blastocysts. Offspring was screened using LacZ internal primers. See Fig. 5.22.

5.3.1.2 Pronucleus Injection

Since injected XLbetageoOpsVen harbouring clones showed no germline transmission, pronucleus injection of supercoiled helper plasmid and transposon vector into fertilized CD1 oocytes was performed (ratio 1:2 transposon:transposase, 2 ng/μl total concentration in 10mM TRIS, pH7,2). Injection was performed by S. Weidemann as described (Nagy et al., 2003).

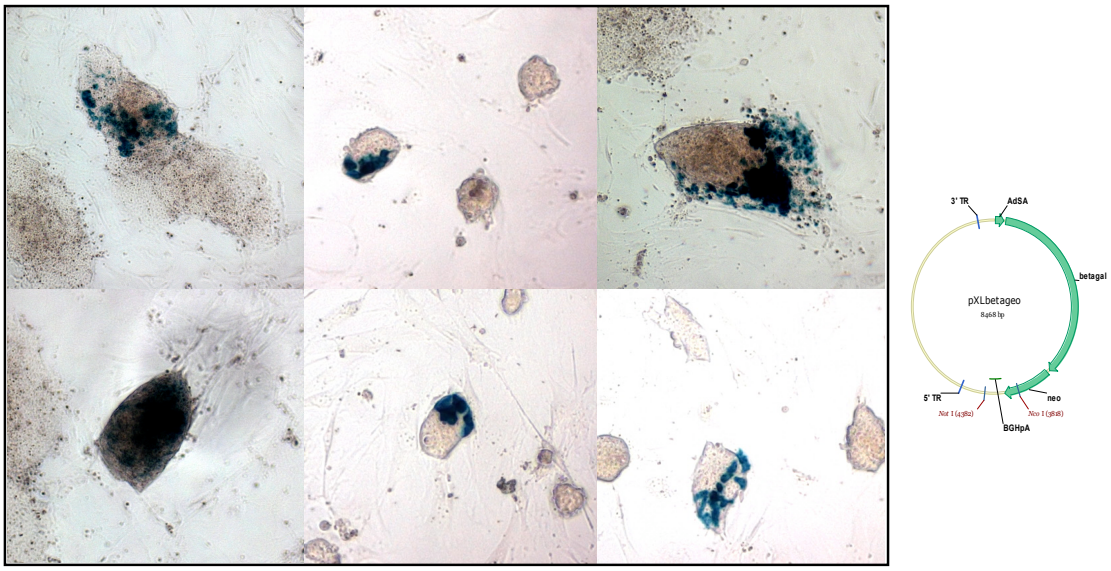


Figure 5.20: *In vitro excision assay of mPB expressing ES cells*
To determine activity of generated mPB transposase expressing cell lines, positive clone 20 and wild type F1 ES cells were expanded and transiently transfected with supercoiled pXLbetageo vector. After three days without selection, LacZ staining was performed and the number of blue colonies was determined. Only the PB positive transfected plate showed blue colonies, which indicated the in vitro activity of mPB to cut the βgeo harbouring transposon out of the transfected plasmid and integrate it in the genome. Shown are examples of clones with positive lacZ staining.

5.4 Mouselines

5.4.1 External Mouselines

- R26SB11
  The endogenous Rosa 26 promoter drives SB11 transposase gene followed by SV40 pA (Dupuy et al., 2005)

- 129-TgTn(sb-T2-Onc)68dla
  Mouseline harbors 25 copies of SB T2/Onc transposon on chromosome 15. TP size 2.2 kb. (Collier et al., 2005)

5.4.2 Generated Mouselines

- TPOPS6 (complete SB transposon in inactive orientation, 2 copies)

- D06, B06 (Caggs driven SBM3a in Rosa26 locus)

- XLbetageo2 (single-copy PB transposon in Car2 gene, contains no in vivo detection cassette, active orientation)

- PBorf8, PB0rf24, PBdsR13 (Caggs driven iPB in Rosa 26 locus PBdsR13 including a 3xP3 driven dsRed cassette)

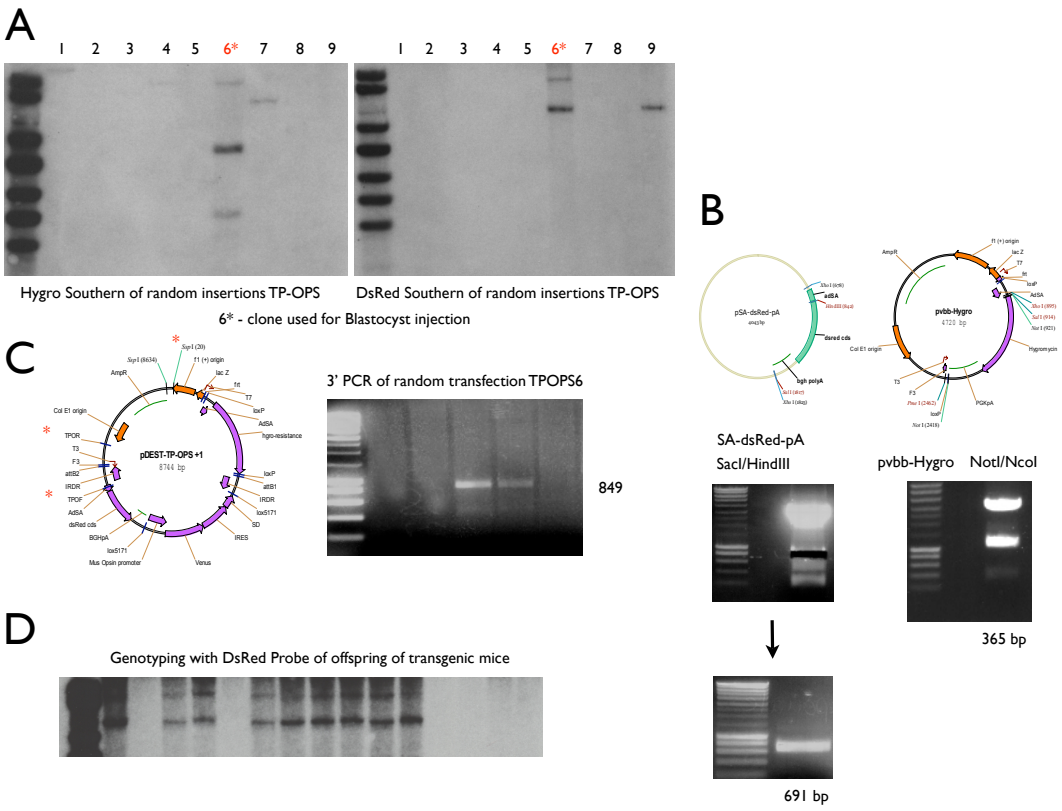


Figure 5.21: *Random integration of pDEST-TPOPS*
Due to problems with Flp RMCE, linear (SspI) pDEST-TPOPS was transfected to TBV ES cells, to generate SB transposon harbouring cell lines and mice. Transfected cells were selected with hygromycin, resistant clones were screened by Southern blot analysis using hygro and dsRed probes (A, B). Also PCR screening of clones was performed using primers TPOF/R to check for the integration of the full length construct (849 bp band), shown in (C). Clone 6 (*) was injected into blastocysts and offspring of transgenic and seed animals (expressing SBM3a or SB11 and harbouring transposon integration) was screened by Southern blot using a dsRed probe (D).

- mPB (Caggs driven mouse codon optimized PB transposase gene in Rosa26 locus)
- XLbetageoOpsVen17 (single-copy PB transposon in Has3 gene, βgeo promoter trap cassette followed by opsin-venus polyA trap cassette, active orientation)

5.4.3 Breeding Strategies

To generate an in vivo mutagenic screen, it was first necessary to breed double transgenic mice, which harbour the transposon and the respective transposase. Then these "seed" animals were backcrossed with wild type C57Bl/6J mice and offspring was screened for transposition during germline and stable reintegration into another genomic locus by Southern blot analysis. Additionally offspring was screened for lack of transposase

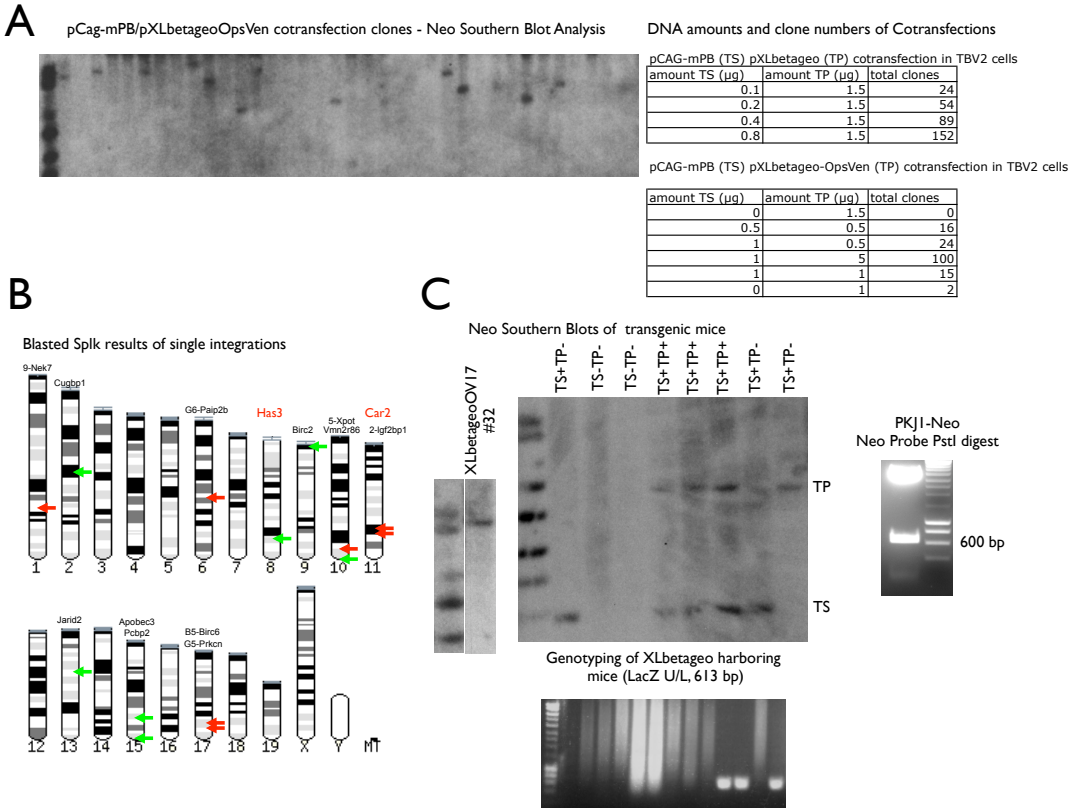


Figure 5.22: *Random integration of PB transposons*
PiggyBac transposons (pXLbetageo, pXLbetageo-OpsVen) were cotransfected with supercoiled mPB expression plasmid, to obtain transposon integrations mediated by piggyBac transposase. DNA amounts of different electroporations, resistant clones after neomycin selection and Southern blot screening of clones are shown in (A). Clones showing a single neo band were screened by SPLK PCR to locate the integration sites. Blasted SPLK results are annotated in (B), green arrows pXLbetageoOpsVen, red arrows pXLbetageo insertions. ES cell clones carrying an integration of XLbetageo on chromosome 11 in Car2 gene and a XLbetageoOV integration on chromosome 8 in Has3 gene were injected into blastocysts. Offspring was genotyped by PCR using LacZ primers and by Southern blot using a neomycin probe (C). Double transgenic seed mice after crossing with transposase expressing mice showed two neo bands in a Southern blot.

by PCR, since otherwise further somatic transposition events could occur.

Mouse line XLbetageo2 harboured the *piggyBac* βgeo transposon on chromosome 11 and was bred to PBdsR13, PBorf24 and PBorf 8 lines. Single transgenic mice for TP and TS were intercrossed, to generate double transgenic offspring, which was screened by PCR (LacZ U/L, ExPGK/Neo). Offspring of double transgenic mice was screened for transposition events by Southern blot analysis (neo probe).

Mouse line TPOPS6 harboured the *Sleeping Beauty* transposon with the dsRed and the Venus cassettes in inactive orientation with respect to the selection marker (probably 2 or 3 copies according to the Southern blot bands indicated in Fig. 5.21). This mouse line was bred to the SBM3a D06 and B06 lines and as a control to the SB11 mouse line. Offspring of

single transgenic breedings and backcrossings of double transgenic mice was screened by PCR (ExPGK/Neo) and by Southern Blot analysis (dsRed probe). The SB11 mouse line was genotyped with the same primers as all other transposase expressing lines, since it represents a knock-in of the En2SA-SB11-SV40pA upstream of a PGK driven neomycin resistance gene (Dupuy et al., 2005).
D06 and B06 were crossed with 129-TgTn(sb-T2-Onc)68dla mouse line to examine in vivo functionality of SB M3a knock-in lines. In both cases the transposition did take place, so inefficient transposition of TPOPS6 might be caused by size or copy number limitations, not by functionality of SBM3a itself. As a control, also SB11 mice were bred to 129-TgTn(sb-T2-Onc)68dla line, to compare efficiency or transposition bias of both *Sleeping Beauty* transposases. Offspring of double transgenic mouselines for T2onc concatemer and *Sleeping Beauty* transposase were screened by Southern blot analysis (T2onc probe, described in Dupuy et al., 2005). T2onc breedings with M3a D06 and SB11 lines were not designed as mutagenic in vivo screen, but only to compare efficiencies of different *Sleeping Beauty* lines, therefore offspring of double transgenic lines was directly screened by Southern blot analysis.
For the inactive TPOPS6 transposon mouse line (SB), offspring of seed mice could be directly screened for transposition. Seed mice harbouring an active primary integration of the transposon (XLbetageoOpsVen insertion), the offspring of seed mice first had to be screened for reintegrations in an inactive state, then next generation of inactive seed mice had to be generated and crossed back to be able to then use the in vivo marker as screen for transposition events in an active state.
Since XLbetageo harbouring mouse lines lacked any in vivo marker, individuals were bred according to TPOPS6 mice and screened by Southern blot analysis for transposon mobilization.
Breeding schemes for active and inactive first integration mouse lines are depicted in Fig. 5.23. Overview of transposon insertions, ongoing breedings, number of screened offspring of backcrossings are summarized in Fig. 5.24.
None of the intercrossed *Sleeping Beauty* expressing mouse lines (SB11 and SBM3a) with the TPOPS6 mouse line showed any cut-and-paste event. About 900 animals (offspring of backcrossing with wild type C57Bl/6J mice) of M3a expressing and TPOPS6 harbouring mice were analysed by Southern blot (dsRed probe) and all blots either showed original bands or no bands at all.
Also the SB11 breedings showed no changes in band pattern in over 250 cases. This indicates that the size of the actual transposable element of 4000 bp might be too large.
Backcrossings of XLbetageo transposon showed so far no transposition events in the Southern blot screen, but only a very limited number could be screened so far and future occurring cut-and-paste events are expected.

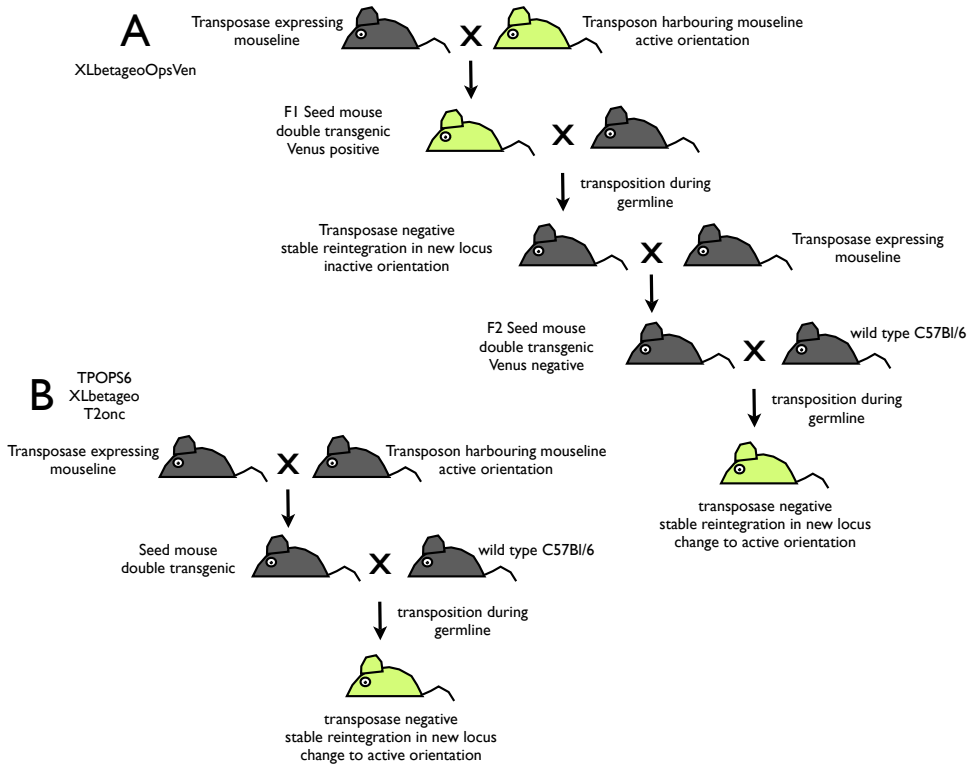
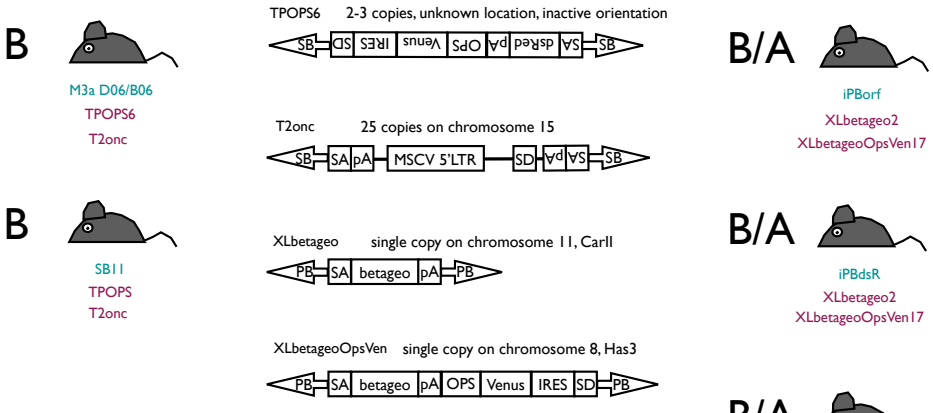


Figure 5.23: *Breeding schemes of in vivo mutagenic transposon screen*
Two different strategies to breed transgenic mice were applied. One starting with the active transposon (A), one starting with the inactive transposon orientation (B). In B, the inactive scheme (TPOPS6, XLbetageo, T2onc transposons), mice were crossed to seed mice expressing also the corresponding transposase, and then backcrossed to wildtype mice. Transposition events of TPOPS would then be detectable by fluorescent retinae. XLbetageo and T2onc lines lack an in vivo marker and were screened by Southern blot analysis. XLbetageoOpsVen crossings did not start yet, but show two additional steps, first to generate stable reintegrations in a venus inactive orientation (transposase negative), followed by crossing those with transposase positive mice, to get a new generation of seed mice, which are venus negative.

Crossings with 129-TgTn(sb-T2-Onc)68dla showed cut-and-paste events. Both versions of *Sleeping Beauty* transposases, SB11 and M3aD06, were able to mobilize transposons out of the concatamer and reintegrate these into a new genomic locus (Southern blot analysis see Fig. 5.25).

SB11 crossings showed in average about 3 times more reintegration bands per mouse than D06 crossings, although M3a is a hyperactive form of *Sleeping Beauty* transposase. A selection of genomic tail DNA of mice of both breedings was screened by Splinkerette PCR (protocol see Dupuy et al., 2005) PCR products were cloned into pCRTOPOII, sequenced and determined by mapping them against the Ensembl mouse genome database using the BLAST algorithm (Schaffer et al., 2001). This was performed to specify reintegration site preferences or local hopping tendencies of the two different *Sleeping Beauty* transposases (results of Splk screen are shown in Fig. 5.26). So far only one DNA sample gave rise to proper PCR



Backcrossing	No. of analysed mice	transposition events
M3aD06 TPOPS6	844	0
M3aB06 TPOPS6	47	0
SB11 TPOPS6	254	0
M3aD06 T2onc	145	3/mouse
SB11 T2onc	140	10/mouse
PBorf8 XLbetageo2	8	0
PBorf8 XlbetageoOpsVen	no crossings yet	
PBorf24 XLbetageo2	no backcrossings yet	
PBorf24 XLbetageoOpsVen	no crossings yet	
PBdsR13 XLbetageo2	30	0
PBdsR13 XLbetageoOpsVen	no crossings yet	
mPB20 XLbetageo2	no crossings yet	
mPB20 XLbetageoOpsVen	no crossings yet	

Figure 5.24: *Overview of breedings and screened mice*
All different breedings are shown, transposase indicated in green, transposon in red. The applied breeding scheme is indicated in letter A (active venus cassette) and B (inactive venus cassette) like described before. All used transposon constructs are schematically shown and screened offspring of back crossing per mouseline is indicated in table, including transposition events.

products (tail clip DNA of mouse D06-T2onc 172), two insertions were mapped on chromosomes 1 and 16, so both reintegrations occurred on different chromosomes and not on the donor chromosome.
Double transgenic mice of both breedings (SB11 and SB M3a) died between 16 and 20 weeks of age probably due to somatic transpositions causing cancer like already described in Dupuy et al. (2005).

5.5 RMCE Efficiency Experiments

5.5.1 RMCE using FlEx Gene Trap Clones

All gene trap clones presented in this study had insertions of a retroviral SA-betageo-pA vector, flanked by recombinase target sites in FlEx configuration (Schnütgen et al., 2005). In brief, the cassette consists of a combination of inversely oriented original and mutant target sites for Flp and Cre recombinases.
This configuration allows unidirectional inversion of the βgeo cassette by the respective recombinase. Since the pEX-Flp insert is flanked with one set of heterotypic oppositely oriented target sites for Flp recombinase,

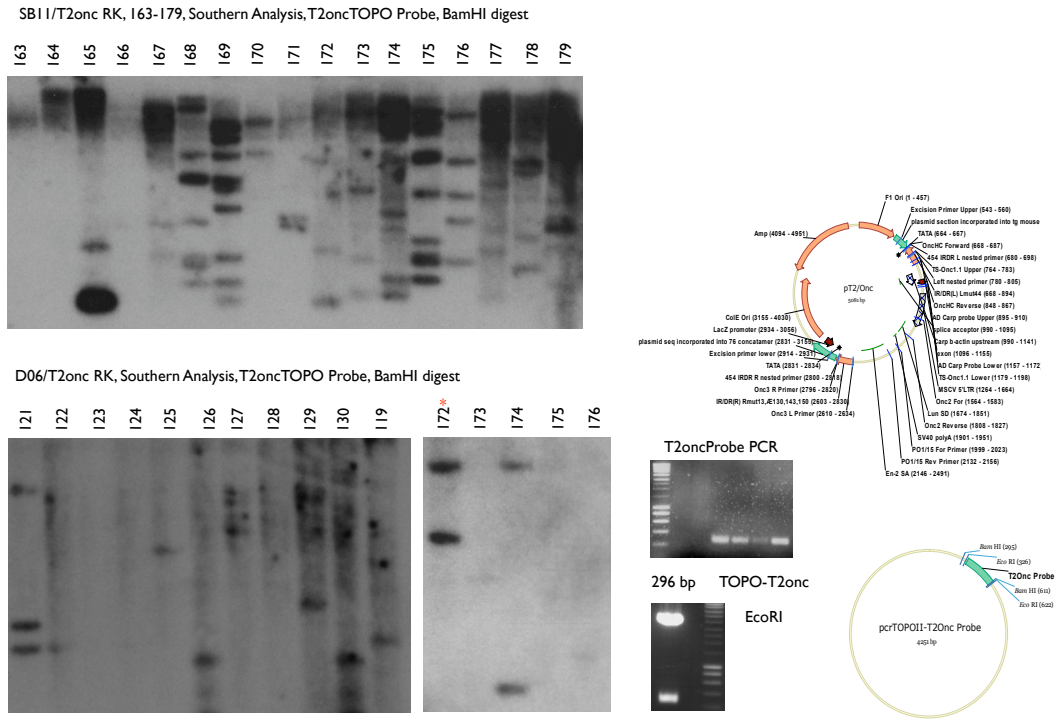


Figure 5.25: *Southern Blot analysis of T2onc breedings, SB11 vs. SBM3a*
Offspring of double transgenic mice for T2onc transposon and SB transposases were screened by Southern blot analysis for transposition events with a T2onc probe, amplified with primers pT2oncProbe F/R and cloned in pCR-TOPOII. The whole T2onc sequence is shown as vector sheet. The upper blot shows transposition events of an assortment of SB11/T2onc back crossings, the lower of M3a D06/T2onc back crossings. Every band indicates a single transposition event.

transient co-transfection of pEX-Flp and FlpO into FlEx gene trap clones allows recombination between the homotypic RRS. The RMCE strategy is outlined in Fig. 5.27.

Different outcomes after co-transfection of pEX-Flp and FlpO and hygromycin selection are possible: (i) recombination of pEX-Flp and βgeo in identical orientation; (ii) recombination of pEX-Flp in inverse orientation, resulting in hygromycin sensitivity. Inverse orientation is the result of recombination with the "inner" FRT/F3 sites and could be followed by another inversion and excision event, leading to correctly exchanged clones; (iii) inversion of the βgeo cassette combined with a random insertion of the pEX-Flp within a transcriptionally active gene, leading to false positive clones.

Different PCR strategies were employed to identify successfully exchanged clones. Hygromycin resistant clones were first analysed for β-gal activity. β-gal-negative clones were further screened by "exchange PCR" using a 5' primer in the LTR and a nested primer in the splice acceptor, which yielded either the hygromycin band of 239 bp, the βgeo band of 516 bp or no band after βgeo inversion (Fig. 5.27 C).

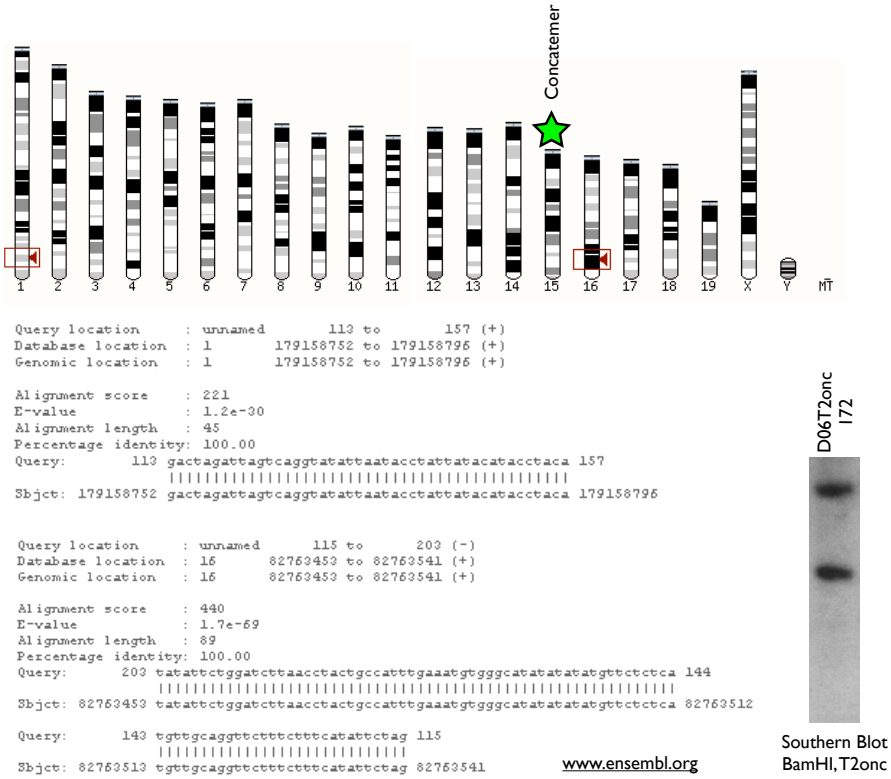


Figure 5.26: *Splinkerette PCR Screen of transposon reintegrations*
Shown are two reinsertions of T2onc transposons from offspring of a double transgenic D06xT2onc mouse backcrossed with wild type C57Bl/6. Southern blot analysis indicated two new insertions, which were confirmed by Splinkerette PCR analysis. T2onc concatemer is located on chromosome 15 (green star), reintegrations happened on chromosome 1 and 15 (red arrow heads). Splinkerette PCR products were cloned into TOPOII vector, sequenced with M13 fwd primer and verified. Sequencing results were blasted against mouse genome (www.ensembl.org) and blast results are shown beneath.

To distinguish inverted βgeo cassettes with random insertions of pEX-Flp and to identify false positive clones as described above, a triplex PCR using primers B045, B048 and B050 was performed which yielded different product sizes depending on the orientation of the gene trap vector. DNA of RMCE clones yielded a slightly larger product (839 bp) as compared to the βgeo inversion (800 bp), whereas original βgeo insertions led to a 631 bp product (5.27 B, left).

An assortment of clones was additionally screened by Southern blot analysis with neomycin and hygromycin probes to detect possible multiple or random insertions. Fig. 5.28 shows the comparison of hygromycin Southern blot and excision PCR results of E224B05 (Nupl 2) clones. The most efficient electroporation of this clone took place with pEX-Flp +2, which matched the gene trap insertion. Only clones picked of this electroporation

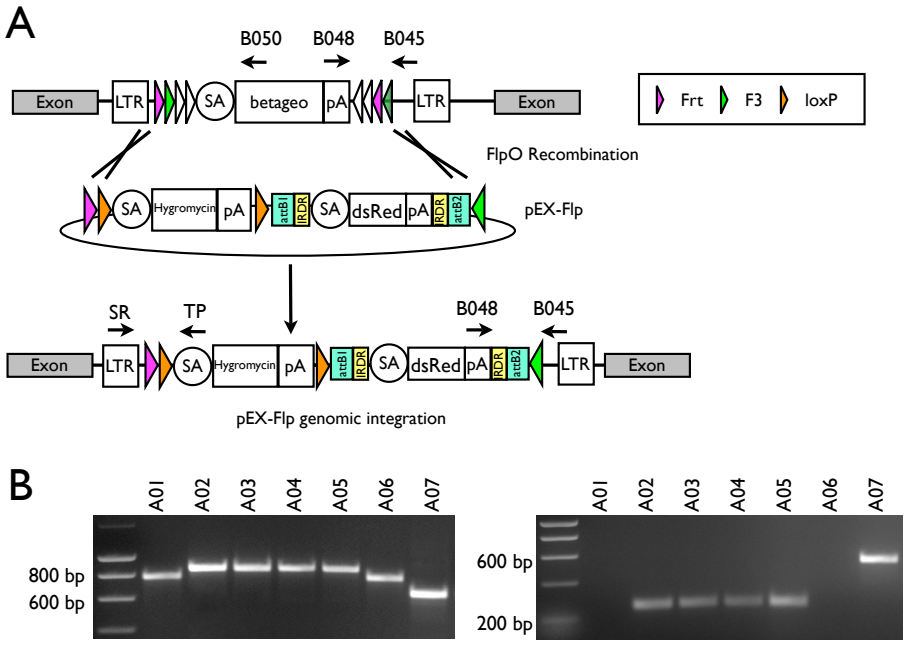


Figure 5.27: *Flp mediated RMCE of pEX-Flp in conditional gene trap clones*
(A) Schematic illustration of the FlpO RMCE in ES cells. pEX-Flp harbors two heterospecific flippase recombination sites, therefore, co-transfection of FlpO and pEX-Flp to any FlEx gene trap ES cell clone allows replacement of β-geo cassette. (B) Analysis of successfully exchanged locus with pEX-Flp containing the adSA-dsRed-BGHpA sequence flanked by IRDR of Sleeping Beauty transposase. Example of a PCR screen of 8 hygromycin resistant clones (E307D01 derivates, A01-A08) concerning orientation of the genetrap vector and successful exchange (left: primers B045, B048, B050; right: primers SR, TP). A01 and A06 are inverted β-geo insertions, they show an 800 bp band on the left and no band on the right. A02-A05 are successfully exchanged clones, they show an 839 bp band on the left and the small 239 bp band on the right. Clone A07 carries the original β-geo insertion and gives rise to the 631 bp band on the left and the 516 bp band on the right.

showed the correct sized hygromycin band (4, 13, 15, 23), which matched to the PCR results. Clones 50 and 52 (pEX-Flp +0 electroporation) showed multiple hygromycin bands, which indicates random integrations or unspecific recombination, since in the PCR these clones still showed the βgeo band of 516 bp.

In Figure 5.29 the results of a neomycin Southern blot of Scpep1 exchange clones were compared with their PCR results. Only clones which already showed the inversion or the βgeo band in the PCR screen (primers B045, B048, B050), also showed a neo band in the Southern blot. Even the difference between βgeo and inverted βgeo was distinguishable in the blot. Additionally also PCR bands of the excision PCR were verified and no controversial results could be seen.

13 different, randomly chosen conditional gene trap clones with insertions in 8 independent genes were co-transfected with pEX-Flp in three reading frames (see Fig. 5.30). The Scpep1 clones were electroporated twice and

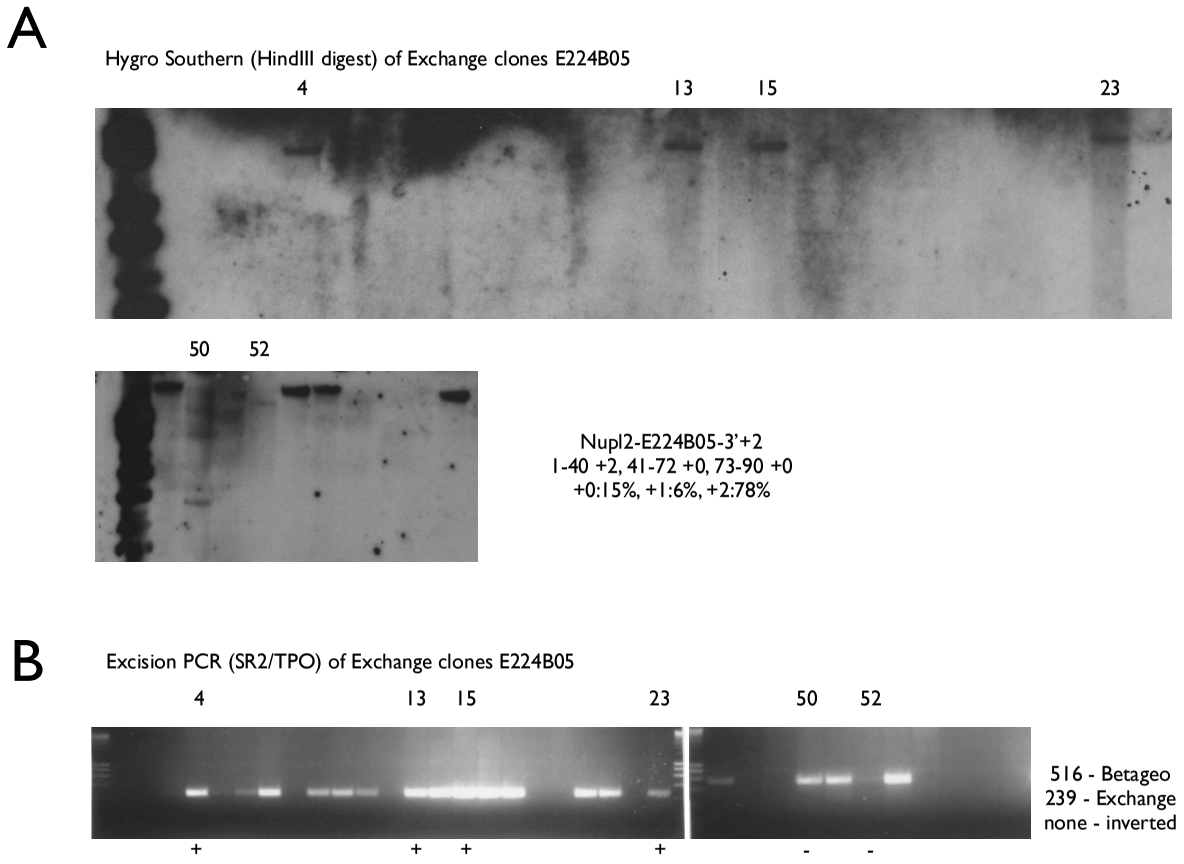


Figure 5.28: *Southern Blot and PCR screen screen of Nupl2 exchange clones*
(A) Exchange clones of three electroporations of Nupl2 clones (E224B05) were screened by Southern blot analysis to verify PCR results. Clones 4, 13, 15, 23 (from pEX-Flp +2 electroporation) showed correct Hygromycin band and also showed the exchange band of 239 in PCR screen (B). Clones 50 and 52 (from pEX-Flp +1 electroporation) showed in Southern screen multiple bands, which corresponds with βgeo band in PCR screen (-). This showed reliability of PCR results and importance of matching frame to receive positive exchange clones in this case, since Nupl2 clone harboured gene trap insertion in frame +2.

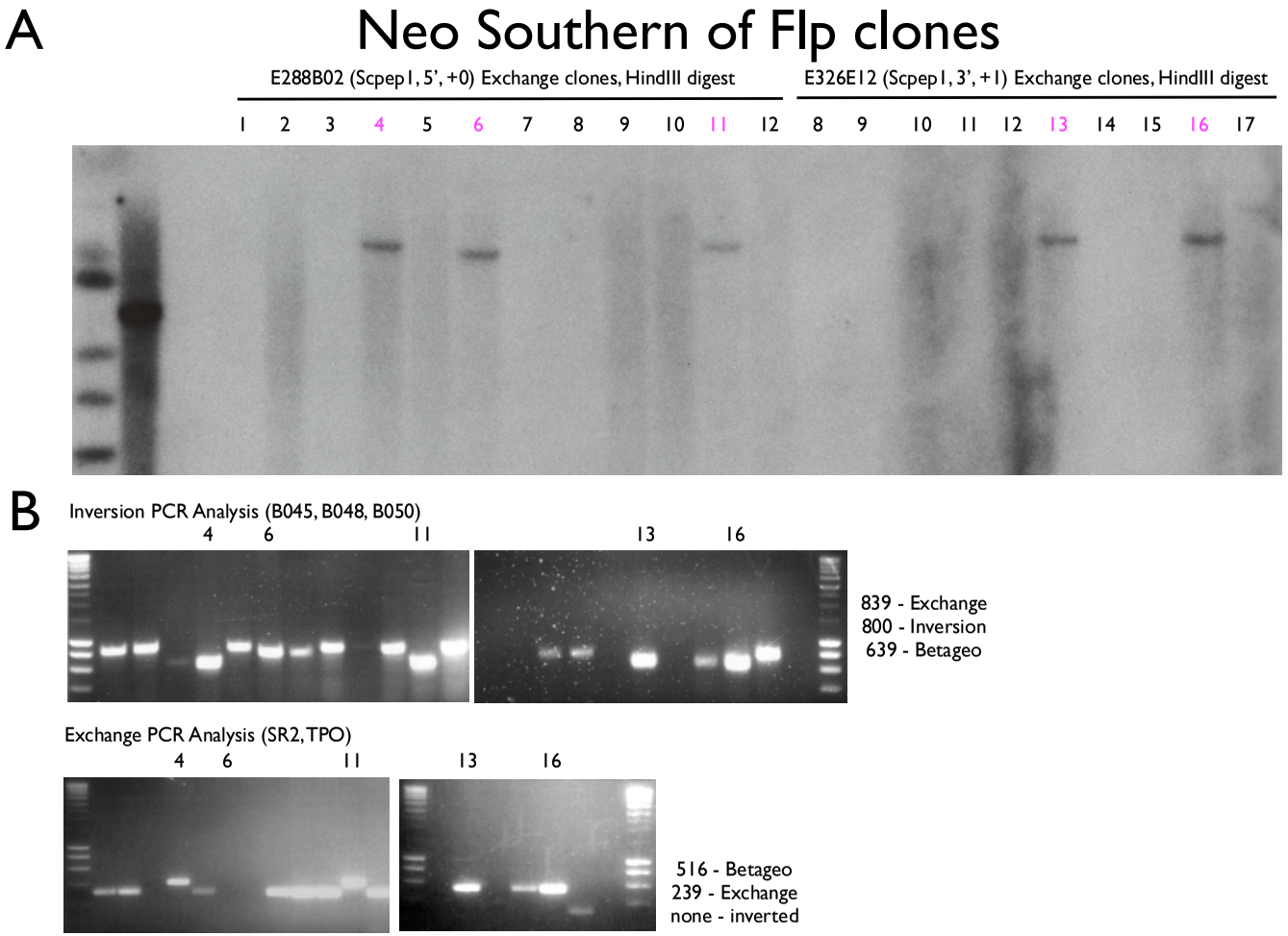


Figure 5.29: *Southern Blot and PCR screen of Scpep1 exchange clones*
An assortment of exchange clones of both electroporations of Scpep1 clones were screened by Southern blot analysis using a neomycin probe to confirm PCR results (A). Only clones 4, 6, 11, 13 and 16 showed a band, matching with PCR results (B), clones were still harbouring the βgeo insertion in original (4, 11, 13, 16) or inverted (6) orientation. Correctly exchanged clones show no neomycin band and the 239 bp band in the PCR.

results were averaged.
Insertions in the same gene were either chosen for different gene trap vector reading frames or different introns (Ahdc1, Msi2, Etl4, Scpep1), to determine possible differences in exchange efficiencies between 5′ and 3′ insertions in the same gene and between different reading frames of pEX-Flp. Cell number and DNA amount per electroporation were similar in all cases to get comparable results.
The efficiencies of RMCE were calculated by dividing correctly exchanged clones by total picked clones for each electroporation. Of the gene trap insertions used for exchange reactions, seven were in frame 0, four in frame +1 and two in frame +2, ten in more 5′ introns and 3 in more 3′ introns of the respective gene (overview of efficiencies, genes and insertion introns indicated in Figure 5.30). Exchange efficiencies of the matching frame of pEX-Flp to the corresponding genetrap insertion are indicated in red numbers.
Total clone number of all 38 electroporations varied from less than 10 clones in 17 cases up to several hundred clones per plate, average clone number per electroporation was 124. The exchange efficiency of the matching frame varied from 0 to 93 % and was 40 % in average. The efficiency of both nonmatching frames per clone varied between 0 and 100 %, but the average efficiency was only 25 %.
In 8 out of the 13 different electroporated genetrap clones, the matching frame also worked most efficient, while in 5 cases a nonmatching frame did.
In five cases (Etl4 3′, Fnbp1, Scpep1 5′ and both Msi2 insertions), only one electroporated pEX-Flp frame led to hygromycin resistant clones, in four out of these, it represented a frame matching the b-geo insertion. The only exception was the Msi2 insertion in intron 2, where only frame 0 yielded positive clones, while the βgeo insertion was in frame +1.
A summary of all electroporations and results is shown in Table 5.1

5.5.1.1 *In vitro* Excision of Selection Marker

All further screens were performed using the successfully exchanged clone A03 (Fig. 5.27 B) derived from the E307D01 (Msi2) gene trap clone. To minimize secondary effects, the selection marker of pEX-Flp vector was designed removable by Cre recombinase in vitro or in vivo.
To demonstrate excision in vitro, clone A03 was transiently transfected with Cag-Cre-IRES-Puro plasmid with and without subsequent puromycin selection. Genomic DNA of plates without selection were pooled and screened by PCR with primers located within the splice acceptor sequence (hygromycin and dsRed) and in the LTR. This PCR yielded different sized products prior to (239 bp) or after (619 bp) excision of the selection marker (Fig. 5.31 A). Lanes marked 1-5 (pooled transfected plates) show both

Clonename	Gene	Insertion	Frame	LacZ original	Expression	Frame pEX-Flp	total clones	picked clones	Efficiency %
E079H11	Gtf2ird1	5' (2 von 30)	0	blue	62	0	27	11	54.55
						1	19	6	66.67
						2	7	3	100.00
E068C09	Etl4	3' (5 von 16)	0	blue	1770	0	24	16	12.50
						1	8	4	0.00
						2	13	5	0.00
E311D09	Etl4	5' (2 von 16)	1	white	1770	0	25	18	27.78
						1	15	6	50.00
						2	16	8	62.50
E224B05	Nupl2	3' (5 von 6)	2	white	249	0	430	34	14.71
						1	120	16	6.25
						2	2080	40	77.50
D045A10	Tardbp	5' (1 von 5)	0	blue	328	0	45	22	45.45
						1	22	10	40.00
E287F07	Fnbp1	5' (1 von 17)	0	blue	165	0	40	4	25.00
						1	2	1	0.00
						2	4	2	0.00
E326E05	Msi2	5' (2 von 14)	1	white	39	0	122	16	87.50
						1	0	0	0.00
						2	5	0	0.00
E307D01	Msi2	5' (4 von 14)	0	blue	39	0	128	20	55.00
						1	2	0	0.00
						2	6	1	0.00
E326E12	Scpep1	3' (10 von 12)	1	white	1186	0	24	13	7.69
						1	5	1	0.00
						2	8	3	33.33
E326E12	Scpep1	3' (10 von 12)	1	white	1186	0	48	18	11.11
						1	7	4	0.00
						2	8	2	50.00
E288B02	Scpep1	5' (1 von 12)	0	white	1186	0	95	32	68.75
						1	4	1	0.00
						2	5	2	0.00
E288B02	Scpep1	5' (1 von 12)	0	white	1186	0	240	24	41.67
						1	6	1	0.00
						2	11	5	20.00
E224H09	Ahdc1	5' (2 von 6)	2	blue	100	0	917	32	75.00
						1	396	32	78.13
						2	224	32	93.75
E326C04	Ahdc1	5' (2 von 6)	1	blue	100	0	25	4	25.00
						1	6	3	0.00
						2	6	1	0.00
E284H06	Ahdc1	5' (2 von 6)	0	blue	100	0	145	27	77.78
						1	52	10	20.00
						2	77	11	9.09

Table 5.1: *Overview of all RMCE electroporations*
Shown is gene trap insertion (name, gene, frame of gene trap vector, insertion intron and relative position, lacZ stain result. Total clone number, number of picked clones and efficiency of RMCE are indicated for every frame of pEX-Flp that was electroporated. All electroporations were performed under similar conditions.

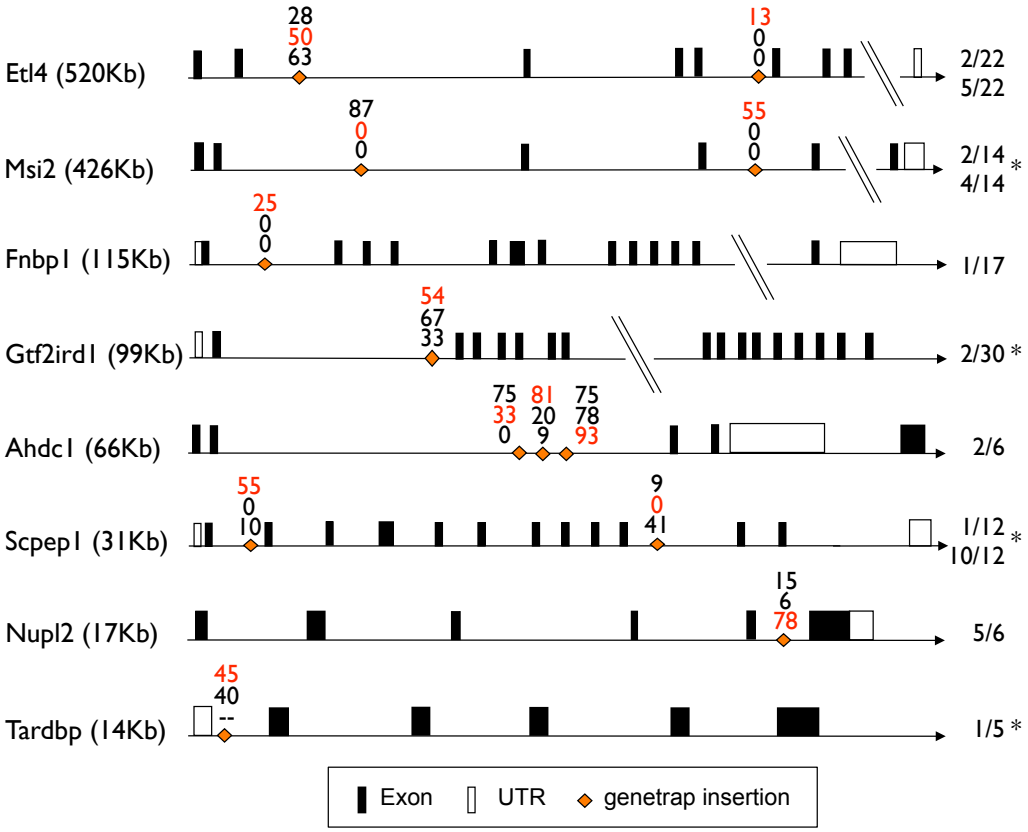


Figure 5.30: *Overview of Flp RMCE efficiencies*
Schematic overview of the efficiency of FlpO-mediated RMCE in FlEx gene trap ES cell clones. Shown are all transfected gene trap clone insertions and the corresponding rounded efficiency of exchange reaction of pEX-Flp in three different reading frames (0 upper, +1 middle, +2 lowest value in percent, red values indicate the frame matching the gene trap insertion). All gene trap clones used had insertions in an intron of the respective gene, the ratio on the right indicates in which intron out of how many total introns the insertion is located. On the left, the size of the genes is indicated in kilobases, the total size of the genes is not drawn in scale, indicated by double lines. (Etl4, Gtf2ird1, Ahdc1, Nupl2 and Tardbp electroporations were done by H. Grunert and C. Wolf)

band sizes, untransfected A03 only showed the smaller product. Without puromycin selection Cre excision occurred only partially.

To ensure the splicing to the dsRed cassette after Cre excision, the same transfection was performed followed by puromycin selection and cDNA of individual clones was screened by PCR with primers located in Msi2 exon II and an internal primer in the dsRed coding sequence (Fig. 5.31 B). This primer set yielded a 550 bp product in three independent clones (lanes 1-3), which was sequence-verified.

As an internal control, a third primer located in Msi2 exon III was added, which yielded a 94 bp product of the wildtype transcript. As further controls genomic DNA of two gene trap clones (E307D01, E326E12) and the plasmid DNA of pEX-Flp was used. Gene trap genomic DNA templates

yielded the wildtype genomic product (427 bp) including the intron 3/4.

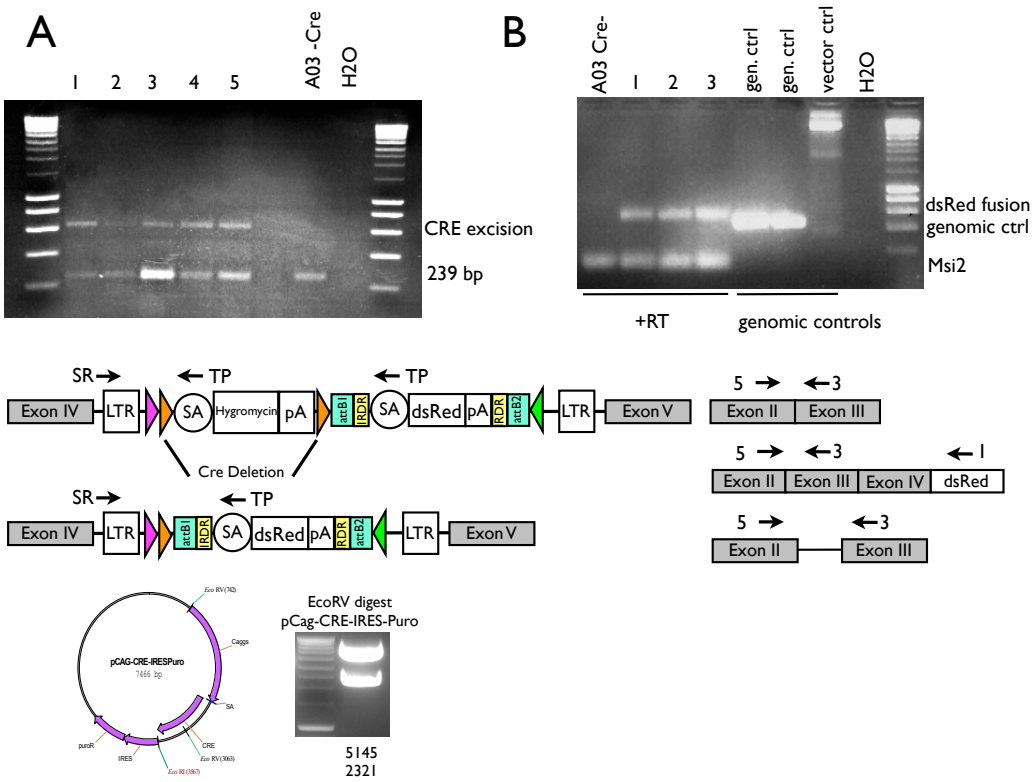


Figure 5.31: *Cre mediated deletion of selection marker of Exchange clones*
(A) Removal of hygromycin resistance upon Cre activity. Cre transfected exchange clone A03 (E307D01 derivative) was analysed using primers located within both the hygromycin/dsRed cassettes (TP) and the LTR of the original gene trap vector (SR). PCR on total genomic DNA of five pooled transfected dishes (lanes 1-5) compared to genomic DNA of clone A03 prior to Cre transfection (A03 Cre-). The internal primer (TP) gives rise to the larger product (619 bp) only after successful excision of the hygromycin cassette. Prior to hygromycin excision, primer TP amplifies a smaller product of 239 bp. (B) RT-PCR and triple PCR analysis to determine splicing to the dsRed after hygromycin excision by using external primers in exon II (5) of the insertion locus (Msi2) and an internal dsRed primer (I) and as internal wildtype control a primer located in exon III (3) of the Msi2 gene. cDNA of individual clones derived from Cre transfected clone A03 after puromycin selection (lanes labelled 1-3) compared to cDNA of clone A03 prior to Cre transfection (lane A03 Cre-). As control genomic DNA of two gene trap clones (E307D01, E326E12) and pEX-Flp plasmid were used.

5.5.1.2 Mobilisation of dsRed by *Sleeping Beauty* Transposase

Clone A03 (E307D01 derivate) was transiently transfected with different amounts (0, 10 and 70 μg) of a Cag-SB100 expression vector. Pooled genomic DNA of transfected plates was screened by PCR. SB100-mediated excision was analyzed by using two primer combinations. Primers were located either in the hygromycin coding sequence (H) or in the BGHpA of the dsRed (B048) cassette (which would be mobilized by SB100) and

with an external primer located 5′ of the F3 site (B045). Templates of plates transfected with 10 and 70 μg SB100 showed both bands, the SB-excision band with primers H/B048 of 1009 bp and the unexcised band of 839 bp with primers B048/B045, whereas the controls 0 μg and A03 without transfection (A03-SB) only yielded the 839 bp product. As further control the gene trap clone E307D01 was used as template and yielded only a 631 bp band with primers B048/B045 (Fig. 5.32). All PCR products were sequence-verified.
As further analysis pooled DNA of SB100 transfected cells was screened by SPLK PCR to screen for reintegration of excised transposon sequence.

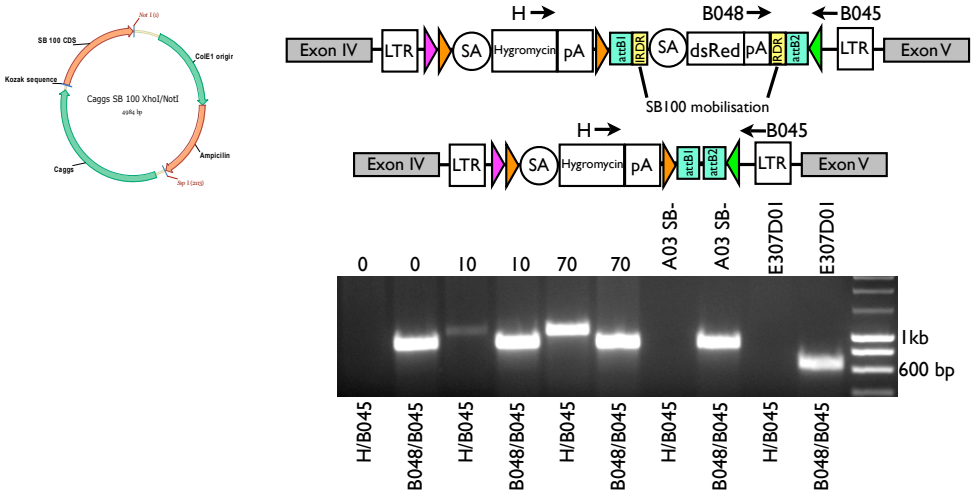


Figure 5.32: *SB100 mediated transposition of Flp exchanged vector*
PCR analysis of SB100 transfected exchange clone to determine mobilization of IRDR flanked cassette. Genomic DNA of pooled SB100 transfected dishes (0, 10 and 70 μg SB100 plasmid) of exchange clone A03 was screened with primers located 5′ of the F3 site (B045), in the pA of the dsRed cassette (B048) and in the hygromycin cds (H). DNA was screened with either primer combination H/45 or B048/B045, as controls genomic DNA of clone A03 (A03 SB-) and E307D01 was used. Primers H/B045 yielded a 1kb product only after successful excision of the IR/DR flanked cassette. Without mobilization, a 839 bp band was amplified by primers B048/B045, the original gene trap insertion led to a 631 bp band.

Chapter 6

Discussion

6.1 General Overview

The aim of this thesis was to establish a phenotype driven mutagenic *in vivo* screen based on transposon technology. Two different transposon systems, *Sleeping Beauty* and *piggyBac*, were chosen to reach that goal. Both previously demonstrated their usefulness in various applications and mutagenic abilities in different organisms (Clark et al., 2004; Izsvak and Ivics, 2005; Miskey et al., 2005; Mates et al., 2007).

In the present work, the transposase expressing vectors containing the Caggs promoter and the respective transposase, were successfully cloned into the C31 RMCE vector and inserted in the targeted Rosa 26 locus with high efficiency. The stable integration and the expression of the respective transposase enzymes were shown *in vitro* as well as *in vivo* in the established mouse lines. In total, three different transposase expressing mouse lines (SBM3a, iPB and mPB) were successfully generated and bred in various different schemes.

The transposon constructs were designed, cloned and successfully integrated into ES cells either by linearized transfection or transposase mediated integration and transgenic mouse lines with stable integrations were generated (TPOPS, XLbetageo and XLbetageoOpsVen).

A Flp-mediated integration of the transposon into conditional gene trap insertions did not work from the start, which led to the secondary project, the establishment of an efficient protocol for Flp mediated RMCE for multipurpose applications. Therefore a minimal transposon vector was designed and efficiently exchanged into various different conditional gene trap clones.

Also subsequent experiments with successfully exchanged clones were done, including the single copy mobilization of the minimal transposon construct by transient SB100X transfection and Cre excision of the selection cassette, which led to expression of the introduced sequence by the endogenous promoter.

6.2 Flp-mediated Cassette Exchange

It could be demonstrated in this work that Flp-mediated exchange of any given sequence with conditional gene trap clones can be highly efficient. Several different conditional gene trap clones, flanked with engineered and wild type RRS for Cre and Flp recombinases, were co-electroporated under optimized conditions with FlpO expression plasmid and the exchange vector pEX-Flp. In this work not only total efficiency of RMCE was investigated, also insight on the importance of matching reading frames and the role of the insertion site were gained.
Based on the results of this work, Flp-mediated RMCE opens up the door to the modification of a whole conditional gene trap library of ES cell clones, and every single insertion represents a potential target to integrate any cDNA of choice.
An universal two component vector system was generated, based on the advantages of one-step Gateway reactions instead of laborious cloning techniques. An exchange vector was designed that contained a selection marker and a Gateway cassette, so that any desired cDNA can easily be introduced. Additional features like loxP sites that flank the selection marker were added, to enable further modifications after RMCE.

6.2.1 Efficiency of Flp-mediated RMCE

The typical exchange efficiencies ranged between 50%-70% with a clear preference towards 5′ integrations (mainly in intron 1 of a gene). This agrees with the insertion preference of promoter-trap vectors in large-scale screens (Floss and Wurst, 2000; Hansen et al., 2003).
Initial attempts to establish the RMCE were unsuccessful and the reason for these problems were the low Flp amounts used. At first 50 μg total DNA per electroporation were inserted, only 1-30 μg of FlpO plasmid were used and basically no successful exchange could be accomplished. Only by repeating the exchange transfections and changing the variables continuously, the final efficient protocol could be established. The crucial factor is a high amount of Flp recombinase (70 μg), whereas exchange vector size or amount played a minor role for efficient RMCE.
This reflects the comparably low efficiency of Flp recombinase in general, which shows only 10% of Cre activity. The predecessor of the codon optimized and thermo-stable FlpO (Raymond and Soriano, 2007) has three times elevated efficiency compared to Flpe (Buchholz et al., 1996). But Caggs promoter (Flpe plasmid) has a three-fold activity compared to PGK promoter (FlpO plasmid), so overall activity of both available versions should be the same (A. F. Stewart, personal communication).
Cobellis et al., 2005 used only 10 μg Flpe plasmid per reaction in their RMCE co-transfections and were also able to reach about 70% exchange efficiencies. One obvious reason for that is the multifunctionality of FlEx

gene trap vectors. Prior to the intended exchange, Flp can invert the gene trap vector and excise the resulting head-to-tail flanked Cre RSS. In only in a limited amount of cases, the exchange reaction will take place without any further inversions and/or deletions (see Fig. 6.1).

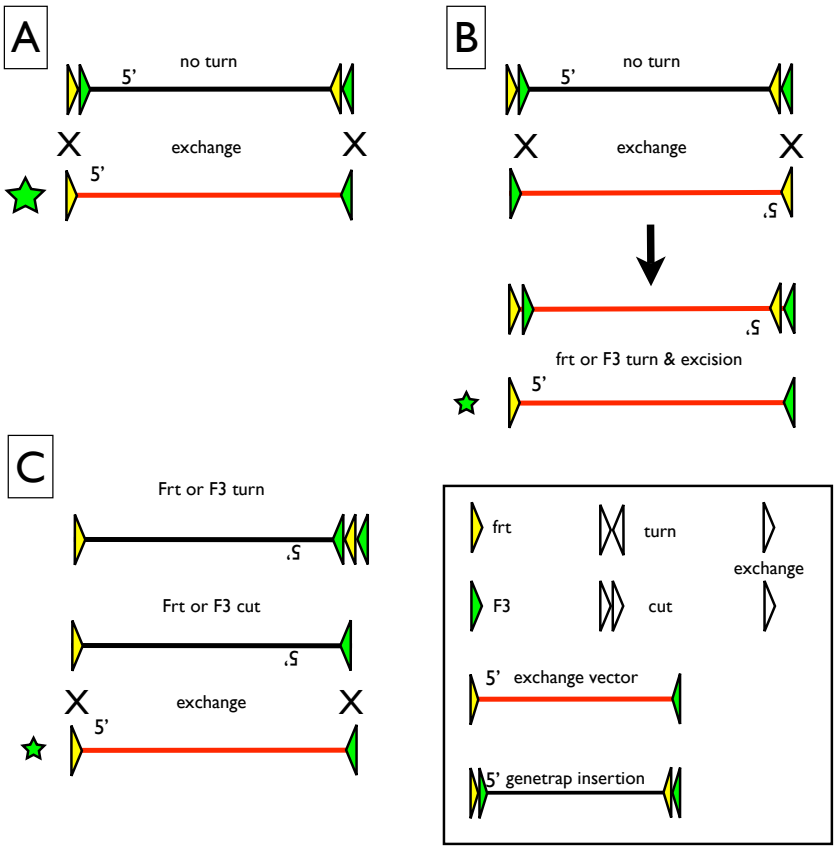


Figure 6.1: *Possible Flp mediated events after RMCE co-transfection.*
Flp recombinase can invert, excise or exchange sequences that are flanked by either F3 or Frt sites. Flp can mediate various reactions with flanked sequences in FlEx array, depending on their orientation towards each other. (A) Shows the correct integration of the exchange vector with the outermost F3/frt sites. (B) Shows exchange in opposite orientation (RMCE with inner F3/frt sites) and requires subsequent inversion to lead to functional integration. (C) Shows exchange reaction after prior inversion and excision event of the gene trap vector. Successful exchange events are marked with green stars, size of star indicates chance due to possible prior Flp reactions. Gene trap insertion indicated in black, exchange vector in red.

Three different cases are possible. The first is the correct exchange reaction between 5′ frt and 3′ F3 of both vectors (A). The second is the successful exchange, but in inverse orientation (with 3′frt and 5′F3 of the gene trap insertion), which leads to two remaining RSS at both ends of the vector instead of only one. Subsequent inversion and excision will lead to a stable integration and a correctly oriented vector. Case three is the prior inversion of the gene trap insertion and excision of RSS before the exchange reaction takes place. This wide range of possibilities and the thermodynamic

preference of each reaction (Baer and Bode, 2001) might lead to comparable efficiencies despite much higher DNA amounts used here.

6.2.2 Reliability of RMCE

One concern arose because of the broad range of clone numbers between single electroporations (Nupl2, Ahdc1) with respect to others, which led to much more resistant clones than the average. The question came up if unspecific secondary integrations of pEX-Flp might have occured. Therefore, 90 hygromycin resistant clones were screened by Southern blot analysis using a hygromycin probe, but only 2% of clones showed secondary insertions.
The gene expression level of each trapped gene that was used was checked by using a recently published Affymetrix Chip Array data set (Nord et al., 2007), to see eventual connections between exchange efficiency and absolute gene expression values, but no obvious correlation could be determined (see Table 5.1). In an assortment of successfully exchanged clones, the loss of the neomycin and lacZ marker were checked by Southern blot analysis using a neomycin probe, results showed that the here established PCR screens are highly reliable.

6.2.3 Importance of Frame

Overall exchange results surprisingly showed the small influence of the reading frame of the exchange vector. Gene trap vectors are generated in three different reading frames and since they trap a endogenous transcript, only one frame should result in a fusion transcript after insertion in the intron. The assumption was therefore that only an exchange vector in the matching frame to the gene trap insertion could lead to resistant ES cell colony. All conditional gene trap clones were electroporated with all three reading frame exchange vectors to prove this hypothesis. Surprisingly the matching frame did not have the expected importance.
Only the minority of gene trap clones could be successfully exchanged with only one electroporated reading frame. The major amount of electroporations led to resistant colonies and successfully exchange reactions with all of the frames. This effect is based on various possibilities.
One likely reason could be the initiation of translation starting at the Kozak site of the hygromycin resistance cassette after exchange with a 5′ integration and a untranslated first exon, like in the Tardbp gene trap. Additionally the role of alternative promoters could lead to resistant clones even after exchange of a non-matching reading frames. For about 50% of all human and mouse genes these alternative promoters were predicted recently, but resulting fusions with endogenous peptides can not be predicted (Baek et al., 2007).

A closer look at current conditional gene trap insertions supports this theory. 3794 genes (5220 different introns) are FlEx trapped at the moment (J. Hansen, personal communication), taking only the longest transcripts into account, the number of introns that have an insertion is 3684. Out of these trapped introns, 2195 (60%) are trapped by one frame, 40% are trapped by two or even three different gene trap vector frames. These multiple traps occur with much higher chance in 5′ regions of endogenous genes, over 80% of multi-trapped introns are in 5′ regions, 40% of the uniquely trapped are in 3′ regions.
These facts point out the less important role of the reading frame in 5′ regions of genes if Kozak-containing vectors are used, which can be confirmed by the results of this work.

6.2.4 Subsequent *in vitro* Approaches

6.2.4.1 Cre-mediated Deletion

Since this approach not only offers the opportunity to integrate any sequence into defined locations very efficiently, but also should offer the possibility for further modifications, two subsequent experiments with exchanged clones were performed.
The removal of the selection marker seemed not only reasonable because of eventual effects on surrounding areas, also the resulting expression of the introduced sequence driven by the endogenous promoter of the trapped gene needed to be shown.
Therefore Cre excision of the flanked marker gene was performed by transient Cre expression *in vitro*. It could be shown that the deletion of the marker gene was highly efficient and the resulting expression of the inserted sequence (SA-dsRed-pA) driven by the endogenous promoter was shown by subsequent RT-PCR (Msi2 gene). This excision can also be performed in vivo, by breeding with Cre expressing mouse lines to the mouse line received from the exchange clone. The Cre deletion can provide additional possibilities for modification, by choosing tissue specific or inducible Cre mouse lines.

6.2.4.2 *Sleeping Beauty* Mobilization

The sequence introduced in this work was flanked by SB binding sites to perform mobilization of the sequence in neighboring locations. Therefore a transient SB100X transfection was performed *in vitro*, to check for mobilization of the introduced sequence. It could be shown that SB100X was able to excise the single copy transposon out of its location by PCR screens. This feature not only provides the possibility of excision, but also of reintegration into another locus *in vitro* or *in vivo*.

As pointed out in the introduction, the additional transposon system compatibility can be used to generate allelic series of mutations in critical loci for human disease *in vitro* and *in vivo*. However, one should clearly take into account which transposon system is best suited for a given quest.

6.2.5 Summary

The RMCE studies performed throughout this work point out a whole new approach for conditional or non-conditional gene expression, that is only restricted by the availability of FlEx gene trap clones.
It represents a highly efficient method to generate transgenic lines, in a directed single-copy manner and therefore circumvents epigenetic silencing because of high copy number integration (Bingham, 1997). It further offers information about gene expression in advance and also allows the replacement of upstream transcriptional regulators with potential target gene.
But most strikingly it offers the possibility to replace mouse genes with the human counterpart in wild type or mutated variations on a transcriptional level, which can play a crucial future role in understanding human disease and establishing mouse models for further research. The GGTC FlEx library already contains more than 620 independent candidate genes for human genetic diseases and the already available Cre mouse line library presents a manifold spectrum of more detailed modifications.

6.3 *In vivo* Transposon-based Mutagenic Screen

6.3.1 *Sleeping Beauty M3a*

The generated SBM3a mouse lines have shown their in vivo activity and germline transposition competence in crosses with the T2/onc harboring mouse line, which carries a concatemer transposon integration. Offspring of double transgenic mice showed cut-and-paste events as determined by Southern blot analyses.
The splinkerette PCR screen for reintegration site localization could be reproduced and chromosomal locations could be determined successfully. Surprisingly neither could the described local hopping preference be observed for M3a in this work, nor was any hyperactive efficiency seen in side-by-side comparison with the SB11 mouse line. To the contrary, SB11 showed at least three times more transposition bands in Southern blot analysis than SBM3a. According to unpublished results of Z. Ivics, M3a should be at least 30% more efficient than SB11.
Since the TP donor mouse line was identical, the transposase expressing mouse line must be responsible for differences in efficiency. One explanation could be overexpression inhibition, due to the strong Caggs promoter

(SB11 expression is driven by the endogenous Rosa26 promoter). Another clue for the "hidden" hyperactivity of *Sleeping Beauty M3a* could be multiple jumps of the same copy and not necessarily the excision of many copies out of the concatemer. Multiple hops could also mask the local hopping preference, which was noticed in various independent studies.
The TP-OPS transposon could not be mobilized *in vivo* at all, neither SB11 nor SBM3a could manage the transposition, although the entire transposon had been inserted. Multiple factors like increased size, lack of methylation and singe-copy integrations are probably reasons for that.

6.3.2 *PiggyBac*

The mutagenic screen of iPB expressing mice, which harbor the XLbetageo transposon, did not lead to transposition events yet, however, only a very limited number of offspring could be screened by now, therefore the efficiency of this screen is not certain to date.
The mPB screen has not yet started, but transgenic transposase expressing mice are viable and mPB efficiency was tested highly efficient *in vitro*.
The XLbetageoOpsVen mice that contain the *in vivo* detection marker were generated, but not crossed yet with PB expressing lines.
A transposon-based mutagenic phenotypical *in vivo* screen can still be established with mPB and single copy integration of XLbetageoOpsVen. Both mouse lines are generated and the reported high efficiency and fewer limitations of *piggyBac* transposase can make the goal of this work still achievable. Also the presence of the *in vivo* detection marker will then lead to an easy and fast identification of new insertions and mutations.

6.3.3 Comparison with Recent Experimental Studies

As mentioned in the introduction, many factors can influence transposition efficiency. Cargo size, methylation, concatemer integrations, expression level of the transposase and also host factors can inhibit cut-and-paste events. To analyze the probable reasons for the inefficiency, especially of the established SBM3a mouse line, the following questions should be taken into account.

- Is the transposon construct too large in size (4 kb; wild type SB: 2.3 kb)?

- Is the transposase expression too strong? (overexpression inhibition)?

- Is the copy number of the transposon too low (methylation missing)?

- Does *piggyBac* show the same limitations as SB *in vivo*?

These questions can only be discussed in the context of previous studies, the following table 6.2 is a brief summary of transposon screens done using SB, PB or both. It lists the organism (ES cell culture, human cells or in mice), the transposases used (and their promoter), the functional parts of the transposon and its size (if known), the arrangement at the donor site (single or multiple integration) and the observed efficiency of the system. The goals of these studies were different, some were focused on activity of the respective transposase during transient transfection, some during stable expression, some focused on reintegration preferences, some on the influence of certain factors, so it seems difficult to reduce all the results to a common denominator but it should be possible to integrate the own results into the picture to be able to optimize these in the future.

6.3.3.1 Limitations of the *Sleeping Beauty M3a* Screen

The first surprising fact is, that the only publication which could show single copy mobilization *in vivo*, is not only one of the oldest, but also used the most "inactive" version of the transposase (according to *in vitro* assays from Liang et al., 2009), the SB10 (Fischer et al., 2001).
All other *in vivo* transposon screens relied on transposon integrations in concatemer formation which, according to the results, appears more efficient due to methylation of the transposon sequences (Park et al., 2006) in the case of SB. The influence of methylation on the PB system was observed by Wang et al., 2008. They validated the enhancing effect on transposition of methylation in the SB system, but showed the opposite effect for PB.
Therefore one may wonder why methylation obviously played no role in Fischer et al., 2001? Another possible reason could be the Prm-1 promoter that drove transposase expression. This promoter is active during spermiogenesis in haploid round spermatids (Braun et al., 1989) and might be just strong enough to lead to an optimal transposase amount before overexpression inhibition occurs. The size of the used transposon was not mentioned explicitly, but was probably around 2 kb and therefore corresponded approximately the wild type size of the SB element (Plasterk et al., 1999).
According to Izsvak et al., 2000 (see Fig. 6.3), the transposon size of 4 kb used in this work would lead to a four-fold decrease of transposition efficiency. By only taking the cargo size into account, this should still lead to transposition events during germline in at least 5% of the offspring since Fischer et al., 2001, reported germline transposition of 20%.
The Caggs promoter which was used for transposase expression may result in overexpression inhibition and be the reason for such low efficiency, but Caggs was the most frequently used promoter in 7 other publications

Publication	Organism	TS	Promoter	TP	Size kb	copies	Efficiency
Fischer et al., 2001	Mouse	SB10	Prm-1	SV40neo	<2	1	20 percent
Dupuy et al., 2001	Mouse	SB10	Caggs	rosa egfp	<3	many	2 new insertions per male gamete
Horie et al., 2001	Mouse	SB10	Caggs	Cag-GFP	4.8	20	1 per 1.5 cell
Dupuy et al., 2002	Mouse	SB10	RNA	pT/K14AG	4	many	no backcrossing
Horie et al., 2003	Mouse	SB10	Caggs	Cag-GFP	4.8	20	7% pos offspring
Carlson et al., 2003	Mouse	SB10	Caggs	rosa egfp		many	only reintegration preference
Keng et al., 2005	Mouse	SB10	Caggs	lacZ/GFP	4.5	100	1.1 transposition per GFP pos. mouse
Geurts et al., 2006	Mouse	SB10	Caggs	tTA	<2	30	3 events per gamete
Luo et al., 1998	ES	SB10	CMV, 50µg	SV40neo	<2	1	0.000035 events per cell
Fischer et al., 2001	Hela	SB10	Prm1	SV40neo	<2		10 times more than other Tc1
Wu et al., 2006	Human cells	iPB/SB11	CMV	SV40Hygro	<2		iPB 2-3 fold more efficient
Wilson et al., 2007	HEK	iPB/SB12	CMV	Kan/Neo	3.1		iPB 2 times more efficient
Wang et al., 2008	ES	iPB/SB11	Caggs/Rosa	PGK-Neo	<2		iPB 2-3 tmes more efficient
Liang et al., 2009	ES	PB/SB	20µg	3 exons	1.2	1	sb100 25x sb11, iPB 10x sb100
Ding et al., 2005	Mouse	iPB	Prm-1	RFP	<2	8	1.1 new insertions per gamete
Wu et al., 2007	Mouse	iPB	Prm-1	bgal egfp	>4	many	1 new insertion per offspring
Schebelle	Mouse	SBM3a	Caggs	dsRed/Venus	4	2	no transposition events
Schebelle	Mouse	iPB	Caggs	betageo	4	1	no transposition events

Figure 6.2: *Summary of an assortment of publications about transposon screens.*
Listed are the organism tested, the used transposase and its promoter, the transposon sequence and its size, the copy number of donor insertions and the efficiency of transposition observed. The last two listings show the used conditions and results of this work.

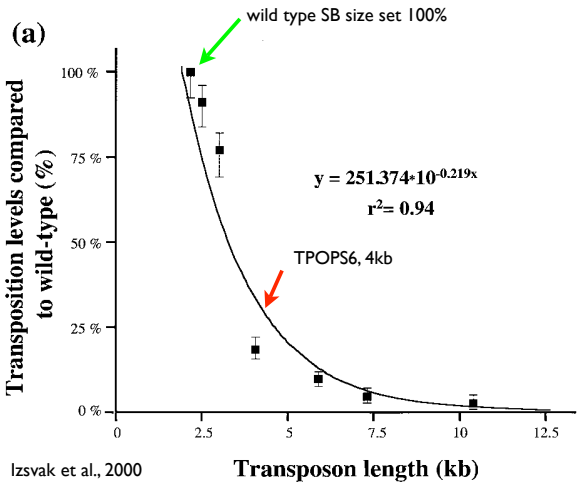


Figure 6.3: *Dependence of transposition efficiency on transposon size in SB system. Transposition efficiency of a 2.2 kb cargo was set 100% (green arrow). Different sized transposons were co-transfected with CMV-SB expression plasmid in human HeLa cells. With every additional kb, transposition efficiency dropped about 30%. Size of used transposon in this work is indicated with red arrow. (Izsvak et al., 2000)*

in vitro and *in vivo* and these studies could show transposition events. Hence, overexpression inhibition can more or less be ruled out as the main reason for inefficiency. The only difference left is that all other studies relied on concatemer integrations of the transposon, which seems to close the circle again.

In case of SBM3a under similar conditions as described here, the resulting inefficiency is definitely a cocktail of all suppressing factors taken together. Since concatemer integrations do not appear appropriate for this screen, the only factor left that could be optimized is the size of the transposon.

The size could be reduced by choosing different marker cassettes, which still would fulfill their assignment, the disruption of genes to cause mutation and the *in vivo* detection of cut-and-paste events, to easily screen the mice. Since all the transposase expressing mouse lines are established already, by generating new transposon harboring mouse lines harboring a smaller transposon, the goal of an efficient mutagenic screen can still be reached.

Two other studies showed single copy mobilization *in vitro*. Liang et al., 2009 and Luo et al., 1998, could mobilize single copy transposons with different versions of SB and PB. Both studies transfected transposon harboring cell lines with high amounts of transposase and selected on excision events, not primarily on reintegration. This selective pressure could lead to higher transposition efficiency, also the high transposase amounts of 20 μg and 50 μg respectively may have played crucial roles.

6.3.3.2 Limitations of the *piggyBac* Screen

In all studies PB showed higher transposition efficiencies and lower limitations caused by cargo size and transposase expression level. PB even showed the contrary effect caused by methylation of the transposon sequence, SB could increase its efficiency 9-fold, PB efficiency was decreased 12-fold (Wang et al., 2008). Single copy mobilization should therefore be possible more easily, even with larger cargo (Ivicz et al, 2009). PB never showed single copy mobilization *in vivo*, but could efficiently show its *in vivo* capabilities in a concatemer arrangement of the transposon (Ding et al., 2005).
Results about iPB of different studies were not uniform, efficiency seemed to be 10 times more efficient than SB100X (which is 25 times more efficient than SB11) according to Liang et al. (2009), but other works showed only a 2-3 fold increase of efficiency (Wilson, Wang and Wu). One reason for this wide span could be the selection pressure, which arose by selecting on excision out of the HPRT locus, also the high amount of transposase (20 μg per electroporation) could lead to overexpression inhibition of SB, but not PB. A third factor was be the comparably small transposon size of 1,2 kb that might also result in increased efficiency.
In this work, the XLbetageo single integrated transposon (4kb) could not be mobilized so far by iPB transposase. A higher amount of offspring should be screened, to finally answer the question whether iPB is efficient *in vivo*. The XLbetageo (with and without the *in vivo* detection cassette) transposon was integrated successfully by the transposase *in vitro* and soon these mouse line can also be bred with the optimized mPB, which can be assumed to function more efficient.

6.3.3.3 *In vivo* Detection

The qualification of the used *in vivo* detection marker, the opsin driven venus fluorescence gene, could not be verified by now. Transgenic mice with a single copy integration of the cassette on chromosome 8 showed no detectable fluorescence in their eyes so far. The reason for that could be the pigmented retinae of agouti mice, which may mask a visible signal of the venus protein. Another possible reason for lack of fluorescence detection could be the nonsense-mediated mRNA decay (NMD). PolyA-trap insertions mainly occur in 3′ regions of genes, since integrations in 5′ regions most often activate the NMD, which prevents the expression.
The integration of the internal ribosome entry site (IRES) into the cassette should have prevented the activation of the NMD, even in 5′ integration sites (Shigeoka et al., 2005). But the IRES may still not work 100% efficient, so in case of 5′ integrations, NMD could lead to a significant lower expression of the *in vivo* detection marker and therefore further complicate the detection. Another possibility to optimize *in vivo* detection would be to

use the Caggs promoter for venus expression, which could then rule out difficulties arising from pigmented retinae and lead to a ubiquitous expression of the detection marker as in previous studies (Horie et al., 2001; 2003).

6.3.3.4 *In vitro* Application

By transposase-mediated integration of the PB transposon randomly in ES cells, the high efficiency of mPB was proven. Also by using various amounts of TS and TP, insights could be gained which proportion of TP and TS are optimal to get stable single integrations of the transposon. By using splinkerette PCR techniques for the fast and precise localization of the insertion, a big library of transposon-mediated gene trap clones could be established. Since *piggyBac* excises exactly and does not leave any footprints behind, the integration is not the end, the sequence is removable by retransfection of transposase. This feature has been used recently to produce transgene-free induced pluripotent stem cells (Stadtfeld and Hochedlinger, 2009).

6.4 Perspectives of Mouse Insertional Mutagenesis

The functional annotation of every gene is definitely highest priority in mouse genetics and one way to achieve this goal is by causing mutations. Insertional mutagenesis plays a crucial role to gain more insight into function and phenotype of genes and various different approaches are currently used (see Fig. 6.4).
Actual methods can be divided in targeted or random insertional mutagenesis. Targeted mutagenesis inserts a mutation specifically in a determined locus, methods which follow this idea are gene targeting via homologous recombination, the Velocigene BAC vector system to generate knock-outs and the use of MICER vectors to generate duplications (Carlson and Largaespada, 2005). All gene targeting methods are limited to *in vitro* application, which has generally been a disadvantage in insertional mutagenesis. The advantages are clearly the mutation of a target gene, so a defined mutation can be introduced and one can focus on genes related to human disease.
The second group is random insertional mutagenesis, like gene trap technology (Stanford et al., 2001). Gene trap is a highly efficient method to mutate active genes in ES cells and due to its randomness, a saturated mutagenesis was the goal. A negative side effect of the use of splicing signals in the gene trap vectors is the resulting bias of integration. The retrovirus delivery shows a clear 5′ integration preference and integration hotspots

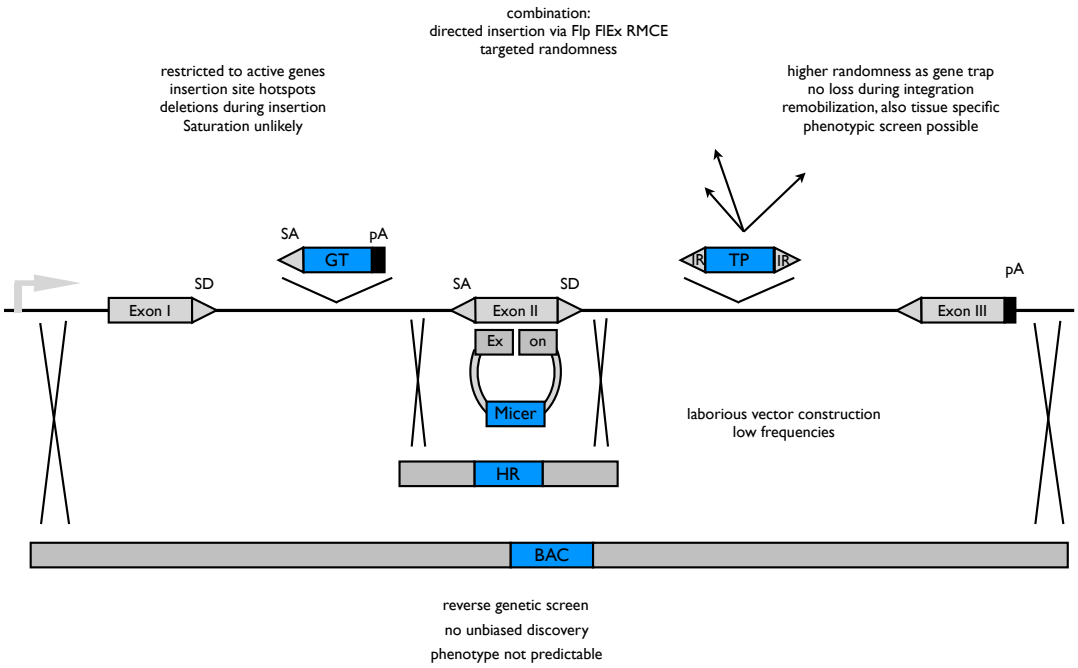


Figure 6.4: *Overview of insertional mutagenic screens.*
Shown is a schematic endogenous locus with three exons. In the lower half, the gene targeting approaches are shown, which all rely on homologous recombination (HR). The upper half shows the random techniques like gene trapping (GT) and transposon mutagenesis (TP). Advantages and problems of approaches are indicated briefly. SD: Splice Donor, SA: Splice Acceptor, pA: polyadenylation signal. Blue boxes represent mutagenic sequence which is inserted.

(Bushman, 2003), so by now the trapping of new genes has declined, even if this technology is much less laborious than gene targeting.

Another random screen is transposon-based insertional mutagenesis. The advantage as compared to gene trapping is the higher randomness of insertions. Transposon systems like *piggyBac* or *Sleeping Beauty* show nearly no insertion preferences and also the risk of sequence deletions during insertion is lower than by retroviral transfection. One striking feature of a transposon-based insertional mutagenesis is clearly the continuance of mobility. *In vitro* or *in vivo* expression of transposase enzyme can excise or reintegrate the transposon sequence. This makes transposon mutagenesis capable for phenotype-driven screens, if germ line transposition works efficiently enough.

The most prominent idea behind the functional annotation of genes are clearly human genetic diseases. By trying to generate phenotypes in model organisms like mice, which show significant alikeness with symptoms known from human, the underlying genes can be identified and their function can be studied. This knowledge can then offer starting points for diagnosis or treatment. Candidate genes are known for a variety of human disease, and so a targeted approach seems reasonable. However

genes in human disease are often dominant which can not be predicted for the model organism, neither can the resulting phenotype be estimated. OMIM (Online Mendelian Inheritance in Man) shows a clear summary: Nearly 13,000 genes are known on the sequence level, but not even 400 of them have a known phenotype. So the importance of phenotype-driven approaches is clear and a transposon-based insertional screen has many features to play an important role in reaching that goal.
Transposons can reinsert randomly or with local hopping preference, to either cause random mutations or with a higher chance mutations in a target gene networks or critical regions. Also the donor site can be established randomly or directed, as this work showed very impressive.
All these approaches have their inherent advantages and difficulties, by combining them, a insertional mutation coverage of the genome should be achieved in the future.

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Appendix A

Abbreviations

A	purine base adenine
Ac	acetate
adSA	adenoviral splice acceptor
ATP	adenosine triphosphate
AMP	ampicilline
attB	attachment site in the donor vector (RMCE, C31 Integrase)
attP	attachment site in the acceptor sequence (RMCE, C31 Integrase)
attL	attachment site in the donor vector (Gateway system)
attR	attachment site in the acceptor vector (Gateway system)
βgeo	β-galactosidase/neomycinphosphotransferase
BCA	bicinchoninic acid
BGH	bovine growth hormone
bp	basepair
BSA	bovine serum albumin
°C	degree Celsius
C31Int	integrase from phage ϕC31
C	pyrimidine base cytosine
CAGGS	β-actin promoter with CMV IV enhancer upstream
CaCl2	calcium chloride
ccdB	ccdB protein is a poison of the DNA-topoisomerase II complexes
cDNA	complementary desoxyribonucleic acid
Ci	Curie; $1\text{Ci} = 3.7 \cdot 10^{10}\text{Bq}$
CMP	Chloramphenicole
CNS	central nervous system
CO2	carbon dioxide
cpm	counts per minute
CRE	causes recombination
Da	Dalton

DEPC	diethylpyrocarbonate
DH5α	E.coli strain
DMEM	modified Eagle Medium after Dulbecco
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotide triphosphate
dsRed	red fluorescent protein from Discosoma spec.
dsRNA	double stranded RNA
DTT	1,4-dithiothreitol
EB	elution buffer
E.coli	Escherichia coli
EF	embryonic fibroblast cells
e.g.	exempli gratia, for example
EDTA	ethylendiamintetraacetate
ES cells	embryonic stem cells
EtOH	ethanol
E14	ES E14 Tg2A.4 cells
F	Farad
FCS	fetal calve serum
Fig.	figure
Flp	Flippase
FlpO	optimized Flippase
FRT	Flp recombinase target
g	gram
G	purinbase guanine
GFP	green fluorescent protein
GGTC	german gene trap consortium
G418	geneticin
h	hour(s)
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HPRT	hypoxanthine phosphoribosyltransferase
Hygro	Hygromycine
IgG	Immunglobuline Class G
IRDR	inverted and direct repeat
IRES	internal ribosomal entry site
KAN	kanamycine
kB	kilo base pairs

kD	kilo dalton
l	liter
LacZ	β-Galactosidase
LB	Luria Broth
LIF	leukemia inhibiting factor
loxP	locus of crossing over of P1 phage
μ	micro (10^{-6})
m	milli (10^{-3})
M	molar (mol/l)
$MgCl_2$	magnesium chloride
min	minute(s)
mut	mutant
n	nano (10^{-9})
n	sample size
NaCl	sodium chloride
NaAc	sodium acetate
NEO	neomycin
nm	nanometer
no.	number
nt	nucleotides
OD	optical density
ON	over night
ORF	open reading frame
pA	polyadenylation signal
PB	piggyBac transposase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGK	phosphor glycerate kinase
PI	protease inhibitor
Puro	puromycine
RIPA	protein lysis buffer
RMCE	recombinase mediated cassette exchange
RNA	ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	rotations per minute
RRS	recombinase recombination site
rs	repaired spacer
RT	room temperature

RT-PCR	reverse transcription PCR
SA	splice acceptor
SAP	shrimp alkaline phosphatase
SB	Sleeping Beauty transposase
SD	splice donor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophorese
Ser (S)	Serine
Splk	splinkerette PCR
SSC	sodium saline citrate
SSR	site specific recombinases
T	pyrimidine base thymine
Tab.	table
TAE	tris acetate with EDTA
TBE	tris borate with EDTA
TBS	tris buffered saline
TBS(T)	tris buffered saline (with Tween)
TBV2	wild-type ES cell line from a 129S2 mouse embryo
TE	tris-EDTA
temp.	temperature
Tris	trishydroxymethyl-aminoethane
U	unit(s)
UTP	uracil triphosphate
UTR	untranscribed region of a gene
UV	ultraviolet
V	volt
Vol.	volume or volumetric content
wt	wild type
x	Symbol for crosses between mouse lines

Appendix B

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