理學博士學位論文

piggyBac Transposon 의 Imprecise Excision 에 의한 초파리 세피압테린 환원효소 돌연변이체 제작

Mutagenesis of *Sepiapterin Reductase* by Imprecise Excision of the *piggyBac* Transposon in *Drosophila melanogaster*

2013年 2月

서울大學校 大學院

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

Mutagenesis by transposon-mediated imprecise excision is the most extensively used technique for mutagenesis in *Drosophila*. Although *P*-element is the most widely used transposon in *Drosophila* to generate deletion mutants, it is limited by the insertion coldspots in the genome where *P*-elements are rarely found. The *piggyBac* transposon was developed as an alternative mutagenic vector for mutagenesis of non-*P*-element targeted genes in *Drosophila* because the *piggyBac* transposon can more randomly integrate into the genome. Previous studies suggested that the *piggyBac* transposon always excises precisely from the insertion site without initiating a deletion or leaving behind an additional footprint. This unique characteristic of the *piggyBac* transposon facilitates reversible gene-transfer in several studies, such as the generation of induced pluripotent stem (iPS) cells from fibroblasts. However, it also raised a potential limitation of its utility in generating deletion mutants in *Drosophila*. In this study, I report multiple imprecise excisions of the *piggyBac* transposon at the *sepiapterin reductase* (*SR*) locus in *Drosophila*. Through imprecise excision of the *piggyBac* transposon inserted in the 5′-UTR of the *SR* gene, I generated a hypomorphic mutant allele of the *SR* gene which showed markedly decreased levels of SR expression. My finding suggests that it is possible to generate deletion mutants by *piggyBac* transposon-mediated

imprecise excision in *Drosophila*. However, it also suggests a limitation of *piggyBac* transposon-mediated reversible gene transfer for the generation of induced pluripotent stem (iPS) cells.

Keywords: Transposon; *P*-element; *piggyBac* transposon; Imprecise excision; Mutagenesis; *Drosophila melanogaster*; Reversible gene transfer; Sepiapterin reductase

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ABBREVIATIONS

AR	aldose reductase
BH_2	7,8-dihydrobiopterin
BH_4	tetrahydrobiopterin
CR	carbonyl reductase
DHFR	dihydrofolate reductase
DHPR	dihydropteridine reductase
DSB	double strand break
dsRNA	double-stranded RNA
EMS	ethylmethane sulfonate
FRT	Flp recombination target
HDR	homology directed repair
H_2-NTP	7,8-dihydroneopterin triphosphate
HSP	heat shock protein
IR	inverted repeat
iPS cell	induced pluripotent stem cell
NHEJ	non-homologous end-joining
PAH	phenylalanine hydroxylase
PCD	pterin-4α-carbinolamine dehydratase
PD	Parkinson's disease
PKU	phenylketonuria
6-PTP	6-pyryvoyltetrahydropterin
REV	revertant
RFP	red fluorescent protein
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction

S.D.	standard deviation
SDS-PAGE	sodiumdodecylsulfate polyacrylamide gel electrophoresis
SR	sepiapterin reductase
SSA	single-stranded annealing
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
UAS	upstream activation sequence
UTR	untranslated region
VDRC	Vienna *Drosophila* RNAi center

I. Introduction

1. Mutagenesis methods in *Drosophila*

1-1. EMS mutagenesis

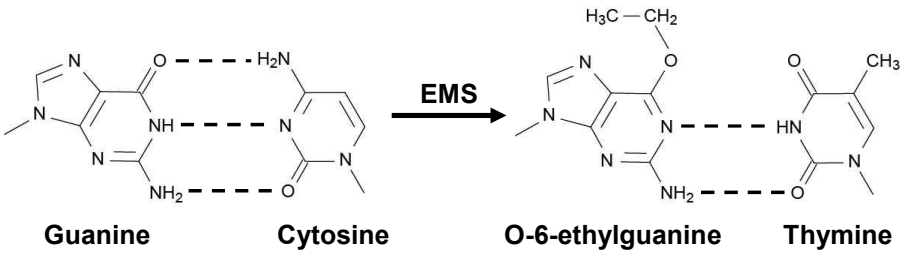
In 1968, Lewis and Bacher developed a *Drosophila* mutagenesis method using Ethylmethane sulfonate (EMS) for forward genetics studies (Figure 1B) (Lewis and Bacher, 1968). EMS is a mutagenic compound with fomula $CH_3SO_3C_2H_5$. It typically produces a random point mutation by guanine alkylation (Pastink *et al.*, 1991), which can cause missense or nonsense mutations in a certain gene. The ethyl group of EMS forms the abnormal base O-6-ethylguanine by reacting with guanine in DNA. During DNA replication, O-6-ethylguanine adducts frequently mispair with thymine instead of cytosine (Figure 1A). Following subsequent rounds of replication, the original G/C base pair can become an A/T base pair. Accordingly, most G/C base pairs in the genome can be potential targets for EMS mutagenesis and therefore the probability of mutation in a specific gene largely depends on the size of the gene. In a typical EMS mutagenesis, G/C to A/T transition occurs ~4.8 times/kb for every 1000 mutagenized chromosomes (Bentley *et al.*, 2000).

Despite the advantage of EMS mutagenesis in a high mutation rate and a broad target range, EMS mutagenesis has not been widely used in *Drosophila.* Lack of efficient and high-throughput methods for detecting mutations limited the use of EMS mutagenesis.

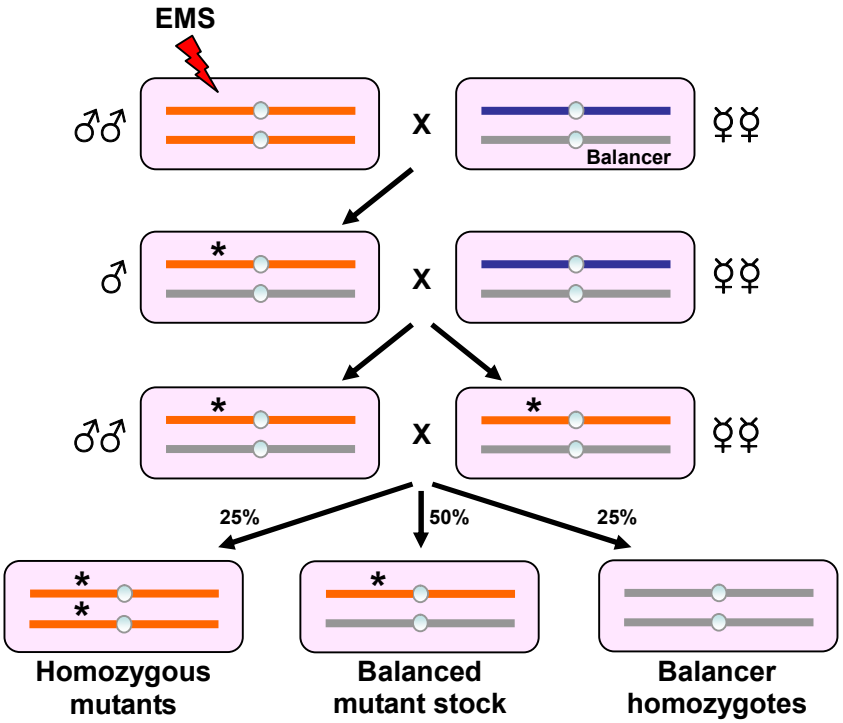
Figure 1. EMS mutagenesis in *Drosophila*

(A) EMS induces transition of a G/C base pair to an A/T base pair. (B) Male flies are fed EMS to induce mutations and are crossed to balancer (grey) virgin flies. In the F_1 generation, each male fly carrying the balancer is backcrossed to balancer virgin flies. If a mutation was successfully induced in the germlines of the paternal flies, the F_1 male flies will carry chromosomes with the mutation (asterisk). In the F_2 generation, male and female flies carrying both the same mutagenized chromosome and the balancer were crossed to each other to maintain an independent balanced mutant stock. In the F_3 generation, 25% of the progenies will be homozygous for the mutation unless the homozygote has a lethal phenotype. Figure 1 was adopted and modified (Johnston, 2002).

A


EMS
Guanine
Cytosine
O-6-ethylguanine
Thymine

B


EMS
Balancer
25%
50%
25%
Homozygous mutants
Balanced mutant stock
Balancer homozygotes

It is time-consuming and labor-intensive to identify genetic alterations by EMS because most mutations are single nucleotide substitutions. Thus, EMS mutagenesis is being replaced by other mutagenesis methods such as genome-wide transposon insertional mutagenesis, which has a major advantage over EMS mutagenesis in molecular tagging by the transposon.

1-2. Mutagenesis by *P*-element-mediated imprecise excision

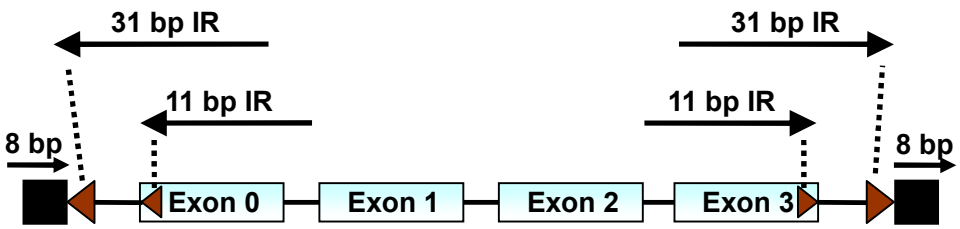
Transposon-mediated imprecise excision is the most extensively used technique for mutagenesis in *Drosophila* (Hiesinger and Bellen, 2004). Transposons are mobile DNA segments that insert in and excise from the genome through the action of transposase. Transposons can escape from the genome with or without genomic alteration at the original insertion site, termed imprecise and precise excision, respectively. In imprecise excision, transposons usually generate a genomic deletion by excising a local genomic region near the original insertion (Preston *et al.*, 1996; Robertson *et al.*, 1988). Although transposon insertions sometimes result in hypomorphic mutations, tansposon-mediated imprecise excisions are often induced to generate complete loss-of-function mutants. Among a variety of transposable elements, the *P*-element is the most popular vehicle to disrupt genes in *Drosophila* because its excision is imprecise at high rates and is completely dependent on *P*-transposase, which allows the control of its excision (Adams and Sekelsky, 2002; Ryder and Russel, 2003).

The wild type *P*-element is 2907 bp in length and carries a 4 exon (0, 1, 2, 3) transposase gene, two 31 bp inverted terminal repeats and two 11 bp inverted subterminal repeats (Figure 2) (Rio, 1990; Hummel and Klambt, 2008). The 31 bp inverted repeats are the binding sites of the inverted repeat binding protein and the 11-bp inverted repeats are regulatory elements. The promoter of the *P*-element is constitutively active and the expression of the transposase is modulated by differential splicing (O'Hare and Rubin, 1983; Amarasinghe *et al.*, 2001). The intron between exons 2 and 3 is properly removed only in the germlines, resulting in a functional transposase of 87 kDa. On the other hand, splicing of the intron does not occur by inhibition of a splicing repressor protein in the somatic cells, resulting in a truncated transposase of 66 kDa that functions as a repressor of *P*-element mobility (Karess and Rubin, 1984). Accordingly, transposition of the wild type *P*-element occurs only in the germlines, and therefore we now utilize genetically engineered Δ2-3 constructs to constitutively express an active transposase for efficient mutagenesis, in which the last intron has been deleted.

Action of *P*-element mobilization has been well studied (Beall and Rio, 1997; Tang *et al.*, 2005). The *P*-element does not have a specific target sequence. The *P*-element generates 8 bp duplication of a target site upon insertion (O'Hare and Rubin, 1983) and leaves a double strand break (DSB) with two complementing 17 bp 3'-overhang structures upon excision. The DSB can be restored in several different ways by DNA repair mechanisms, which generate different products.

Figure 2. Structure of the wild type *P*-element

The *P*-element consists of a 4-exon transposase gene, two 31 bp inverted terminal repeats, and two 11 bp inverted subterminal repeats. An 8 bp duplication of a target site is generated upon insertion of the *P*-element. Figure 2 was adopted and modified (Hummel and Klambt, 2008).

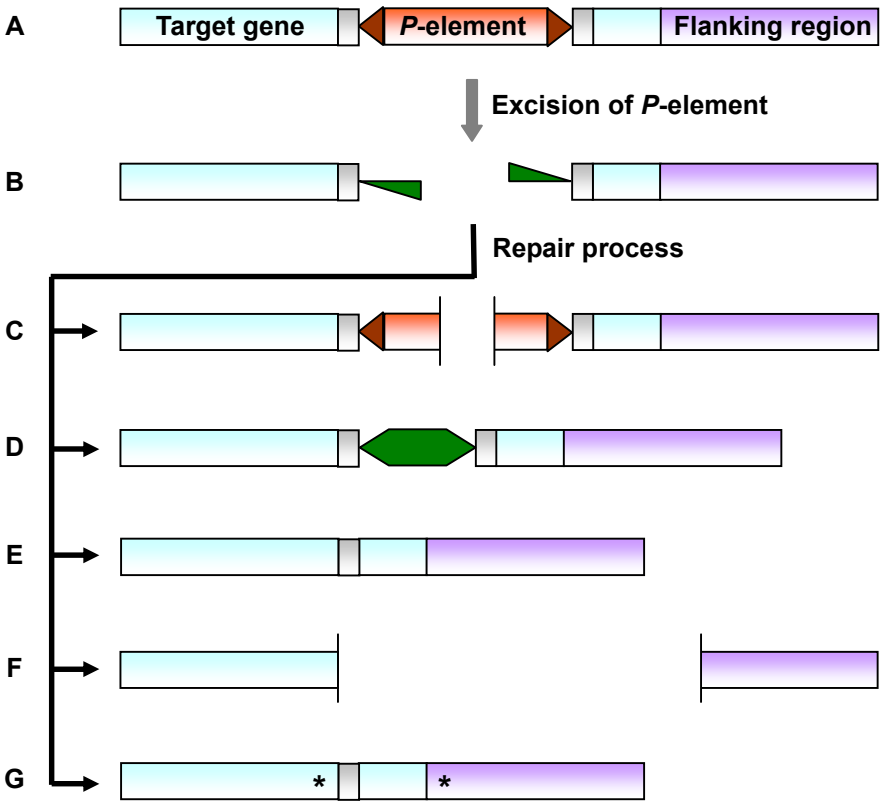

31 bp IR
31 bp IR
11 bp IR
11 bp IR
8 bp
8 bp
Exon 0
Exon 1
Exon 2
Exon 3

DSB repair mechanisms are comprised of two major pathways (Adams and Sekelsky, 2002): homology directed repair (HDR), in which a DSB is restored by synthesis from homologous templates; and non-homologous end-joining (NHEJ), in which the ends of a DSB are directly ligated to one another. HDR from the sister chromatid can result in restoration of an intact or internally deleted *P*-element (Figure 3C). HDR from the homologous chromosome can result in restoration of the original chromosome sequence without the *P*-element (Figure 3E) (Engels *et al.*, 1990). NHEJ can leave a footprint (Figure 3D) (Stavely *et al.*, 1995). An imprecise excision is a product of incomplete gap repair. A DSB can be enlarged to a gap by exonucleases before repair, and aberrant repair of the gap can generate a deletion that extends in one or both directions from the original insertion site (Figure 3F). Alterations in sequence were occasionally found near the original insertion site after excision of the *P*-element (Figure 3G), which are the results of gap repair with an ectopic template. HDR generally uses homologous templates such as the sister chromatid or the homologous chromosome, however, it sometimes uses an ectopic template such as a homologous sequence that is present elsewhere in the genome. When a DSB is restored by gap repair using an ectopic template, changes in sequence can be introduced.

If a *P*-element insertion is present in the vicinity of a gene of interest, loss-of-function mutations can be easily obtained by *P*-element-mediated imprecise excision (or more accurately, imprecise repair) (Voelker *et al.*, 1984; Daniels *et al.*, 1985). Over the last 20 years, mutagenesis by *P*-element-mediated imprecise excision allowed generation of a number of loss-of-function mutants.

Figure 3. Mutagenesis by *P*-element-mediated imprecise excision

(A) *P*-element is inserted in the target gene (Blue). The grey regions represent 8 bp duplication of the target site, and the violet region represents the flanking region. (B) Excision of the *P*-element generates a DSB with 17 bp 3'-overhang structures (Green) derived from the 31 bp inverted repeats of the *P*-element. (C) HDR using the sister chromatid can regenerate an intact or internally deleted *P*-element (D) HDR using the homologous chromosome can reproduce the original chromosome sequence without the *P*-element. (E) NHEJ leaves a footprint. (F) Incomplete gap repair can generate a deletion. (G) Gap repair with an ectopic template can introduce changes (asterisk) in sequence near the original insertion site. Figure 3 was adopted and modified (Adams and Sekelsky, 2002).


A
Target gene
P-element
Flanking region
Excision of P-element
B
Repair process
C
D
E
F
G
*
*

However, the mutagenesis method can not mutate all the genes in *Drosophila* because of the insertion biases of the *P*-element in the genome. Genes located in the coldspots where the *P*-element is barely inserted can not be subjected to mutagenesis by *P*-element-mediated imprecise excision.

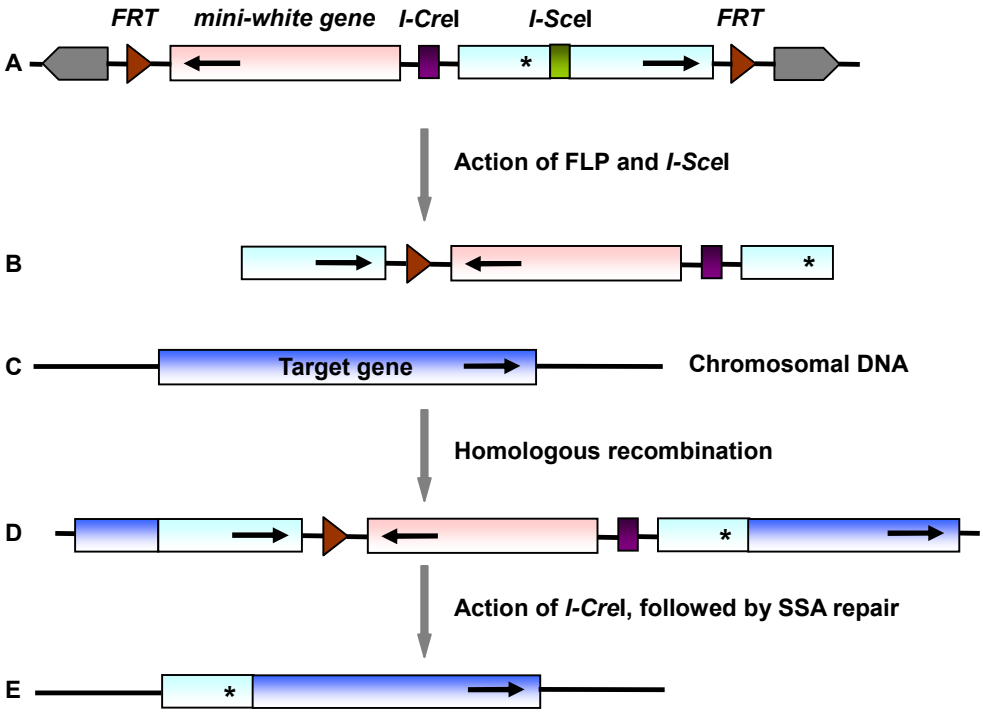
1-3. Targeted gene replacement mutagenesis

Targeted gene replacement, also called gene targeting, is a mutagenesis method that substitutes an endogenous gene with an introduced copy carrying a mutation. This method utilizes homologous recombination, in which nucleotides are exchanged between two similar or identical sequences of DNA. Homologous recombination occurs during meiosis and produces chromosomes comprising new combinations of genes, providing offspring with genetic diversity. Homologous recombination is also used to repair DNA damage such as a DSB.

It is important to introduce linear DNA into cells because linear DNA is more recombinogenic than closed circular DNA. In *Drosophila*, it is achieved by generating linear targeting DNA *in vivo*. In 2000, Rong and Golic developed a method to generate linear DNA in *Drosophila* using a transgenic construct (Rong and Golic, 2000). The construct was designed to have four key features (Figure 4A): a copy of a target gene carrying an engineered mutation and an restriction site for *I-Sce*I, a restriction endonuclease recognizing an 18 bp sequence absent in the *Drosophila* genome; a *mini-white* marker gene; two 34 bp Flp recombination target (*FRT*) sequences; an restriction site for *I-Cre*I, a restriction endonuclease recognizing an 22 bp sequence.

Figure 4. Targeted gene replacement mutagenesis in *Drosophila*

(A) The donor construct is integrated in the genome by *P*-element-mediated transformation. The donor construct has a copy (light blue) of a target gene carrying an engineered mutation (asterisk) and an *I-Sce*I site (green), a *mini-white* marker gene (pink), two *FRT*s (brown arrowheads), an *I-Cre*I site (violet). (B) Linear targeting DNA can be produced by expression of Flp and *I-Sce*I. (C) The target gene is present in the genome. (D) The linear DNA can be integrated into the genome by homologous recombination, thereby generating a tandem duplication of the target gene. (E) Single-stranded annealing (SSA) pathway can be induced by expression of *I-Cre*I, thereby one copy of the duplication can be retained by random reduction. Figure 4 was adopted and modified (Adams and Sekelsky, 2002).


FRT
mini-white gene
I-CreI
I-SceI
FRT
A
*
Action of FLP and I-SceI
B
*
C
Target gene
Chromosomal DNA
Homologous recombination
D
*
Action of I-CreI, followed by SSA repair
E
*

Expression of Flp can produce free circular DNA from the integrated construct by catalyzing recombination between the two *FRT*s. Expression of *I-Sce*I can subsequently cut the circular DNA to generate linear DNA with ends homologous to the target gene (Figure 4B). Then, the linear DNA can be integrated into the genome by homologous recombination, thereby forming a tandem duplication of the target gene (Figure 4D). Next, expression of *I-Cre*I can produce a DSB between the duplication. The DSB can induce SSA pathway, in which recombination can occur between the two copies (Rong and Golic, 2001). Consequently, only one copy can be retained in the genome (Figure 4E).

Targeted gene replacement mutagenesis is becoming more popular and replacing the conventional mutagenesis by *P*-element-mediated imprecise excision. However, this method is lengthy. It requires about six months for the entire procedure comprising generation of the donor construct and transgenic flies. Moreover, different integration loci of the same donor construct in the genome severely affect the frequency of targeted gene replacement.

1-4. Knockdown using RNAi

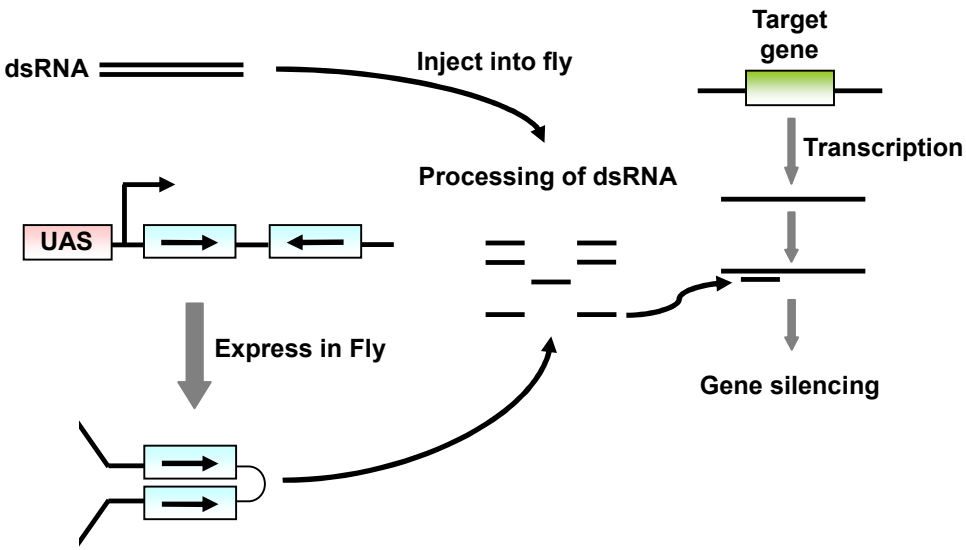
RNAi interference (RNAi) is a powerful method to reduce expression of genes at the post-transcriptional stage. RNAi induces silencing of a gene without mutating the endogenous gene (Fire *et al.*, 1998). Administration of double-stranded RNA (dsRNA) corresponding to either a portion or the entire coding region of a particular gene into embryonic cells can effectively and specifically interfere with expression of the gene

(Montgomery *et al.*, 1998), resulting in a phenotype essentially equivalent to genetic mutations of the gene. Two methods have been developed to deliver dsRNA to *Drosophila*. The first method is microinjection of dsRNA into *Drosophila* embryo (Figure 5) (Misquitta and Paterson, 1999). This method has an advantage in degrading both maternally inherited mRNA and zygotically expressed mRNA. However, suppression of gene expression is transient so introduction of dsRNA by microinjection does not efficiently induce silencing of genes later in development (Misquitta and Paterson, 1999). Consequently, microinjection method has been limited to study function of genes in the late stages of development.

In order to circumvent this problem, Kennerdell and Carthew developed an alternative method to express RNA with long inverted repeats in *Drosophila* (Figure 5) (Kennerdell and Carthew, 2000). RNA transcribed from a transgene becomes double-stranded by forming hairpin-loop structures through the inverted repeats and an in-between spacer, which was demonstrated to successfully interfere with gene expression in *Drosophila* (Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Piccin *et al.*, 2001). Furthermore, dsRNA expression by the GAL4-UAS system makes it feasible to induce temporal and spatial suppression of gene expression in *Drosophila* (Kennerdell and Carthew, 1998; Piccin *et al.*, 2001). Vienna *Drosophila* RNAi Center (VDRC) was established in April 2007 and it currently comprises 31,892 *UAS-RNAi* transgenic flies covering 13,264 *Drosophila* genes (93.8%). Suppression of gene expression can be simply induced by crossing *Gal4* lines with *UAS-RNAi* transgenic lines obtained from VDRC. Accordingly, knockdown using RNAi has become the most convenient mutagenesis method in *Drosophila*.

Figure 5. Knockdown using RNAi in *Drosophila*

Introduction of dsRNA can be achieved by either of two methods: Microinjection of *in vitro*-transcribed RNA into embryos (upper left), or expression of RNA with inverted repeat sequences *in vivo* using a transgenic construct (lower left). The introduced dsRNA is processed into 21-25 nucleotide small interfering RNA (siRNA) by the Dicer, and is used to degrade mRNA of a target gene. Figure 5 was adopted and modified (Adams and Sekelsky, 2002).


dsRNA
Inject into fly
Target gene
Transcription
Processing of dsRNA
UAS
Express in Fly
Gene silencing

However, knockdown using RNAi also has a disadvantage. Unlike other mutagenesis methods, knockdown using RNAi can not induce complete inactivation of genes. Thus, it can not be utilized when complete inactivation of a target gene is desired.

2. *piggyBac* transposon

The *piggyBac* transposon was first discovered in cabbage looper moth, *Trichoplusia ni* (Cary *et al.*, 1989). It is a Class II DNA transposable element, which includes the *P*, *hobo* and *mariner* elements (Elick *et al.*, 1996). The *piggyBac* transposon is 2472 bp flanked by 13 bp inverted terminal repeats (Cary *et al.*, 1989; Fraser *et al.*, 1996; Finnegan, 1990). It integrates into the genome at the tetranucleotide TTAA site, which is duplicated upon insertion (Wang and Fraser, 1993). The *piggyBac* transposon is more randomly integrated into the *Drosophila* genome and inserts successfully even in the coldspots of *P*-element integration (Hacker *et al.*, 2003; Horn *et al.*, 2003; Thibault *et al.*, 2004). To mutate genes in the coldspots of the *P*-element, the *piggyBac* transposable element was developed as an alternative. Exelixis Inc. (San Francisco, CA) has generated genome-wide *piggyBac* transposon-inserted *Drosophila* libraries, which are available through Harvard Medical School (Thibault *et al.*, 2004). Despite these attractive advantages, the *piggyBac* transposon is not widely used for gene disruption because it was thought that the *piggyBac* transposon always excised precisely by the *piggyBac*-specific transposase and generated no local deletions (Elick *et al.*, 1996, Fraser *et al.*, 1996; Witsell *et al.*, 2009). This feature limits the use of the

piggyBac tansposon in generating deletion mutants by imprecise excision. Consequently, only the flies carrying a *piggyBac* transposon insertion that directly disrupts a gene or alters specific gene expression have been used as mutant alleles (Hiesinger and Bellen, 2004).

In addition to the utility of the *piggyBac* transposon as a mutagenic vector in *Drosophila*, the *piggyBac* transposon has recently been used as a gene delivery vector. In contrast to the *P*-element, the *piggyBac* transposon is also functional in mammalian cells and mice (Ding *et al.*, 2005). The *piggyBac* transposon was demonstrated to transfer exogenous genes successfully into human and mouse cell lines as well as into mice (Ding *et al.*, 2005; Wilson *et al.*, 2007). Moreover, the unique features of the *piggyBac* transposon enable reversible gene transfer through precise excision of the transposon after gene delivery, leaving no transgene and no genomic alteration at the original integration locus (Chen *et al.*, 2010; Lacoste *et al.*, 2009). Due to these advantages of the *piggyBac* vector compared with viral vectors, the *piggyBac* transposon has been recently utilized to establish transgene-free induced pluripotent stem (iPS) cells (Woltjen *et al.*, 2009; Yusa *et al.*, 2009). Fibroblasts were successfully reprogrammed to iPS cells by *piggyBac* transposon-mediated introduction of four key genes (*c-Myc*, *Klf4*, *Oct4* and *Sox2*) that were clearly removed from the genome without genomic alteration after pluripotent cells were established (Woltjen *et al.*, 2009).

3. Sepiapterin reductase and tetrahydrobiopterin

Sepiapterin reductase is an enzyme that catalyzes the terminal reaction in the biosynthesis of tetrahydrobiopterin (BH_4). BH_4 is an essential cofactor for nitric oxide synthase and aromatic amino acid hydroxylases, including phenylalanine hydroxylase (PAH; EC 1.14.16.2), tyrosine hydroxylase (TH; EC 1.14.16.3), and tryptophan hydroxylase (TPH; EC 1.14.16.4). PAH catalyzes the conversion of phenylalanine to tyrosine, which is the first and rate-limiting step of phenylalanine degradation in the liver. Mutations in the *PAH* gene cause phenylketonuria (PKU), a metabolic disease displaying mental retardation and an accumulation of phenylalanine. TH and TPH are the rate-limiting enzymes for the synthesis of dopamine and serotonin in the brain, respectively (Fitzpatrick, 1999). Mutations in the *TH* gene have been implicated in brain disorders such as Segawa's disease, Parkinson's disease (PD), and schizophrenia (Goodwill *et al.*, 1997). Mutations in the *TPH* gene have been associated with depression. BH_4 deficiency can lead to the similar phenotypes (Blau *et al.*, 1996).

In addition to the function of BH_4 as cofactors, BH_4 has been thought to have antioxidant effects because of its fully reduced structure. Several studies reported that BH_4 can reduce oxidative stress. It was demonstrated that BH_4 scavenges superoxide in dopaminergic neurons (Nakamura *et al.*, 2001) and that BH_4 attenuates NO-induced endothelial cell death (Shimizu *et al.*, 1998).

The *de novo* pathway of BH_4 biosynthesis is well established (Figure 6). The first step of *de novo* biosynthesis of BH_4 is the conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2-NTP) by GTP cyclohydrolase I (GTPCH I; EC 3.5.4.16), which is the rate-limiting step of the BH_4 biosynthesis.

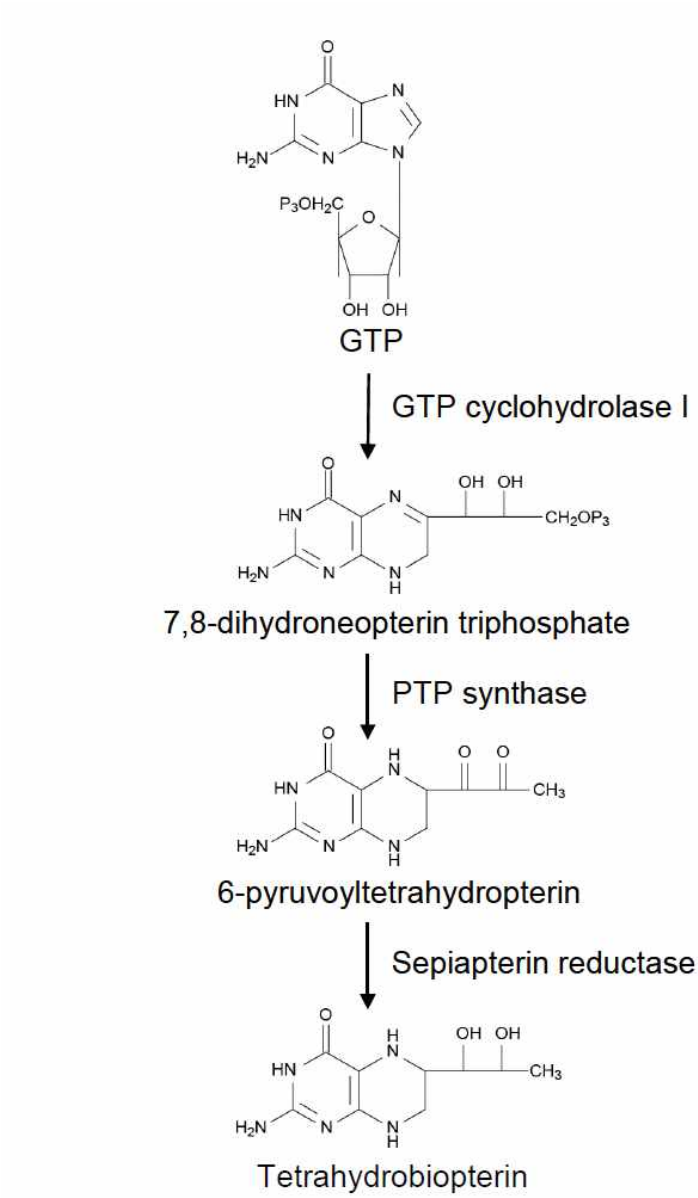


Figure 6. The *de novo* biosynthetic pathway of tetrahydrobiopterin

H_2-NTP is then converted to 6-pyruvoyltetrahydropterin (6-PTP) by 6-pyruvoyltetrahydropterin synthase (PTP synthase; EC 4.6.1.10). Next, 6-PTP is converted to BH_4 by sepiapterin reductase (SR; EC 1.1.1.153). The *de novo* biosynthetic pathway of BH_4 is well conserved in *Drosophila*.

Several studies suggested that *SR* is related to PD. PD is a progressive neurodegenerative disorder characterized by the selective degeneration of dopaminergic neurons.

PARK3 locus harboring *SR* was shown to be associated with both the development and onset age of PD (DeStefano *et al.*, 2002; Gasser *et al.*, 1998). Single nucleotide polymorphism (SNP) located 196 bp upstream of *SR* was shown to be linked to age of onset of PD (Karamohamed *et al.*, 2003). The 4-fold increase of SR expression was observed in the brain of patients with PD (Tobin *et al.*, 2007), supporting implication of *SR* in PD.

Two human patients with mutations in the *SR* were first reported in 2001 (Bonafe *et al.*, 2001). They displayed a BH_4-dependent monoamine neurotransmitter deficiency without hyperphenylalaninemia.

A murine model for SR deficiency was generated in 2006 (Yang *et al.*, 2006). The $SR^{-/-}$ mice showed reduced levels of BH_4 and BH_4-dependent neurotransmitters including dopamine, norepinephrine, and serotonin. The animal model also exhibited PKU, dwarfism, and impaired body movement. In 2011, it was reported that autophagy is induced in the $SR^{-/-}$ mice (Kwak *et al.*, 2011).

4. Purpose of this study

Previous studies suggested that the *piggyBac* transposon always excises precisely from the insertion site. This unique characteristic of the *piggyBac* transposon raised a potential limitation of its utility in generating deletion mutants in *Drosophila*. However, it facilitates reversible gene-transfer in several studies, such as the generation of iPS cells from fibroblasts. In this study, I report multiple imprecise excisions of the *piggyBac* transposon inserted in the 5'-UTR of the *SR* gene. I generated a hypomorphic mutant allele of the *SR* gene which showed markedly decreased levels of *SR* expression. My findings suggest that it is possible to generate deletion mutants by *piggyBac* transposon-mediated imprecise excision in *Drosophila*. However, it also suggests a limitation of *piggyBac* transposon-mediated reversible gene transfer for the generation of iPS cells.

II. Materials and Methods

1. *Drosophila* strains

All stocks were maintained and raised on standard fly food at 25°C unless otherwise specified. The following fly stocks were obtained from Bloomington Stock Center: $SR^{piggyBac}$ (No. 18753); *CyO,PBAC\T* (No. 8285); *FM6* (No. 4327); *Actin-Gal4* (No. 4414); y^{1}, w^{1118}, *FRT19A* (No. 1744); *Ubi-mRFP, w*, hs-FLP, FRT19A* (No. 31418). W^{1118} was a gift from J. Chung (Seoul National University). W^{1118} and SR^{REV} were used as control strains.

2. Southern blot analysis

Genomic DNA was extracted from 1-day-old male flies using G-spin™ (iNtRON Biotechnology, Korea). Genomic DNA (40 μg) was digested with *Hin*dIII (Fermentas), separated by 1% agarose gel, and transferred onto Nytran SuPerCharge membrane (Schleicher and Schuell). Hybridization was performed in ExpressHyb hybridization solution (Clontech) with [α-^{32}P]dCTP-labeled DNA probes specific for the *piggyBac* transposon. The *piggyBac* transposon fragment was amplified using *piggyBac* transposon-specific primers (forward, 5'-GTCTGCGTAAAATTGACGCATGCATTC-3' and reverse, 5'-CTACCGCTTGACGTTGGCTGCAC-3') from *BL18753* genomic DNA. Radioactive probes were prepared using Megaprime DNA labeling systems (GE Healthcare). Radioactive signals were visualized using a BAS-2500 Bio-image

analyzer (Fujifilm).

3. Sequence analysis of *piggyBac* transposon integration loci

Genomic DNA of *BL18753* was prepared using DNAzol (Invitrogen). Genomic PCR was performed with specific primer sets. For the *piggyBac* transposon 5' end, 5'-TTGGCTGTCTTTTTGCCTTAACTCGCTTTG-3' and 5'-TCCAAGCGGCGACTGAGATG-3' primers were used. For the *piggyBac* transposon 3' end, 5'-CCTCGATATACAGACCGATAAAAC-3' and 5'-GCTGACCTTGACATTGACATTCGATCACACA-3' primers were used (Thibault *et al.*, 2004). PCR products were separated by 1% agarose gel, extracted from the agarose gel, and sequenced to confirm the exact insertion site.

4. Induction of *piggyBac* transposon excision in *Drosophila*

piggyBac transposon excision was induced in *Drosophila* by following the cross scheme under 25°C culture conditions (Figure 9). Male flies carrying *piggyBac*-specific transposase (*BL8285*) were mated to virgin flies carrying the *piggyBac* transposon insertion at the *SR* locus (*BL18753*) in bottles. Male progenies carrying both the *piggyBac* transposon insertion and *piggyBac*-specific transposase were selected by a phenotypic selection marker and mated to *FM6* first balancer (*BL4327*) virgin flies in vials. A total of 208 matings were established, each with one male and five virgins. From the cross, *piggyBac* transposon excised virgins were collected and maintained by mating to *FM6* first balancer male flies. To test the effect of culture

temperature on *piggyBac* transposon-mediated imprecise excision, the same cross scheme was also performed at 29°C. A total of 193 matings were established.

5. Analyses of *piggyBac* transposon excision type at *sepiapterin reductase* locus

Fly genomic DNA was prepared using DNAzol (Invitrogen). PCR was performed with the forward primer sequence 5'-TTGGCTGTCTTTTTGCCTTAACTCGCTTTG-3' and the reverse primer sequence 5'-GCTGACCTTGACATTGACATTCGATCACACA-3', to obtain a 542 bp product using wild type genomic DNA as the template. Used primers are indicated by solid arrows in Figure 7A. PCR products were analyzed on 2.5% agarose gel. Shifted bands compared with the control band were extracted from agarose gel and sequenced to verify the exact deletion region. Sequencing was performed using a 3730 DNA analyzer (Applied Biosystems) at the Genome Research Facility in Seoul National University.

6. RT-PCR

For RT-PCR, total RNA was isolated using easy-spin^{TM} (iNtRON Biotechnology, Korea) and reverse transcribed using M-MLV reverse transcriptase (Promega) and oligo-dT (18mer) primers. The resulting cDNA was amplified by PCR with specific primer sets. For *sepiapterin reductase*, 5'-ATGGACCTGAAACAGCGCACATA-3' and 5'-CTAGAACTGCTCATCCCTGTAAT-3' primers were used to obtain a 786 bp

product. Used primers are indicated by dashed arrows in Figure 7A. For the *GAPDH* control, 5'-GTCAACGATCCCTTCATCGA-3' and 5'-TGTACGATAGTTTTGGCTAG-3' primers were used (Kim *et al.*, 2010).

7. Real-time RT-PCR

cDNA was prepared by the same method described in the RT-PCR section. Real-time RT-PCR was conducted using *sepiapterin reductase* specific primers with the forward sequence 5'-AACGGATCAAAGCCGAGGGTTCC-3' and the reverse sequence 5'-TTTCCACCGGATGCCTCCAGAAT-3'. The *rp49* control was amplified with the forward primer 5'-AGATCGTGAAGAAGCGCACCAAG-3' and the reverse primer 5'-CACCAGGAACTTCTTGAATCCGG-3' (Ha *et al.*, 2009). The values were normalized to *rp49*. The results are expressed as arbitrary units. *SR* gene expression in W^{1118} was taken arbitrarily as 10.

8. Sepiapterin reductase assay for *Drosophila* extracts

3-day-old male flies were collected, homogenized in lysis buffer (100 mM Kpi, pH 6.0) containing 2 mM PMSF, and followed by sonication. After centrifugation at 13,500 rpm for 15 minutes, the supernatants were dialyzed twice in 500 ml of lysis buffer for 12 hours. Proteins were quantified by the standard Bradford method. All procedures for protein preparation were performed at 4°C. SR enzyme assay was conducted in 100 mM Kpi (pH 6.0), 50 uM sepiapterin, 50 uM NADPH and 50 ug of total protein at 30°C for 30 minutes under the dark condition. Reaction was stopped by

adding 30% trichloroacetic acid (TCA), and further incubated for 1 hour under the dark condition after adding 1% I_2/ 2% KI. Excessive iodine was reduced by adding 2% ascorbic acid. A portion of the reaction mixture was analyzed by HPLC with a reverse phase C18 column (Inertsil ODS-3: 4.6 x 250 mm, GL Sciences) and a fluorescence detector (Waters 474). The final reaction product biopterin was monitored by fluorescence detector with excitation at 362 nm and emission at 435 nm.

9. Quantification of content of tetrahydrobiopterin

The level of BH_4 was quantified by differential oxidation method described previously (Kaneko *et al.*, 2001) with some modifications. 3-day-old male flies were homogenized in extraction buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 10% (v/v) glycerol and 0.1% (v/v) tween 20) containing 1 mM DTT and processed by following the protocol. The final reaction product biopterin was analyzed by HPLC with the same condition described in the sepiapterin reductase assay for *Drosophila* extracts section.

10. Site-directed mutagenesis

The catalytically inactive $SR^{T153D,\ Y166V}$ mutant was generated by site-directed mutagenesis (Cosmo Genetech, Seoul, Korea). Tyrosine 153 at the active site was mutated to aspartate by changing the GAC codon to ACC. Tyrosine 166 was mutated to valine by changing the GTT codon to TAT. For generation of T153D, 5'-GTGGTTAATCTCAGCGACTTGGCAGCCATTGCA-3' and 5'-

TGCAATGGCTGCCAAGTCGCTGAGATTAACCAC-3' primers were used. For generation of Y166V, 5'-TCCTCGATGGCACACGTTTGCACGGTGAAGGCA-3' and 5'-TGCCTTCACCGTGCAAACGTGTGCCATCGAGGA-3' primers were used. Mutated DNA was sequenced to verify the alteration of two codons.

11. Cloning, expression, and purification of SR and $SR^{T153D, Y166V}$

Wild type *SR* was cloned into pET15b vector as *Nde*I-*Bam*HI fragments, and transformed into BL21 (DE3) cells. Transformants were cultured in LA media, and expression of recombinant SR-6xHis protein was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside when A600 reached 0.5. After 4-hour-induction at 37 °C, SR-6xHis fusion protein was purified from cell extracts with His•Bind Resin (Novagen) according to the manufacturer's protocol. Cloning, expression and purification of recombinant $SR^{T153D, Y166V}$-6xHis protein were performed using the same method with SR-6xHis.

12. *in vitro* sepiapterin reductase assay for recombinant proteins

Reaction mixture was incubated at 30°C in 100 mM Kpi (pH 6.0), 50 uM sepiapterin, 50 uM NADPH and 5 μg of recombinant SR or $SR^{T153D, Y166V}$. Consumption of the substrate, sepiapterin, was monitored at various time points by scanning spectrophotometer with a range of 325 nm to 525 nm.

13. Generation of transgenic flies

SR and inactive $SR^{T153D, Y166V}$ transgenic flies were generated. The cDNAs for the genes were amplified by PCR and cloned into the pUAST-HA vector (Brand and Perrimon, 1993). *P*-element-mediated germline transformation was performed according to the standard protocol (Spradling and Rubin, 1982). The parental strain for all germline transformations was W^{1118}.

14. Immunoblot analysis

Protein extracts were prepared by homogenizing 3-day-old male flies in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing a 1X protease inhibitor cocktail (Calbiochem-Merck4Biosciences) and a 1X phosphatase inhibitor cocktail (Pierce). The extracts were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked in Tris-buffered saline (TBS) with 0.1% tween 20 and 5% BSA for 1 hour. I used the following primary antibodies: rabbit anti-phospho-Akt (1:1000; Cell signaling), rabbit anti-Akt (1:1000; Cell signaling), mouse anti-β-tubulin (1:3000; Sigma-Aldrich), and rat anti-HA (1:1000; Roche). Detection of the primary antibodies was carried out with HRP-conjugated secondary antibodies (goat anti-rabbit, Pierce; donkey anti-mouse, Jackson ImmunoResearch Laboratories; and goat anti-rat, Jackson ImmunoResearch Laboratories) and an ECL-Plus detection kit (GE Healthcare). The images were processed on a LAS-3000 (Fujifilm).

15. Paraquat-sensitivity assay

230 male flies (3-day-old) of each genotype were collected and divided into 23 vials of 10 flies. The flies were starved for 5 hours and then transferred to vials with 3M filter paper soaked with 5% sucrose solution containing 20 mM paraquat (Methyl viologen, Sigma-Aldrich). The flies were kept in the dark and their survival was checked every 3 hours.

16. Generation of the SR^{Ex234} mosaic clones

For generation of the SR^{Ex234} mitotic recombination clones in larval fat body, eggs were collected for 8 hours and followed by heat-shock at 38°C for 90 minutes. The larval genotype was *SR^{Ex234}, FRT19A / Ubi-mRFP, w*, hs-FLP, FRT19A*.

III. Results

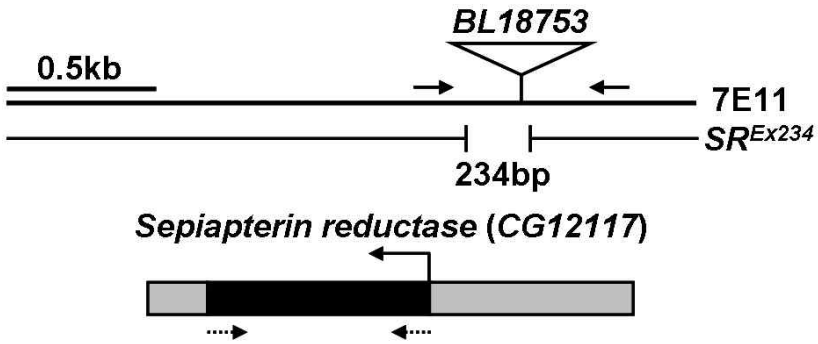
1. Characterization of *BL18753*, a fly line with *piggyBac* transposon insertion at the *sepiapterin reductase* locus

The *D. melanogaster* ortholog of the human *sepiapterin reductase* is encoded by *CG12117* and was named *SR* (Seong *et al.*, 1998). Sepiapterin reductase is a key enzyme in the biosynthesis of BH_4, an essential cofactor required for the synthesis of catecholamine neurotransmitters (Thony *et al.*, 2000). The *SR* gene is located at 7E11 on the X chromosome in *Drosophila* (Figure 7A). The *BL18753* fly line carrying a *piggyBac* transposon insertion at the *SR* locus was obtained from the Bloomington Stock Center. The *piggyBac* transposon is the only transposable element available at the *SR* locus. To determine the number of *piggyBac* transposon integrations in *BL18753*, I performed Southern blot analysis. I confirmed that *BL18753* carries only one *piggyBac* transposon integration in the genome (Figure 8). Next, I examined the exact *piggyBac* tranposon insertion site and flanking sequence. The *piggyBac* transposon integration locus was sequenced by PCR product sequencing. This revealed the *piggyBac* transposon insertion at a TTAA tetranucleotide in the 5'-UTR of the *SR* gene (Figure 7A and B), which is consistent with the fly line information provided from the Bloomington Stock Center. No further mutation was observed near the integration locus (Figure 7B).

Figure 7. Schematic representation of the *sepiapterin reductase* genomic locus

(A) Schematic representation of the *SR* genomic locus. The original *piggyBac* transposon insertion site is indicated by a triangle above the map. SR^{Ex234} was obtained by *piggyBac* transposon-mediated imprecise excision, and the excised region is represented by vertical lines. (B) Genomic sequence of *piggyBac* transposon insertion locus in *BL18753*. A triangle indicates the *piggyBac* transposon insertion. Bold letters (TTAA) designate the insertion site of the *piggyBac* transposon.

A

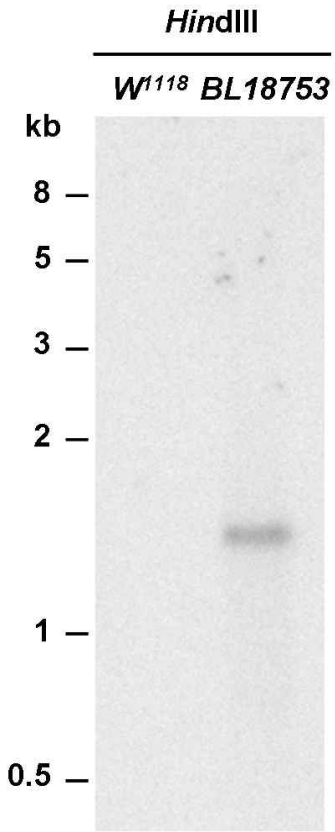

BL18753
0.5kb
7E11
SREx234
234bp
Sepiapterin reductase (CG12117)

B


5´
3´
TATTTGTAGAAACAATTAATTATTATTTTTGGAA

Figure 8. *BL18753* carries only one *piggyBac* transposon integration.

Southern blot analysis of W^{1118} and *BL18753* hybridized with radioactive DNA probes specific for the *piggyBac* transposon.


HindIII
W1118
BL18753
kb
8
5
3
2
1
0.5

2. Induction of *piggyBac* transposon excision in *Drosophila*

To induce *piggyBac* transposon excision in *Drosophila*, I designed a cross scheme (Figure 9) to obtain male flies carrying both the *piggyBac* tranposon insertion and the *piggyBac*-specific transposase. *piggyBac* transposon excision occurs in the germlines of these flies that constitutively express a *piggyBac*-specific transposase under the control of the *αTub84B* promoter. The excision of the *piggyBac* transposon is easily detected in the next generation by loss of eye color, a selection marker of the *piggyBac* transposon. In total, I obtained 131 *piggyBac* transposon excised lines from 208 independent male germlines at 25°C and 74 *piggyBac* transposon excised lines from 193 independent male germlines at 29°C (data not shown).

3. Identification of *piggyBac* transposon-mediated imprecise excision

To identify whether the excision occurred precisely or imprecisely, I conducted genomic PCR, as described in the Materials and Methods section. I identified three imprecise excisions (SR^{Ex17a}, SR^{Ex135} and SR^{Ex198}) and 66 precise excisions (data not shown) from the 25°C culture condition (Figure 10A). I also identified three imprecise excisions (SR^{Ex17b}, SR^{Ex22} and SR^{Ex41}) and 43 precise excisions (data not shown) from 29°C culture condition (Figure 10B). The percentage of imprecise excision to total excision was 4.3% at 25°C and 6.5% at 29°C. The SR^{Ex234} line from 29°C culture condition was excluded from statistical analysis because it was obtained from an experiment without statistical control.

Figure 9. Cross scheme used to induce *piggyBac* transposon excision at the *SR* locus in *Drosophila*

♂, a male; ♀, a virgin female; ♂♂, males; ♀ ♀, virgin females.

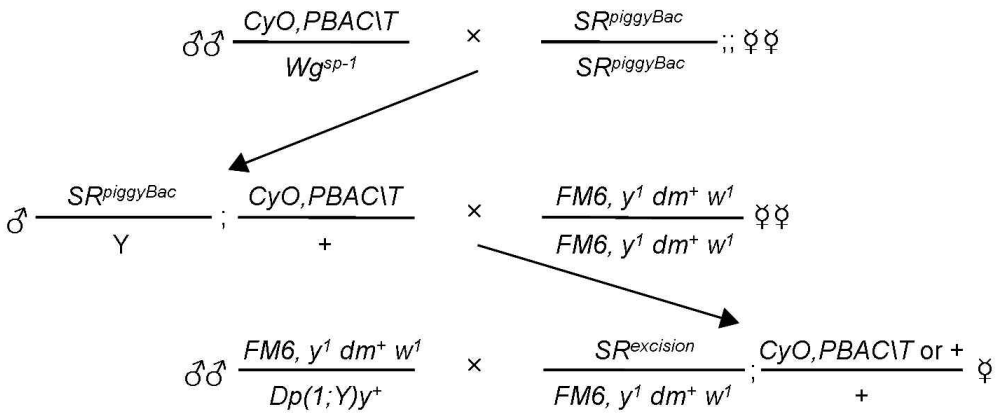
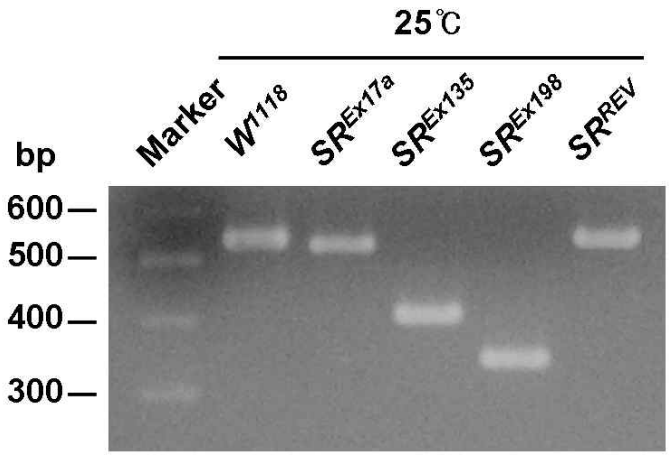

♂♂ CyO,PBAC\T / Wg^sp-1 × SR^piggyBac / SR^piggyBac ;; ♀♀
♂ SR^piggyBac / Y ; CyO,PBAC\T / + × FM6, y^1 dm^+ w^1 / FM6, y^1 dm^+ w^1 ♀♀
♂♂ FM6, y^1 dm^+ w^1 / Dp(1;Y)y^+ × SR^excision / FM6, y^1 dm^+ w^1 ; CyO,PBAC\T or + / + ♀

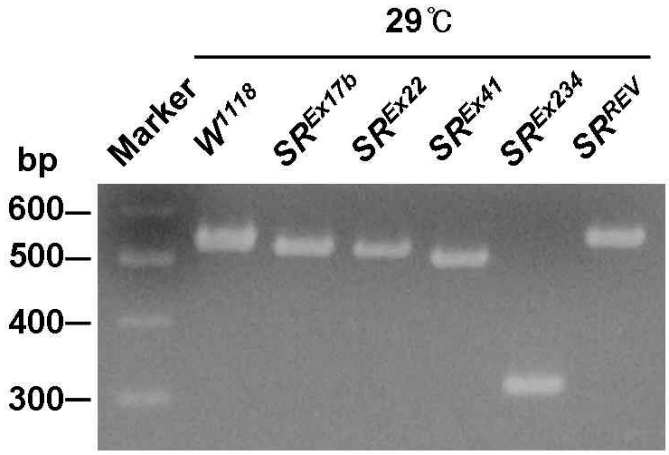
Figure 10. *piggyBac* transposon-mediated imprecise excision at the *SR* locus in *Drosophila*

(A and B) Genomic PCR analyses of imprecise excisions were carried out under 25°C and 29°C culture conditions, respectively. W^{1118} and SR^{REV} were used as controls. REV, revertant. Superscript numbers on *SR* excision lines indicate size of deletion (bp) in each line. 100 bp DNA ladder (Bioneer) was used as the size marker.

A


25℃
Marker
W1118
SREx17a
SREx135
SREx198
SREV
bp
600
500
400
300

B


29℃
Marker
W1118
SREx17b
SREx22
SREx41
SREx234
SREV
bp
600
500
400
300

Multiple precise excisions and identical imprecise excisions obtained from the same male germline were counted as one because one gonialblast develops into 64 spermatids during *Drosophila* spermatogenesis (Fuller, 1998). I then determined genomic lesions by sequence analysis of the genomic PCR products. Sizes of deletions varied from 17 to 234 bp (Table 1 and 2). Among seven imprecise excision alleles, six alleles displayed bidirectional deletions from their insertion site, and one allele displayed an unidirectional deletion (Table 1 and 2). Intriguingly, two alleles gained random footprints after excisions. The SR^{Ex17a} has a single nucleotide change (A to T) at the 4th nucleotide position downstream from the breakpoint (Table 1), and the SR^{Ex234} has a new TAT trinucleotide sequence at the breakpoint (Table 2).

4. Characterization of the SR^{Ex234} mutant

I characterized the SR^{Ex234} mutant, the allele carrying the longest (234 bp) deletion at the *SR* locus among the seven genome-deleted lines. The genomic lesion in the SR^{Ex234} mutant disrupts the 5'-UTR of the *SR* gene but not the coding sequence of the *SR* gene (Figure 7A). To characterize the SR^{Ex234} mutant, I first examined the transcript level of *SR* by RT-PCR. In the SR^{Ex234} mutant, the transcript level of *SR* was markedly reduced (Figure 11A), suggesting that the SR^{Ex234} is a strong hypomorphic mutant of *SR*. I also confirmed the expression of *SR* mRNA by real-time RT-PCR. Consistent with RT-PCR data, the expression of *SR* mRNA was significantly reduced in the SR^{Ex234} (Figure 11B). Because the transcript level of *SR* was decreased in the SR^{Ex234} mutant, I next investigated whether the SR activity was also reduced.

Table 1. Analysis of alleles created by *piggyBac* transposon-mediated imprecise excision under 25°C culture conditions

*, Single nucleotide change (A to T) at 4th nucleotide position downstream from the breakpoint.

Allele	Size of deletion (bp)	Direction of deletion	Footprint
SR^{Ex17a}	17	Bidirectional	*
SR^{Ex135}	135	Unidirectional	None
SR^{Ex198}	198	Bidirectional	None

Table 2. Analysis of alleles created by *piggyBac* transposon-mediated imprecise excision under 29°C culture conditions

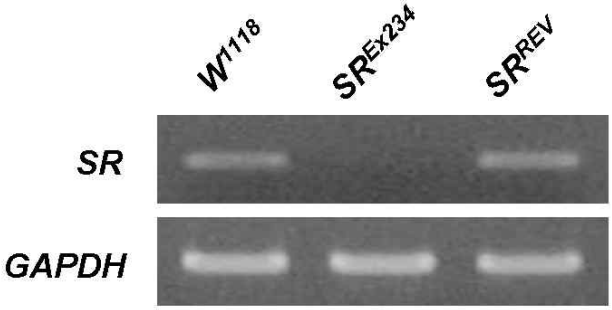
#, Gain of new TAT trinucleotide sequence at the breakpoint.

Allele	Size of deletion (bp)	Direction of deletion	Footprint
SR^{Ex17b}	17	Bidirectional	None
SR^{Ex22}	22	Bidirectional	None
SR^{Ex41}	41	Bidirectional	None
SR^{Ex234}	234	Bidirectional	#

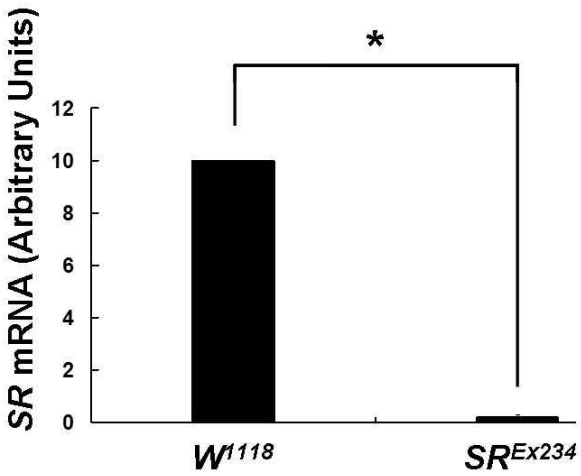
Figure 11. Expression of *SR* in the SR^{Ex234} mutant

(A) *SR* mRNA expression analysis by RT-PCR in 3-day-old male flies. The SR^{Ex234} mutant showed significantly reduced expression of *SR*. *GAPDH* was used as a loading control. (B) *SR* mRNA expression analysis by real-time RT-PCR. mRNA levels are expressed by arbitrary units. Error bar indicates the standard deviation (S.D.) of three independent experiments. Asterisk indicates significance by Student's *t*-test (*$p<0.0001$).

A


W1118
SREx234
SREV
SR
GAPDH

B


SR mRNA (Arbitrary Units)
12
10
8
6
4
2
0
*
W1118
SREx234

Due to the lack of SR antibody, the protein level of SR could not be determined. In agreement with the mRNA data, the specific activity of SR was also dramatically reduced in the SR^{Ex234} mutant (Figure 12). I then examined whether the reduction of SR activity successfully induced deficiency of BH_4, the reaction product of SR. Despite the depletion of SR mRNA and activity, the SR^{Ex234} mutant showed mild decrease in the level of BH_4 (53% of wild type) (Figure 13).

5. Activation of Akt pathway in the SR^{Ex234} mutant

Since elevation of p-Akt in the muscle and liver of the $SR^{-/-}$ mice was reported (Kwak *et al.*, 2011), I tested whether the level of p-Akt was also increased in the SR^{Ex234} mutant. As observed in the $SR^{-/-}$ mice, the level of p-Akt was also enhanced in the SR^{Ex234} mutant (Figure 14). Interestingly, the level of of Akt, which was unchanged in the $SR^{-/-}$ mice, was also increased in the SR^{Ex234} mutant (Figure 14). Ubiquitous expression of SR by *Actin-Gal4* driver restored elevation of both p-Akt and Akt in the SR^{Ex234} mutant (Figure 14). I next investigated whether the catalytic activity of SR is important for the increase. Based on the sequence alignment of *Drosophila* SR with rat SR (Figure 15), in which serine 158 and tyrosine 171 is important for catalytic activity (Fujimoto *et al.*, *1999*), two conserved corresponding amino acids (threonine 153 and tyrosine 166) were deduced to be key amino acids in the active site of *Drosophila* SR.

Figure 12. Relative sepiapterin reductase specific activity

Relative NADPH-dependent SR specific activity in 3-day-old male flies. Error bars indicate the standard deviation (S.D.) of three independent experiments. Asterisk indicates significance by Student's *t*-test (**p*<0.0001).

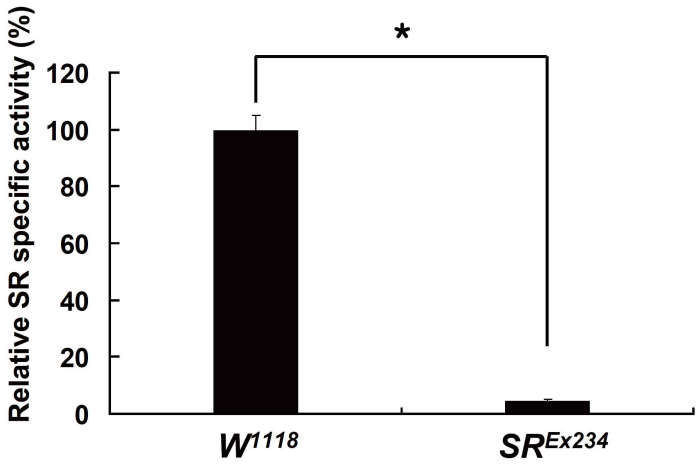

Relative SR specific activity (%)
120
100
80
60
40
20
0
*
W1118
SREx234

Figure 13. Relative tetrahydrobiopterin contents

Relative BH_4 contents in 3-day-old male flies. Error bars indicate the standard deviation (S.D.) of three independent experiments. Asterisk indicates significance by Student's *t*-test (*p<0.05).

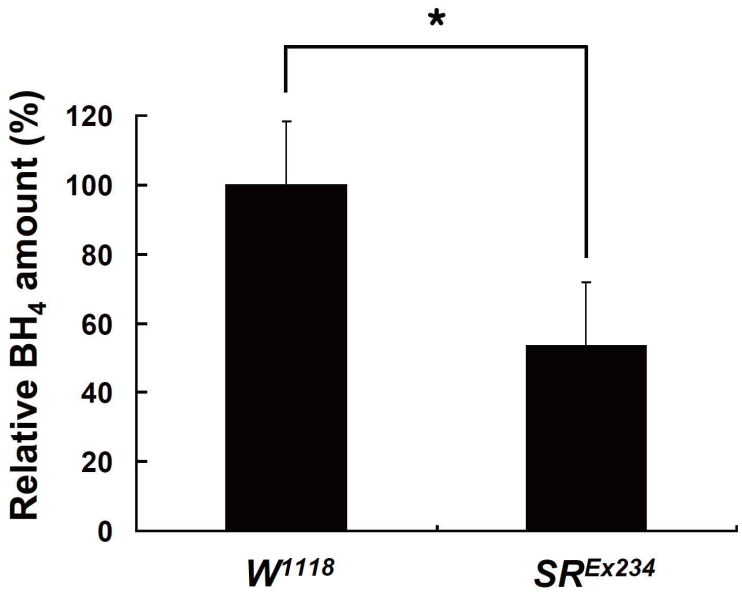

*
Relative BH4 amount (%)
120
100
80
60
40
20
0
W1118
SREx234

Figure 14. Akt pathway is affected in the SR^{Ex234} mutant.

Western blot analysis with anti-p-Akt, anti-Akt, anti-β-tubulin and anti-HA antibodies. The levels of both p-Akt and Akt were elevated in the SR^{Ex234} mutant. The increases were restored by expression of SR. Expression of catalytically inactive $SR^{T153D, Y166V}$ showed a small rescue effect compared to that of active SR. β-tubulin was used as a loading control. HA was used to detect expression of exogenous genes.

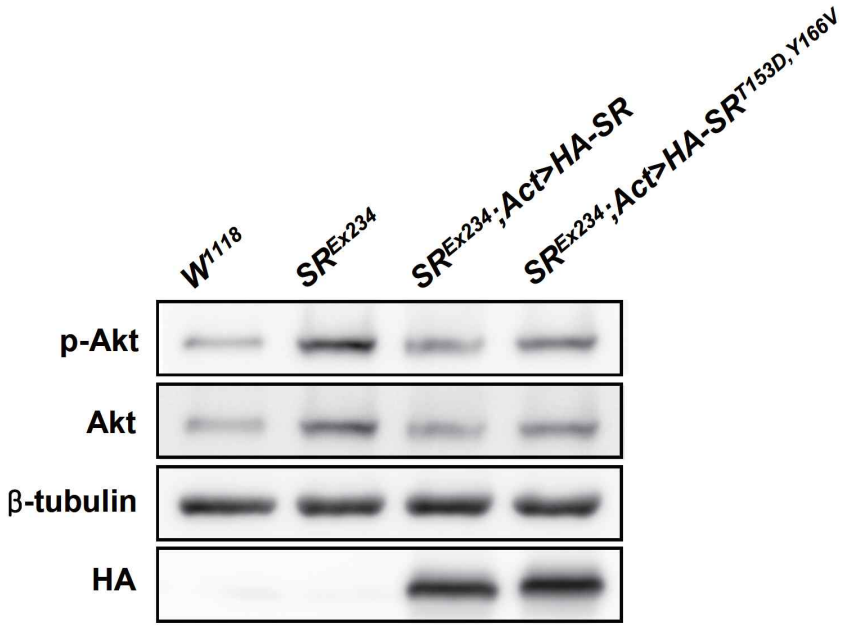
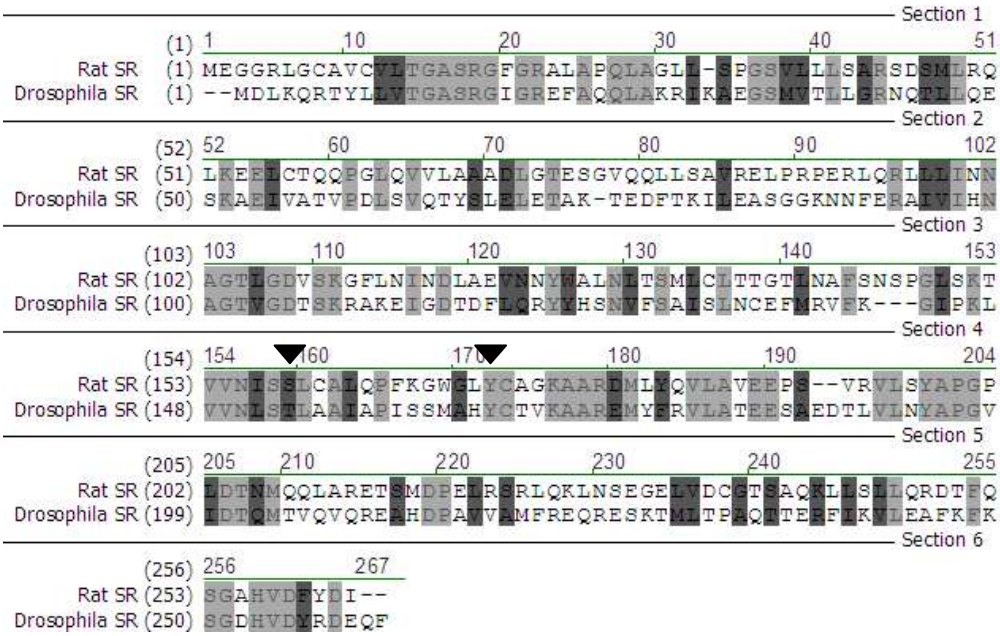

W1118
SREx234
SREx234;Act>HA-SR
SREx234;Act>HA-SRT153D,Y166V
p-Akt
Akt
β-tubulin
HA

Figure 15. Amino acid sequence alignment

Amino acid sequence alignment was performed with rat SR and *Drosophila* SR using *vetor NTI Advance*^{TM} *10* program (Invitrogen). Two conserved amino acids of the active site were indicated by triangles.


Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Rat SR
Drosophila SR

I constructed a catalytically inactive form of $SR^{T153D, Y166V}$, in which threonine 153 and tyrosine 166 were mutated to aspartate and valine, respectively. $SR^{T153D, Y166V}$ was confirmed to be catalytically inactive by an enzyme assay with recombinant proteins (Figure 16). Expression of $SR^{T153D, Y166V}$ by *Actin-Gal4* exhibited a small rescue effect compared to that of functional SR, suggesting that the catalytic activity of SR is responsible for the increase of both p-Akt and Akt (Figure 14).

6. Hyposensitivity of the SR^{Ex234} mutant to oxidative stress

Since it was reported that BH_4 can function as an antioxidant (Nakamura *et al.*, 2001; Shimizu *et al.*, 1998), I examined whether the SR^{Ex234} mutant is more susceptible to oxidative stress. When exposed to 20 mM paraquat, its survival rate was monitored (Figure 17). Contrary to expectations, the SR^{Ex234} mutant appeared to be less sensitive to paraquat compared to wild type. The time required for 50% lethality of the SR^{Ex234} mutant (51 hours) was longer than that of wild type (29 hours), suggesting that the SR^{Ex234} mutant is hyposensitive to oxidative stress.

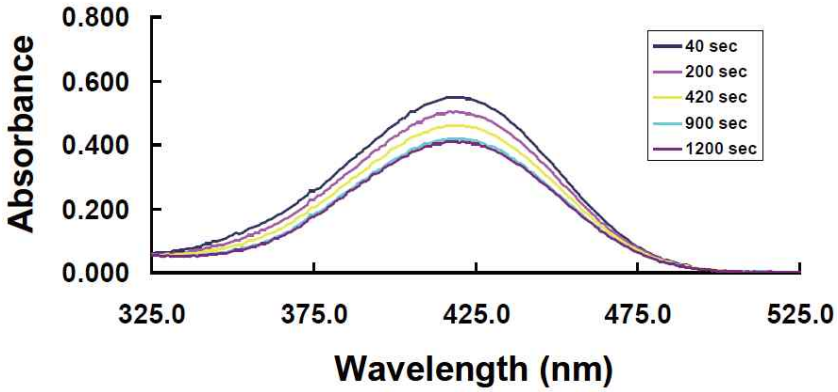
7. Generation of the SR^{Ex234} mosaic clone

The SR^{Ex234} mosaic clones were generated for further studies. I checked whether the SR^{Ex234} mosaic clones in larval fat body were successfully generated. The SR^{Ex234} clones were observed in the fat body and marked by the absence of RFP (Figure 18).

Figure 16. Validation of catalytic inactivity of $SR^{T153D, Y166V}$

(A) Spectrum of reaction by recombinant SR. Consumption of sepiapterin, a substrate of SR, was monitored. (B) Spectrum of reaction by recombinant $SR^{T153D, Y166V}$. Consumption of sepiapterin was not observed.

A


0.800
0.600
0.400
0.200
0.000
Absorbance
325.0
375.0
425.0
475.0
525.0
Wavelength (nm)
40 sec
200 sec
420 sec
900 sec
1200 sec

B

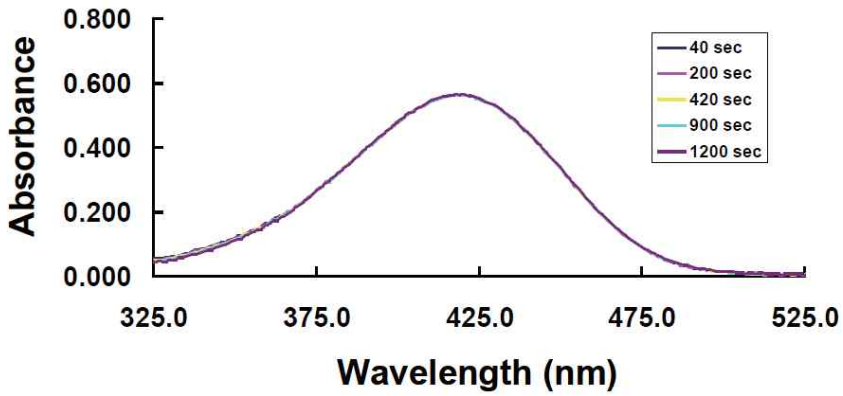

0.800
0.600
0.400
0.200
0.000
Absorbance
325.0
375.0
425.0
475.0
525.0
Wavelength (nm)
40 sec
200 sec
420 sec
900 sec
1200 sec

Figure 17. The *SR*^{Ex234} mutant is hyposensitive to paraquat.

Graph of survival rates of 20 mM paraquat-fed male flies counted every 3 hours.

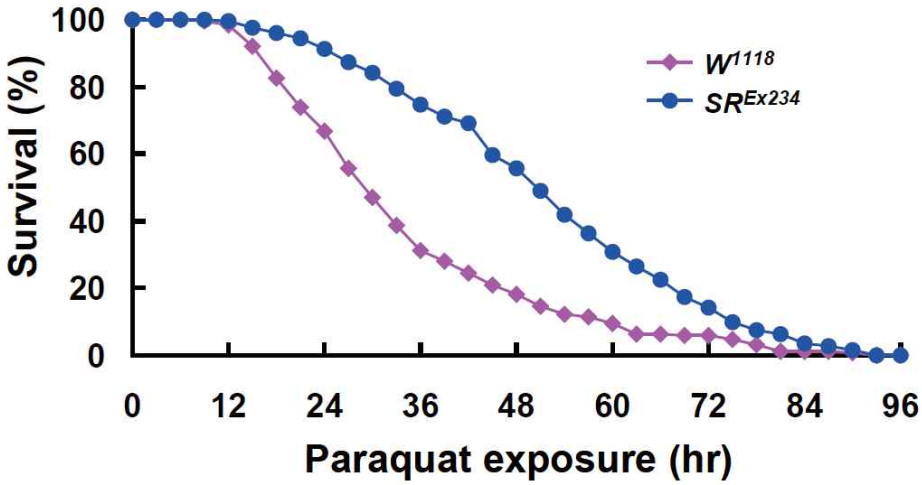
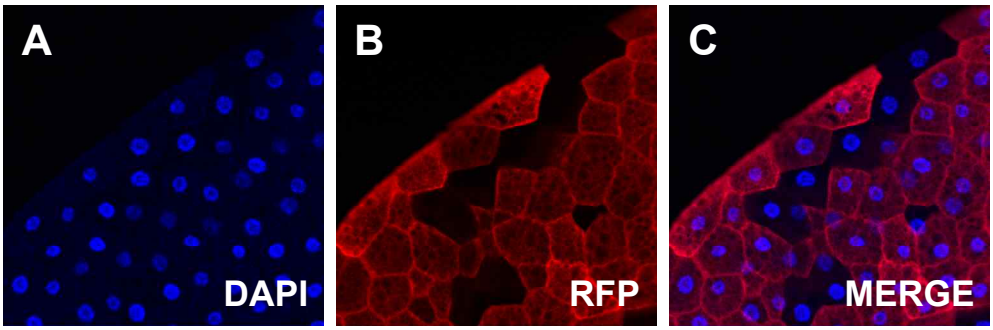

100
80
60
40
20
0
Survival (%)
0
12
24
36
48
60
72
84
96
Paraquat exposure (hr)
W1118
SREx234

Figure 18. Generation of the *SR*^{Ex234} mosaic clones in larval fat body

(A-C) Single confocal sections of larval fat body of the *SR*^{Ex234} mosaic clones. Fat body carrying the *SR*^{Ex234} clones was stained with DAPI and anti-RFP antibody. The *SR*^{Ex234} clones are marked by the absence of RFP.


A
DAPI
B
RFP
C
MERGE

IV. Discussion

Mutagenesis is the first step to elucidate the function of genes in animal models. Several mutagenesis methods have been developed for *Drosophila melanogaster*, which includes EMS, *P*-element-mediated imprecise excision, targeted gene replacement, and RNAi. Among the four methods, mutagenesis by *P*-element-mediated imprecise excision has been the most extensively used and has contributed greatly to elucidate the function of genes in *Drosophila* (Robertson *et al.*, 1988; Hiesinger *et al.*, 2004). However, the *P*-element has a critical limitation because of its insertion bias in the genome (Spradling *et al.*, 1999).

To mutate genes in the coldspots of the *P*-element, the *piggyBac* transposon was developed as an alternative. Newly developed insertional mutagenesis by the *piggyBac* transposon allows us to study genes in the coldspots of the *P*-element due to its random insertion into the genome (Thibault *et al.*, 2004).

However, previous studies suggested that the *piggyBac* transposon always excises precisely from the genome without deleting the genomic region around the original insertion site (Elick *et al.*, 1996; Fraser *et al.*, 1996), preventing the use of the *piggyBac* transposon from generating deletion mutants in *Drosophila*.

I was not in agreement with the conclusion of the studies for two reasons. The first reason is that the number of excision of the *piggyBac* transposon observed in the studies seems to be insufficient to conclude that the *piggyBac* transposon always excises imprecisely. In the initial studies, they reached the conclusion by observing

seven independent precise excisions of the *piggyBac* transposon from the baculovirus genome in the IPLB-SF21AE insect cell line (Fraser *et al.*, 1996) and eleven independent precise excisions from a plasmid carrying a *piggyBac* transposon insertion in the same insect cell line (Elick *et al.*, 1996). The second reason is that it is not the transposon but the repair that can determine the type of excision. Since the *piggyBac* transposon leaves a DSB with 4 bp 3'-overhang structures upon excision (Mitra *et al.*, 2008), I thought that imprecise excisions of the *piggyBac* transposon can occur by aberrant gap repair.

In this study, I generated seven independent *piggyBac* transposon-mediated imprecise excision lines. These are attributed to incomplete gap repair. Among the seven alleles, two mutants had additional footprints. The SR^{Ex17a} had a single nucleotide change (A to T) at 4th nucleotide position downstream form the breakpoint. The SR^{Ex234} gained a new TAT trinucleotide sequence at the breakpoint. These are attributed to gap repair using an ectopic template.

I investigated whether the frequency and the deletion size of *piggyBac* transposon-mediated imprecise excision is affected by temperature conditions. The frequency of imprecise excision by the *piggyBac* transposon at the *SR* locus was 4.3% at 25°C and 6.5% at 29°C, respectively. The frequency at 29°C is higher than that at 25°C. These are higher than by the *P*-element on average (approximately 1%) (Ryder and Russel, 2003). The average size of deletions is 117 bp at 25°C and 79 bp at 29°C, respectively. The mean size of deletions at 25°C is bigger than that at 29°C. However, the longest deletion (234 bp) was obtained from 29°C culture condition. The deletion size of *piggyBac* transposon-mediated imprecise excision is much smaller than that of the *P*-

element, which ranges from a few base pairs to several kilobases (Preston *et al.*, 1996). Nevertheless, the *piggyBac* transposable element still has an advantage in generating deletion mutants of the genes that are located in genomic regions where the *P*-element is rarely found.

It is thought that the frequency and the deletion size of transposon-mediated imprecise excision are largely dependent on the activity of exonucleases that can enlarge a gap. Thus, it will be interesting to test whether overexpression of the exonucleases enhances imprecise excision of the *piggyBac* transposon.

SR is a key enzyme in the biosynthesis of BH_4, an essential coenzyme required for the synthesis of important biogenic amines, including catecholamines and serotonin (Thony *et al.*, 2000). In an effort to establish BH_4-deficient fly models, I successfully generated *SR* mutants by *piggyBac* transposon-mediated imprecise excision. The SR^{Ex234} mutant showed almost complete depletion in *SR* mRNA and SR specific activity, but moderate reduction in BH_4.

The mild decrease of BH_4 can be explained by discovery of enzymes that can compensate the function of SR. SR catalyzes the final reaction of the *de novo* pathway of BH_4 biosynthesis, in which SR converts 6-PTP to BH_4 via a two-step reaction (Figure 19). In the initial step, SR reduces the C-1'-keto group at the side chain of 6-PTP, leading to the formation of 1'-hydroxy-2'-oxopropyltetrahydropterin. The intermediate is then converted to 1'-oxo-2'-hydroxypropyltetrahydropterin by side chain isomerization. 1'-oxo-2'-hydroxypropyltetrahydropterin, commonly called 6-lactoyltetrahydropterin, is finally converted to BH_4 by reduction of the C-1'-keto group at the side chain of 1'-oxo-2'-hydroxypropyltetrahydropterin.

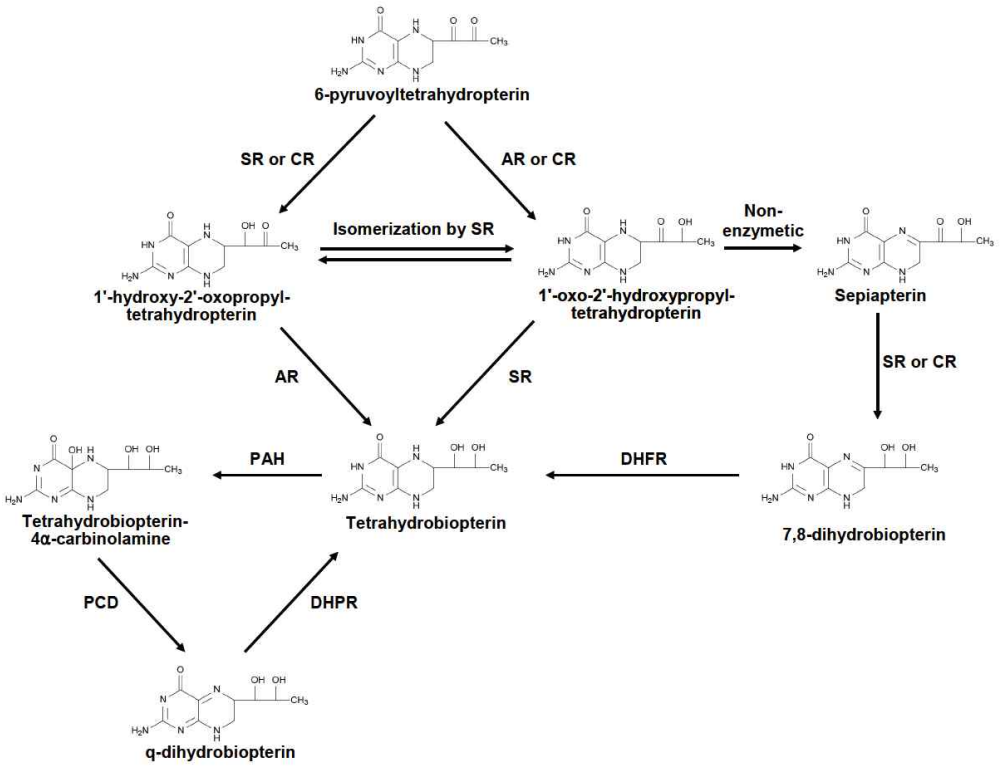


Figure 19. Alternative pathways of tetrahydrobiopterin synthesis

Previous studies suggested that aldose reductase (AR) and carbonyl reductase (CR) also can convert 6-PTP to BH_4 (Figure 19). AR can mediate the conversion of 6-PTP to 1'-oxo-2'-hydroxypropyltetrahydropterin (Milstien and Kaufman, 1989; Levine *et al.*, 1990) and also can catalyze the conversion of 1'-hydroxy-2'-oxopropyltetrahydropterin to BH_4 (Park *et al.*, 1991). CR can mediate the formation of 6-PTP to 1'-hydroxy-2'-oxopropyltetrahydropterin or 1'-oxo-2'-hydroxypropyltetrahydropterin (Park *et al.*, 1991). Therefore, BH_4 can be synthesized in the absence of SR.

The moderate reduction of BH_4 in the SR^{Ex234} mutant also can be explained by alternative pathways of BH_4 biosynthesis such as the salvage pathway and the regeneration pathway. In the salvage pathway (Figure 19), 1'-oxo-2'-hydroxypropyltetrahydropterin is non-enzymatically converted to sepiapterin, which is next catalyzed to 7,8-dihydrobiopterin (BH_2) by SR or CR. BH_2 is finally reduced to BH_4 by dihydrofolate reductase (DHFR). In the regeneration pathway (Figure 19), BH_4 is oxidized to tetrahydrobiopterin-4α-carbinolamine by PAH during the hydroxylation of phenylalanine to tyrosine (Fitzpatrick, 1999). tetrahydrobiopterin-4α-carbinolamine is converted to q-dihydrobiopterin by pterin-4α-carbinolamine dehydratase (PCD). q-dihydrobiopterin is finally regenerated to BH_4 by dihydropteridine reductase (DHPR).

In the previous study with the $SR^{-/-}$ mice (Yang *et al.*, 2006), similar mild decrease of BH_4 (40.5% of wild type) in the brain was reported. On the contrary, a dramatic reduction of BH_4 (1.1% of wild type) in the liver was reported. These data suggest that AR and CR may be present mainly in the brain of mice. I think that I observed moderate reduction of BH_4 in the SR^{Ex234} mutant because I quantified the level of BH_4

from the whole bodies of *Drosophila*.

Interestingly, the SR^{Ex234} mutant displayed elevation of both p-Akt and Akt. This is somewhat inconsistent with data from the $SR^{-/-}$ mice. The $SR^{-/-}$ mice showed elevation of only p-Akt. I think that it is because of difference of the used model organisms. Generally, activation in the Akt pathway is achieved by phosphorylation of Akt. I expressed exogenous SR in the SR^{Ex234} mutant to examine whether it restores the Akt pathway. Expression of SR successfully rescued the Akt pathway. Oral supplementation of BH_4 is infeasible because BH_4 can be readily oxidized to BH_2 in aqueous solution (Cunnington *et al*., 2012). I also expressed catalytically inactive $SR^{T153D,\ Y166V}$ to examine whether the catalytic activity of SR is important for alterations in the Akt pathway. Expression of $SR^{T153D,\ Y166V}$ showed a small rescue effect compared to that of functional SR, suggesting that catalytic activity of SR is responsible for changes in the Akt pathway. Activation of the Akt pathway in the SR^{Ex234} mutant may be due to decrease in the level of BH_4. Reduction in the level of BH_4 can increase oxidative stress because BH_4 can function as an antioxidant compound (Nakamura *et al*., 2001; Shimizu *et al*., 1998). Reactive oxygen species such as H_2O_2 is known to stimulate the Akt pathway (Wang *et al*., 2000). Therefore, decreased level of BH_4 can cause activation of the Akt pathway.

Interestingly, the SR^{Ex234} mutant appears to be hyposensitive to paraquat compared to wild type. It is attributed to activation of the Akt pathway in the SR^{Ex234} mutant. It is known that the Akt pathway protects cells from oxidative stress (Wang *et al*., 2000). Thus, the SR^{Ex234} mutant can be less sensitive to paraquat.

Akt is a hub protein in a variety of cellular signaling pathway and has a critical

role in diverse cellular processes. These include apoptosis, protein synthesis, glucose metabolism, and cell proliferation. The SR^{Ex234} mutant does not show any morphological phenotype so it is difficult to predict the role of the Akt pathway activated by SR deficiency. I will continue to investigate physiological roles of the Akt pathway enhanced in the SR^{Ex234} mutant.

Although I clearly demonstrated that *piggyBac* transposon can excise imprecisely at the *SR* locus in *Drosophila*, it still remains to be determined whether *piggyBac* transposon-mediated imprecise excision generally occurs at other loci in *Drosophila*. Although another group used the same transgenic fly line that constitutively expresses the *piggyBac*-specific transposase under the *αTub84B* promoter, they failed to obtain imprecise excision of the *piggyBac* transposon at the *WRN exonuclease* locus in wild type *Drosophila* (Witsell *et al.*, 2009). They isolated 25 excisions at the *WRN exonuclease* locus, but all of them were precise. Another group used transgenic fly lines that express the *piggyBac*-specific transposase under the *Hsp70* heat shock promoter (Thibault *et al.*, 2004). They recovered 5 or 10 excisions from each of six different loci, but all of them were also precise. I think that it was due to the difference of integration loci of the *piggyBac* transposon that may affect the frequency of *piggyBac* transposon-mediated imprecise excision.

The *piggyBac* transposon has been recently utilized to establish transgene-free iPS cells (Woltgen *et al.*, 2009; Yusa *et al.*, 2009). Four key genes (*c-Myc*, *Klf4*, *Oct4* and *Sox2*) introduced by the *piggyBac* transposon were clearly removed from the genome without any genomic alteration after fibroblasts had been successfully reprogrammed to iPS cells (Woltjen *et al.*, 2009). My findings raise the limitation of the use of the

piggyBac transposon for reversible gene delivery because of the possibility of imprecise excision and thereby, unexpected mutations in the reprogrammed iPS cells. Although imprecise excision of the *piggyBac* transposon has not been reported in the generation of iPS cells, I think that it is because the technique was developed only three years ago and thereby the number of experiments using the method is not enough to discover imprecise excision. Therefore, the *piggyBac* transposon-mediated reversible gene transfer strategy for the generation of iPS cells should be more carefully monitored.

V. References

Adams, M. D., and Sekelsky, J. J. (2002) From sequence to phenotype: reverse genetics in *Drosophila melanogaster*. *Nat. Rev. Genet.* 3, 189-198

Amarasinghe, A. K., MacDiarmid, R. A., Adams, M. D., and Rio, D. C. (2001) An *in vitro*-selected RNA-binding site for the KH domain protein PSI acts as a splicing inhibitor element. *RNA*. 7, 1239-1253

Beall, E. L., and Rio, D. C. (1997) *Drosophila* P-element transposase is a novel site-specific endonuclease. *Genes Dev.* 11, 2137-2151

Bentley, A., MacLennan, B., Calvo, J., and Dearolf, C. (2000) Targeted recovery of mutations in *Drosophila*. *Genetics.* 156, 1169-1173

Blau, N., Barnes, I., and Dhondt J. L. (1996) International database of tetrahydrobiopterin deficiencies. *J. Inherit. Metab. Dis.* 19, 8-14

Bonafe, L., Thony, B., Penzien, J. M., Czarnecki, B., and Blau, N. (2001) Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine-neurotransmitter deficiency without hyperphenylalaninemia. *Am. J. Hum. Genet.* 69, 269-277

Brand, A. H., and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 2, 401-415

Cary, L. C., Goebel, M., Corsaro, B. G., Wang, H. G., Rosen, E., and Fraser, M. J. (1989) Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology.* 172, 156-169

Chen, Y. T., Furushima, K., Hou, P. S., Ku, A. T., Deng, J. M., Jang, C. W., Fang, H., Adams, H. P., Kuo, M. L., Ho, H. N., Chien, C. L., and Behringer, R. R. (2010) *piggyBac* transposon-mediated reversible gene transfer in human embryonic stem cells. *Stem Cells Dev.* 19, 763-771

Cunnington, C., Assche, T. V., Shirodaria, C., Kylintireas, I., Lindsay, A. C., Lee, J. M., Antoniades, C., Margaritis, M., Lee, R., Cerrato, R., Crabtree, M. J., Francis, J. M., Sayeed, R., Ratnatunga, C., Pillai, R., Choudhury, R. P., Neubauer, S., and Channon, K. M. (2012) Systemic and Vascular Oxidation Limits the Efficacy of Oral Tetrahydrobiopterin Treatment in Patients With Coronary Artery Disease. *Circulation*. 125, 1356-1366

Daniels, S. B., McCarron, M. Y., Love, C., and Chovnick, A. (1985) Dysgenesis-induced instability of *rosy* locus transformation in *Drosophila melanogaster*: analysis of excision events and the selective recovery of control element deletions. *Genetics.*

109, 95-117

DeStefano, A. L., Lew, M. F., Golbe, L. I., Mark, M. H., Lazzarini, A. M., Guttman, M., Montgomery, E., Waters, C. H., Singer, C., Watts, R. L., Currie, L. J., Wooten, G. F., Maher, N. E., Wilk, J. B., Sullivan, K. M., Slater, K. M., Saint-Hilaire, M. H., Feldman, R. G., Suchowersky, O., Lafontaine, A., Labelle, N., Growdon, J. H., Vieregge, P., Pramstaller, P. P., Klein, C., Hubble, J. P., Reider, C. R., Stacy, M., MacDonald, M. E., Gusella, J. F., and Myers, R. H. (2002) PARK3 Influences Age at Onset in Parkinson Disease: A Genome Scan in the *Gene*PD Study. *Am. J. Hum. Genet.* 70, 1089-1095

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y., and Xu, T. (2005) Efficient transposition of the *piggyBac* (PB) transposon in mammalian cells and mice. *Cell.* 122, 473-483

Elick, T. A., Bauser, C. A., and Fraser, M. J. (1996) Excision of the *piggyBac* transposable element in vitro is a precise event that is enhanced by the expression of its encoded transposase. *Genetica.* 98, 33-41

Engels, W. R., Johnson-Schlitz, D. M., Eggleston, W. B. and Sved, J. (1990) High-frequency *P* element loss in *Drosophila* is homolog dependent. *Cell.* 62, 515-525

Fraser, M. J., Ciszczon, T., Elick, T., and Bauser, C. (1996) Precise excision of TTAA-specific lepidopteran tranposons *piggyBac* (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Mol. Biol.* 5, 141-151

Finnegan, D. J. (1990) Transposable elements and DNA transposition in eukaryotes. *Curr. Opin. Cell Biol.* 2, 471-477

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature.* 391, 806-811

Fitzpatrick, P. F. (1999) Tetrahydropterin-dependent amino acid hydrozylases. *Annu. Rev. Biochem.* 68, 355-381

Fujimoto, K., Ichinose, H., Nagatsu, T., Nonaka, T., Mitsui, Y., and Katoh, S. (1999) Functionally important residues tyrosine-171 and serine-158 in sepiapterin reductase. *Biochim. Biophys. Acta.* 1431, 306-314

Fuller, M. T. (1998) Genetic control of cell proliferation and differentiation in *Drosophila* spermatogenesis. *Semin. Cell Dev. Biol.* 9, 433-444

Gasser, T., Muller-Myhsok, B., Wszolek, Z. K., Oehlmann, R., Calne, D. B., Bonifati, V., Bereznai, B., Fabrizio, E., Vieregge, P., Horstmann, R. D. (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat. Genet.* 18, 262-265

Goodwill, K. E., Sabatier, C., Marks, C., Raag, R., Fitzpatrick, P. F., and Stevens, R. C. (1997) Crystal structure of tyrosine hydroxylase at 2.3 A and its implications for inherited neurodegenerative diseases. *Nat. Struc. Biol.* 7, 578-585

Ha, E. M., Lee, K. A., Seo, Y. Y., Kim, S. H., Lim, J. H., Oh, B. H., Kim, J., and Lee, W. J. (2009) Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. *Nat. Immunol.* 10, 949-957

Hacker, U., Nystedt, S., Barmchi, M. P., Horn, C., and Wimmer, E. A. (2003) *piggyBac*-based insertional mutagenesis in the presence of stably integrated P element in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 100, 7720-7725

Hiesinger, P. R., and Bellen, H. J. (2004) Flying in the face of total disruption. *Nat. Genet.* 36, 211-212

Horn, C., Offen, N., Nystedt, S., Hacker, U., and Wimmer, E. A. (2003) *piggyBac*-based insertional mutagenesis and enhancer detection as a tool for functional insect

genomics. *Genetics.* 163, 647-661

Hummel, T., and Klambt, C. (2008) P-element Mutagenesis. *Methods in Molecular Biology*. 420, 97-117

Johnston, D. S. (2002) The art and design of genetic screens: *Drosophila melanogaster*. *Nat. Rev. Genet.* 3, 176-188

Kaneko, Y. S., Mori, K., Nakashima, A., Nagatsu, I., Nagatsu, T., and Ota, A. (2001) Determination of tetrahydrobiopterin in murine locus coeruleus by HPLC with fluorescence detection. *Brain Res. Brain Res Protoc.* 8, 25-31

Karamohamed, S., DeStefano, A. L., Wilk, J. B., Shoemaker, C. M., Golbe, L. I., Mark, M. H., Lazzarini, A. M., Suchowersky, O., Labelle, N., Guttman, M., Currie, L. J., Wooten, G. F., Stacy, M., Saint-Hilaire, M., Feldman, R. G., Sullivan, K. M., Xu, G., Watts, R., Growdon, J., Lew, M., Waters, C., Vieregge, P., Pramstaller, P. P., Klein, C., Racette, B. A., Perlmutter, J. S., Parsian, A., Singer, C., Montgomery, E., Baker, K., Gusella, J. F., Fink, S. J., Myers, R. H., Herbert, A. (2003) A haplotype at the *PARK3* locus influences onset age for Parkinson's disease: The *Gene*PD study. *Neurology.* 61, 1557-1561

Karess, R. E., and Rubin, G. M. (1984) Analysis of P transposable element functions in *Drosophila. Cell.* 38, 135-146

Kennerdell, J. R., and Carthew, R. W. (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnol.* 18, 896-898

Kim, S. H., Kim, H. J., Kim, S., and Yim, J. (2010) *Drosophila* Cand1 regulates Cullin3-dependent E3 ligases by affecting the neddylation of Cullin3 and by controlling the stability of Cullin3 and adaptor protein. *Dev. Biol.* 346, 247-257

Kwak, S. S., Suk, J., Choi, J., Yang, S., Kim, J., Shon, S., Chung, J., Hong, Y. H., Lee, D. H., Ahn, J. K., Min, H., Fu, Y., Meadows, G. G., and Joe, C. O. (2011) Autophagy induction by tetrahydrobiopterin deficiency. *Autophagy.* 11, 1323-1334

Lacoste, A., Berenshteyn, F., and Brivanlou, A. H. (2009) An efficient and reversible transposable system for gene delivery and lineage-specific differentiation in human embryonic stem cells. *Cell Stem Cell.* 5, 332-342

Lam, G., and Thummel, C. S. (2000) Inducible expression of double-stranded RNA directs specific genetic interference in *Drosophila. Curr. Biol.* 10, 957-963

Levine, R. A., Kapatos, G., Kaufman, S., and Milstien, S. (1990) Immunological evidence for the requirement of sepiapterin reductase for tetrahydrobiopterin biosynthesis in brain. *J. Neurochem.* 54, 1218-1224

Lewis, E. B., and Bacher, F. (1968) Method for feeding ethyl-methanesulfonate (EMS) to *Drosophila* males. *Drosop. Inf. Serv.* 43, 193

Milstien, S., and Kaufman, S. (1989) Immunological studies on the participation of 6-pyruvoyl tetrahydropterin (2'-oxo) reductase, an aldose reductase, in tetrahydrobiopterin biosynthesis. *Biochem Biophys. Res. Commun.* 165, 845-850.

Misquitta, L., and Paterson, B. (1999) Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci. USA* 96, 1451-1456

Mitra, R., Fain-Thornton, J., and Craig, N. L. Misquitta (2008) *piggyBac* can bypass DNA synthesis during cut and paste transposition. *EMBO J.* 27, 1097-1109

Montgomery, M. K., Xu, S., and Fire, A. (1998) RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA* 95, 11502-15507

Nakamura, K., Bindokas, V. P., Kowlessur, D., Elas, M., Milstien, S., Marks, J. D., Halpern, H. J., and Kang U. J. (2001) Tetrahydrobiopterin Scavenges Superoxide in Dopaminergic Neurons. *J. Biol. Chem.* 37, 34402-34407

O'Hare, K., and Rubin, G. M. (1983) Structures of P transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. *Cell.* 34, 25-35

Park, Y. S., Heizmann, C. W., Wermuth, B., Levine, R. A., Steinerstauch, P., Guzman, J., and Blau, N (1991) Human carbonyl and aldose reductase: new catalytic functions in tetrahydrobiopterin biosynthesis. *Biochem. Biophys. Res. Commun.* 175, 738-744

Pastink, A., Heemskerk, E., Nivard, M., Van Vliet, C., and Vogel, E. (1991) Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and -deficient strains of *Drosophila melanogaster*. *Mol. Gen. Genet.* 229, 213-218

Piccin, A., Salameh, A., Benna, C., Sandrelli, F., Mazzotta, G., Zordan, M., Rosato, E., Kyriacou, C. P., and Costa, R. (2001) Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer. *Nucleic Acids Res.* 29, E55-5

Preston, C. R., Sved, J. A., and Engels, W. R. (1996) Flanking duplications and deletions associated with P-induced male recombination in *Drosophila*. *Genetics*. 144, 1623-1638

Rio, D. C. (1990) Molecular mechanisms regulating *Drosophila* P element transposition. *Annu. Rev. Genet.* 24, 543-578

Robertson, H. M., Preston, C. R., Phillis, R. W. Johnson-Schlitz, D. M., Benz, W. K., and Engels, W. R. (1988) A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics*. 118, 461-470

Rong, Y. S., and Golic, K. G. (2000) Gene targeting by homolgous recombination in *Drosophila. Science.* 288, 2013-2018

Rong, Y. S., and Golic, K. G. (2001) A targeted gene knock down in *Drosophila*. Genetics. 157, 1307-1312

Ryder, E., and Russel, S. (2003) Transposable elements as tools for genomics and genetics in *Drosophila*. *Brief Funct. Genomic Proteomic.* 2, 57-71

Seong, C., Kim, Y. A., Chung, H. J., Park, D., Yim, J., Baek, K., Park, Y. S., Han, K., and Yoon, J. (1998) Isolation and characterization of the *Drosophila melanogaster* cDNA encoding the sepiapterin reductase. *Biochim. Biophys. Acta.* 1443, 239-244

Shimizu, S., Ishii, M., Kawakami, Y., Momose, K., and Yamamoto, T. (1998) Protective effects of tetrahydrobiopterin against nitric oxide-induced endothelial cell death. *Life Sciences*. 18, 1585-1592

Spradling, A., and Rubin. G. M. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science.* 4570, 348-353

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S., Rubin, G. M. (1999) The Berkeley *Drosophila* Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics.* 153, 135-177

Stavely, B. E., Heslip, T. R., Hodgetts, R. B., and Bell, J. B. (1995) Protected *P*-element termini suggest a role for inverted repeat-binding protein in transposase-induced gap repair in *Drosophila melanogaster*. *Genetics*. 139, 1321-1329

Tang, M., Cecconi, C., Kim, H., Bustamante, C., and Rio, D. C. (2005) Guanisine triphosphate acts as a cofactor to promote assembly of initial P-element transposase-DNA synaptic complexes. *Genes Dev.* 19, 1422-1425

Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L., Ryner, L., Cheung, L. M., Chong, A., Erickson, C., Fisher, W. W., Greer, K., Hartouni, S. R., Howie, E., Jakkula, L., Joo, D., Killpack, K., Laufer, A., Mazzotta, J., Smith, R. D., Stevens, L. M., Stuber, C., Tan, L. R., Ventura, R., Woo, A., Zakrajsek, I., Zhao, L., Chen, F., Swimmer, C., Kopczynski, C., Duyk, G., Winberg, M. L., and Margolis, J. (2004) A complementary transposon tool kit for Drosophila melanogaster using P and *piggyBac. Nat. Genet.* 36, 283-287

Thony, B., Auerbach, G., and Blau, N. (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* 347, 1-16

Tobin, J. E., Cui, J., Wilk, J. B., Latourelle, J. C., Laramie, J. M., McKee, A. C., Guttman, M., Karamohamed, S., DeStefano, A. L., and Myers, R. H. (2007) Sepiapterin reductase expression is increased in Parkinson's disease brain tissue. *Brain Res.* 1139, 42-47

Voelker, R. A., Greenleaf, A. L., Gyurkovics, H., Wisley, G. B., Huang, S., and Searles, L. L. (1984) Frequent imprecise excision among reversions of a *P* element-caused lethal mutation in *Drosophila. Genetics.* 107, 279-294

Wang, H. G., and Fraser, M. J. (1993) TTAA serves as the target site for TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insec Mol. Biol.* 1, 109-116

Wang, X., McCullough, K. D., Franke, T. F., and Holbrook, N. J. (2000) Epidermal Growth Factor Recepter-dependent Akt Activation by Oxidative Stress Enhances Cell Survival. *J. Biol. Chem.* 275, 14624-14631

Wilson, M. H., Coates, C. J., and George Jr., A. L. (2007) *piggyBac* transposon-mediated gene transfer in human cells. *Mol. Ther.* 15, 139-145

Witsell, A., Kane, D. P., Rubin, S., and Mcvey, M. (2009) Removal of the bloom syndrome DNA helicase extends the utility of imprecise transposon excision for making null mutations in *Drosophila. Genetics.* 183, 1187-1193

Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H. K., and

Nagy, A. (2009) *piggyBac* transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature.* 458, 766-770

Yang, S., Lee, Y. J., Kim, J., Park, S., Peris, J., Laipis, P., Park, Y. S., Chung, J. H., and Oh, S. P. (2006) A Murine Model for Human Sepiapterin-Reductase Deficiency. *Am. J. Hum. Genet.* 78, 575-587

Yusa, K., Rad, R., Takeda, J., and Bradley, A. (2009) Generation of transgene-free induced pluripotent mouse stem cells by the *piggyBac* transposon. *Nat. Methods.* 6, 363-369

국문초록

트랜스포존이 유전체에서 이탈될 때 삽입 위치의 주변지역에서 결실이 유도되는 현상을 이용하는 돌연변이 제작 방법은 초파리 유전자의 결실 돌연변이를 유도하기 위해 가장 활발히 사용되는 실험 기법이다. *P*-element가 유전자 결실 돌연변이 초파리의 제작에 가장 널리 사용되지만, *P*-element가 유전체에 삽입 될 때 특정 지역을 선호하는 특징으로 인해 활용이 제한적이다. 피기백 트랜스포존은 유전체 상에서 특정 지역을 선호하지 않고 삽입되기 때문에 *P*-element가 삽입되지 않는 지역에 위치하는 유전자의 돌연변이를 유도하기 위해 개발되었다. 기존 연구 결과들은 피기백 트랜스포존이 유전체에서 이탈될 때 항상 주변 지역의 유전자 결실을 유도하지 않음을 보고하였다. 이러한 특징은 만능 유도 줄기세포의 제작과 같은 연구에서 피기백 트랜스포존이 가역적인 유전자 도입의 운반체로 사용될 수 있게 하였다. 그러나 이는 역시 피기백 트랜스포존이 유전자 결실 돌연변이 초파리 제작에 활용되지 못하게 하였다. 본 연구에서 저는 초파리의 세피압테린 환원효소의 유전자 좌에서 피기백 트랜스포존이 이탈될 때 삽입 위치의 주변지역에서 결실이 유도됨을 확인하였다. 이 새로운 현상을 활용하여 세피압테린 환원효소의 발현이 줄어든 세피압테린 환원효소 돌연변이 초파리를 성공적으로 제작하였다. 본 연구 결과는 피기백 트랜스포존이 이탈될 때 삽입 위치 주변지역에서 결실이 유도되는 현상을 활용하여 피기백 트랜스포존이 초파리 유전자 결실 돌연변이 제작에 활용될 수 있음을 제시한다. 또한 이 연구 결과는 피기백 트랜스포존이 만능 유도 줄기세포의 제작 과정에서 가역적인 유전자 운반체로 사용될 경우 예상치 못한 돌연변이를 일으킬 수 있음을 제시한다.

주요어: 트랜스포존; *P*-element; 피기백 트랜스포존; 돌연변이; 초파리; 가역적 유전자 운반; 세피압테린 환원효소

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