Western Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

4-19-2016 12:00 AM

A Genetic Test of a Model for Two Activities of Fushi Tarazu Protein in Drosophila Melanogaster

Alaa Briek
The University of Western Ontario

Supervisor
Dr. Anthony Percival-Smith
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
© Alaa Briek 2016

Follow this and additional works at: https://ir.lib.uwo.ca/etd

 Part of the Cell Biology Commons, and the Developmental Biology Commons

Recommended Citation

Briek, Alaa, "A Genetic Test of a Model for Two Activities of Fushi Tarazu Protein in Drosophila Melanogaster" (2016). *Electronic Thesis and Dissertation Repository*. 3698.
https://ir.lib.uwo.ca/etd/3698

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

Abstract

The important pair-rule segmentation gene *fushi tarazu (ftz)* encodes a homeodomain (HD)-containing protein involved in the establishment of even-numbered parasegments during embryonic development. The *D. melanogaster ftz* is a derived *homeotic selector (Hox)* gene which lost its homeotic function during the evolution of arthropods. Genetic analyses have shown that FTZ has two distinct activities required during development: HD-dependent and HD-independent FTZ activities. The aim of this study was to test the interaction of the two FTZ activities proposed by Hyduk and Percival-Smith (1996), by generating site-specific mutant *ftz* alleles for intragenic complementation. CRISPR-mediated homology directed repair (HDR) was used to introduce engineered *ftz* alleles into the *ftz* locus. Subsequently, four *ftz* engineered alleles were constructed in vectors for reintroduction by Recombinase-mediated cassette exchange (RMCE). Despite using multiple approaches no CRISPR mediated HDR events were detected, and therefore, the model could not be tested.

Keywords

Drosophila melanogaster, pair rule genes, *fushi tarazu*, homeodomain, FTZ-F1, segmentation, CRISPR/Cas9, homologous recombination, RMCE, intragenic complementation.

Co-Authorship Statement

I performed all experimental procedures described in this thesis and drafted the manuscript. My supervisor, Dr. Anthony Percival-Smith assisted in one injection experiment, injecting Cas9 flies with CRISPR DNA, helped in cloning the repair template, made intellectual contributions to the experimental design and provided editorial comments. Dr. Robert Cumming proofread the thesis.

Acknowledgments

By the end of the graduate work, my acknowledgement and many thanks are due to Dr. Anthony Percival-Smith, my supervisor, for the constant guidance, encouragement and advice he has provided throughout my time as his student. His immense knowledge helped me successfully carry out this project. I also extend my gratitude to my advisors, Dr. Robert Cumming and Dr. Sashko Damjanovski, for their help, support and encouragement.

I thank my friends and colleagues, the members of the APS lab: Laura Garofalo, Anirban Banerjee, Samantha Koot, Claire Newman and Sheng Cheng, as well as the volunteers who helped me out, Sina Fararooie and Houshang Azimi. I also acknowledge the help provided by all other honors students and volunteers who offered their time to the APS lab. Also, thanks to all members of the MGU for your assistance and cooperation in the core.

In addition, I thank my family, especially my father, who has empowered me throughout my journey here in Canada. I am really grateful for all the encouragements that you have provided for me until this final stage. Along with that I am really pleased to have a supportive family; my mother, sisters and brothers who always have a tremendous faith in me; I really cannot forget your support in making the decision to follow my dreams. Also, to my “Beesh”, I truly appreciate you being there for me during my ups and downs, and I am really blessed to have your unconditional love and support. Last but not least, I express my appreciation to King Abdullah Scholarship Program, for their financial support during my stay in Canada. I would not have reached this far in following my dreams without all above mentioned people and organization.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

aa	amino acid
act	*actin5c promoter*
AEL	after egg laying
ANT-C	*Antennapedia* Complex
A-P	anterior - posterior
att	attachment
bcd	*bicoid* (gene)
bp	base pair
BX-C	*Bithorax* Complex
Cas	CRISPR-associated
chiRNA	chimeric RNA
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DEPC	diethylpyrocarbonate
DSB	double-stranded break
dsDNA	double-stranded DNA
en	*engrailed (*gene)

eve	*even-skipped* (gene)
EXD	Extradenticle (protein)
Fst	*Frost*
ftz	*fushi tarazu* (gene)
FTZ –F1	Fushi tarazu-factor 1
ftz^{FL}	full length *ftz* (gene)
$ftz^{\Delta HD}$	*ftz* with a complete deletion of the HD (gene)
$ftz^{\Delta FTZ\text{-}F1}$	*ftz* with a deletion of FTZ-F1 binding site (gene)
FTZ^{TT}	Triple-tagged FTZ (protein)
gRNA	guide RNA
HD	Homeodomain
HDR	homology-directed repair
HR	homologous recombination
Hox	*Homeotic selector* (gene)
indels	insertions or deletions
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVT	*in vitro* transcription
LB	Luria Bertani
NHEJ	non-homologous end joining
nos	*nanos promoter*

nt	nucleotide
PAM	protospacer adjacent motif
PBS	phosphate buffered saline
RMCE	recombinase-mediated cassette exchange
TALE/TALEN	transcription activator-like effector / nuclease
tracrRNA	*trans*-activating CRISPR RNA
w^+	*miniwhite*$^+$ (gene)
wg	*wingless* (gene)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
y^+	*yellow*$^+$ (gene)
ZFN	zinc-finger nuclease

1 INTRODUCTION

1.1 *Drosophila melanogaster* as a model system

Drosophila melanogaster commonly known as the "fruit fly", is an attractive model system of great interest to researchers in the fields of molecular biology, genetics and neuroscience. The powerful genetic tools available for *D. melanogaster* allow investigators to elucidate the basis of complex traits, and gene-gene and gene-environment interactions. The *D. melanogaster* genome has been sequenced (Adams *et al*., 2000). In addition, *D. melanogaster* has a short life cycle, can be easily handled in laboratories and females have high fecundity. After fertilization, the egg is laid externally, which allows scientists to study its development very closely thereby offering a key model of development. How genes control development has been studied in great depth in *D. melanogaster* due to its well-understood cell biology, genetics and genome (Rubin, 1988). In addition, research into the genetics of *D. melanogaster* has been greatly assisted by the ability to introduce DNA into the genome. The most used method to introduce DNA into flies is P-element mediated transformation (Spradling & Rubin, 1982; Beall & Rio, 1998; Konev *et al*., 2003). Over the past ten years, methods for gene replacement via homologous recombination (HR) have also been developed (Rong & Golic, 2000; Horn & Handler, 2005), but due to the complexity and inefficiencies of the procedures, they are not used extensively (Huang *et al*., 2009).

1.2 Transgenic techniques used in *Drosophila*

1.2.1 P-element-mediated transformation

The P-element is a *Drosophila* transposable element that has interested researchers for many years (Majumdar & Rio, 2015). They are small transposons that have terminal 31 base pair (bp) inverted repeats, and generate 8 bp direct repeats of target DNA sequence upon insertion (Huang *et al*., 2009). P-elements are used for mutagenesis and the development of genetically modified flies used in genetic research (Venken & Bellen, 2007). P-element-mediated germ-line transformation is a powerful transgenic tool in *Drosophila*, especially when it is employed as an insertional mutagen or when it is

combined with tools such as the GAL4-UAS system (Brand & Perrimon, 1993). One important characteristic of P-elements is the random site of integration into the genome. Although the random nature of P-element integration is vital for generating insertion mutations and deletions (indels), it is not ideal for generating transgenic flies. The random integration of P-elements requires substantial efforts to map the site of insertion, and the genomic position of the insertion can influence the expression of the transgene thereby requiring analysis of multiple insertion lines to ensure that the alteration in phenotype is due to exclusively transgene expression and not an insertion artifact. Another disadvantage of the P-element system is its variable transformation effectiveness, a serious problem for large-scale transgenesis efforts (Bateman *et al*., 2006). Numerous strategies have been developed to overcome this issue of random insertion of transposons using systems based on the FLP and Cre recombinases (Siegal & Hartl, 1996; Venken & Bellen, 2007). These solutions allow precise targeting to genomic landing site, bypassing the need to analyze multiple independent insertions (Bateman *et al*., 2006).

1.2.2 TALENs and Zinc-finger nucleases

Zinc-finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) are important tools in modern biological research (Gaj *et al.*, 2013). These chimeric nucleases are composed of programmable, sequence-specific DNA-binding components associated with a general DNA cleavage domain. ZFNs and TALENs allow a comprehensive variety of genetic alterations by inducing DNA double-stranded breaks (DSBs) that activate error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) at precise genomic positions (**Figure 1**; Liu *et al*., 2012). The adaptability of ZFNs and TALENs emerge from the capability to customize the DNA-binding domain to recognize virtually any sequence (Carlson *et al*., 2012). These DNA-binding modules can be merged with various effector domains, including repressors recombinases, transposases, nucleases, transcriptional activators, histone acetyltransferases and DNA-histone methyltransferases to influence the genomic structure and function (Gaj *et al*., 2013). Therefore, the capacity to modify a gene is

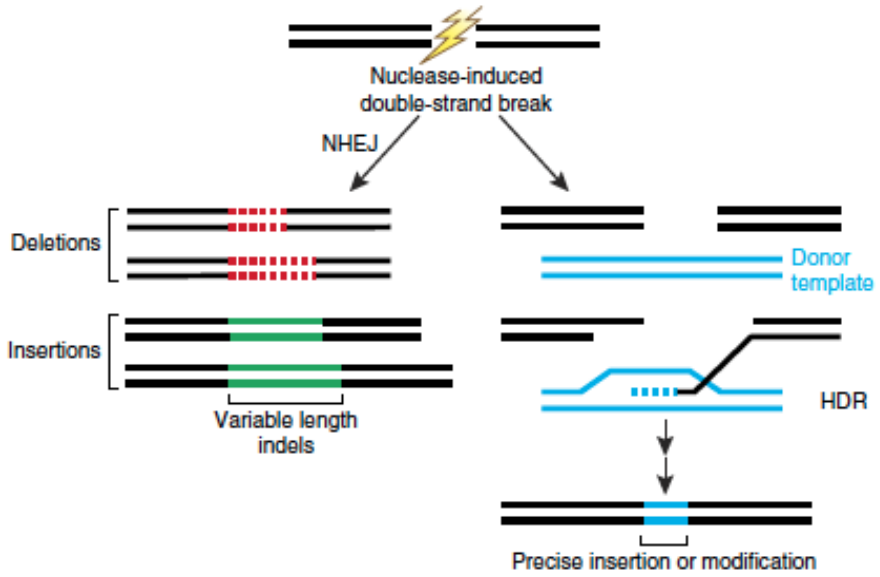


Figure 1. Nuclease-induced double-stranded breaks (DSBs) used for gene editing. The DSBs made in a genomic target site initiate DNA damage response pathways that repair the break either by non-homologous end joining (NHEJ), which result in indel mutations (red and green), or by homology-directed repair **(**HDR) in the presence of a repair template (blue), which lead to precise modification of the genome. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology (Sander & Joung), copyright (2014).

largely based on the DNA-binding affinity and specificity of designed zinc-finger and TALE proteins.

1.2.3 Recombinase-mediated cassette exchange

Recombinase-mediated cassette exchange (RCME) is the exchange of two specific DNA segments between two DNA molecules. The bacteriophage ΦC31 integrase is used to perform RMCE (Groth *et al*., 2004; Venken *et al*., 2011). The enzyme typically catalyzes specific, unidirectional, site-specific recombination between two attachment sites (*att* sites) called *attP* and *attB*. The ΦC31 integrase facilitates recombination between the two 39 base pair sequences, the *attP* site, which is usually pre-integrated into a *Drosophila* chromosome and serves as a target, or landing site, for precise integration of DNA carried on a plasmid with an *attB* site (Bischof *et al*., 2007). After recombination, the *attP* and *attB* sites are converted to *attR* and *attL* sites (**Figure 2**; Groth *et al*., 2004; Bateman *et al*., 2006). The incorporated or exchanged DNA is stably inherited and expressed.

1.2.4 Clustered regularly interspaced short palindromic repeats

Clustered regularly interspaced short palindromic repeats (CRISPR) is a relatively recent and novel genome engineering tool that is being employed to accomplish efficient, targeted, genetic modifications not only in *Drosophila*, but also in the genomes of many other model and non-model organisms (Cho *et al*., 2013; Cong *et al*., 2013; DiCarlo *et al*., 2013; Friedland *et al*., 2013; Gratz *et al*., 2013; Hwang *et al*., 2013; Wang *et al*., 2013). CRISPR was first discovered in 1987 in the *Escherichia coli (E. coli)* genome and has subsequently been shown to participate in adaptive bacterial immunity (Ishino *et al*., 1987; Barrangou *et al*., 2007). The CRISPR/ Cas9 system protects prokaryotes against foreign genetic elements (**Figure 3.A**; Bhaya *et al*. 2011). In the type II CRISPR system short RNA sequences complementary to the invading nucleic acids, the CRISPR RNA (crRNA), and a *trans*-activating CRISPR RNA (tracrRNA) direct the CRISPR-associated nuclease (Cas9) to introduce site specific DSBs in the exogenous invading DNA (Bhaya *et al*. 2011; Gaj *et al*., 2013).

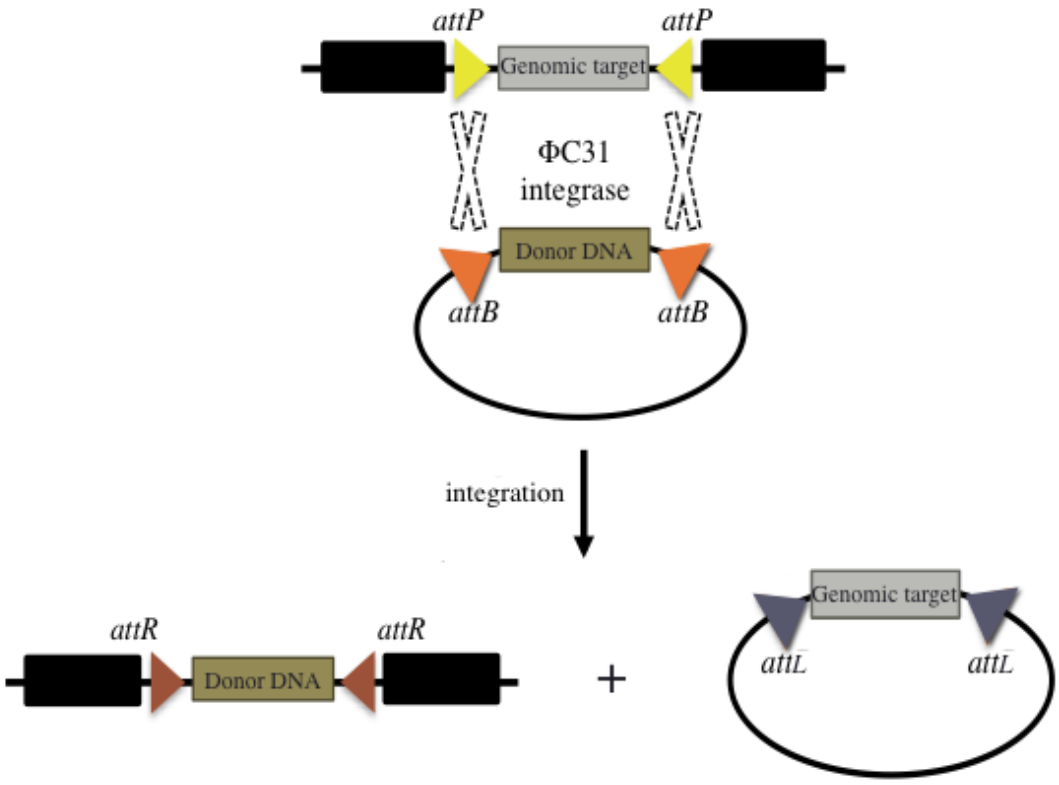


Figure 2. Site-specific integration via φC31 integrase-mediated cassette exchange. The ΦC31 integrase typically catalyzes specific, unidirectional, site-specific recombination between the *attP* sites (yellow triangles), which are usually pre-integrated into a genomic site for precise integration of DNA carried on a plasmid with *attB* sites (orange triangles). After recombination, the *attP* and *attB* sites are converted to *attR* sites (red triangles) in the host, and *attL* sites (grey triangles) in the donor plasmid.

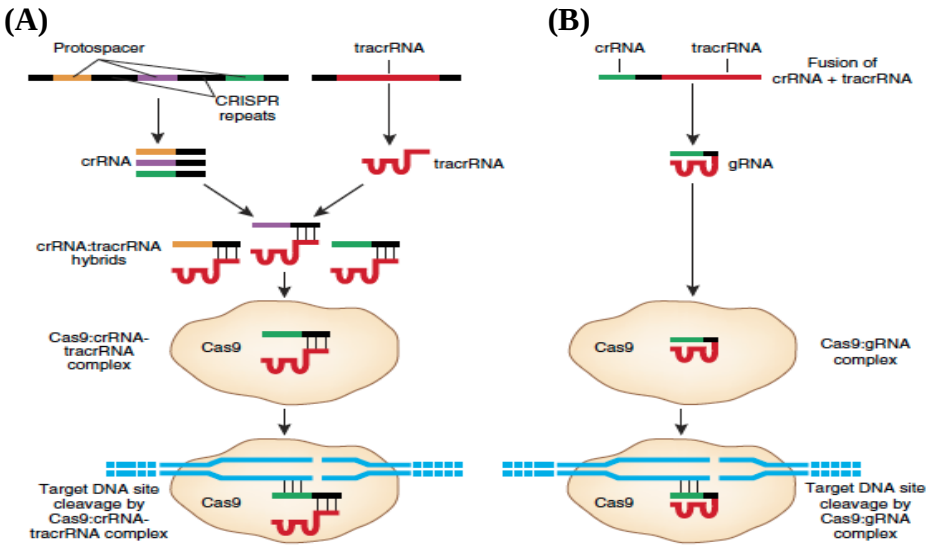


Figure 3. CRISPR/Cas9 system. The type II CRISPR system has been used as a highly efficient method for generating site-specific cleavage of double-stranded DNA. **(A)** CRISPR system in prokaryotes. Small fragments from invading DNA (called spacer) are incorporated between CRISPR arrays within the host genome, and separated by a short palindromic repeats. CRISPR arrays are then transcribed and transcripts produce crRNA, which now contain a sequence complementary to the foreign DNA (protospacer) and part of CRISPR repeats. TracrRNA hybridizes to the crRNA repeat region and complexes with the Cas9 nuclease to introduce site-specific double-stranded breaks next to the PAM sequence in the exogenous invading DNA (Bhaya *et al.* 2011; Gaj *et al.*, 2013; Sander & Joung, 2014). **(B)** The modified CRISPR/Cas9 system only requires RNA-guided Cas9 nuclease isolated from *Streptococcus pyogenes*, a synthetic chimeric RNA (chiRNA) or guide RNA (gRNA) containing both the crRNA and the tracrRNA (Jinek *et al.*, 2012). Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology (Sander & Joung), copyright (2014).

Recently, the type II CRISPR system has been used as a highly efficient method for generating site-specific cleavage of double-stranded DNA (Gratz *et al*., 2013). CRISPR/Cas9 system used is a modification of the original type II system. The modified system still uses an RNA- guided Cas9 nuclease isolated from *Streptococcus pyogenes*, but has introduced a synthetic chimeric RNA (chiRNA) containing both the crRNA and the tracrRNA (**Figure 3.B**; Jinek *et al*., 2012). This advance established a simple two-component CRISPR system for genome editing, as it only requires a binary Cas9/chiRNA riboprotein to create site-specific DSBs in the host DNA. The target site recognition of CRISPR/Cas9 depends on the chiRNA that contains 20 bases of sequence complementary with the targeted genomic DNA sequence, and targets DNA in the genome for cleavage when it is followed by a protospacer adjacent motif (PAM) sequence, "NGG". The 20-base chiRNA recognition sequence and the PAM sequence make the CRISPR system very specific because the chance that an identical sequence of greater than 20 bases occurs twice in a genome is low (Horn & Handler, 2005; Gratz *et al*., 2013). The DSBs made in a genomic target site initiate DNA damage response pathways that repair the break either by NHEJ, which is error-prone, or by HR in the presence of a repair template, which can lead to precise modification of the genome (Capecchi, 1989; Banga & Boyd, 1992; Rong & Golic, 2000; Gratz *et al*., 2013). TALENs and ZFNs are chimeric nucleases that induce DSBs (Bibikova *et al*., 2002). However, these methods are laborious, time consuming and and the specificity is mediated through proteins not complementary nucleotide sequences relative to the simpler CRISPR system (Huang *et al*., 2009; Gaj *et al*., 2013).

1.3 Development of *Drosophila melanogaster*

D. melanogaster has an average life span of 30 days. Development of an adult fly takes 10 days in a complex life cycle composed of four major stages: embryonic, larval, pupal and adult. First, embryogenesis, the development of a larva from a fertilized egg, is composed of a number of sub-stages: syncytial blastoderm, cellular blastoderm, gastrulation, germband extension and germband retraction. Embryogenesis takes one day after which a first instar larva hatches. This larva eats, grows and molts to give the second instar larva, which grows and molts to give the third instar larva. The three larval stages

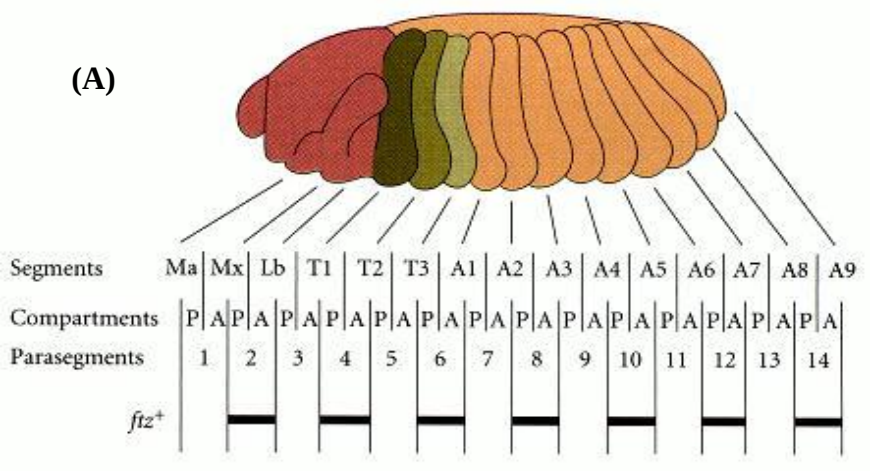
take around 4-5 days to complete. After this, the larva forms a pupal case, entering the pre-pupal and the pupal stages. In the pupal stage, the fly transforms from the larval form to an adult in a process called metamorphosis. Finally, a sexually reproductive, adult fly ecloses from the pupal case.

1.4 Segmentation in *Drosophila melanogaster*

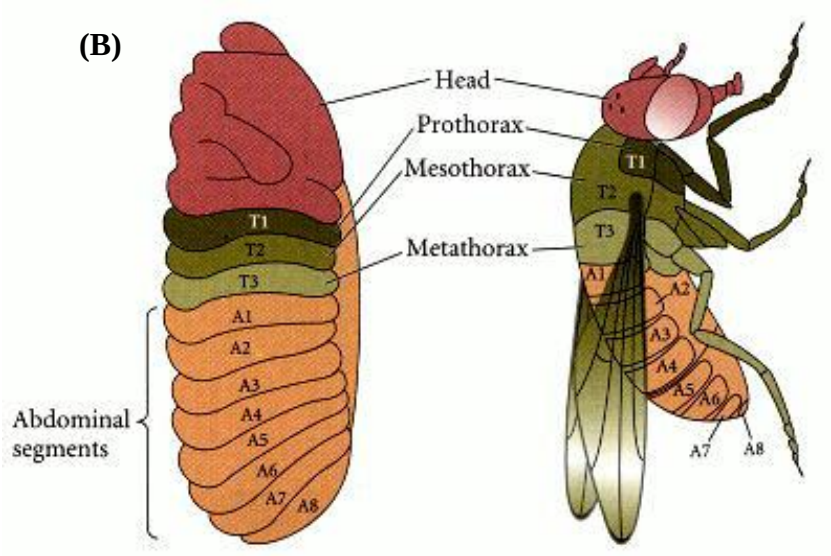
The development of the fertilized *D. melanogaster* egg into a larva and then an adult fly depends on proper pattern formation along the anterior-posterior (A-P) axis. Much research has been done on body pattern formation in *Drosophila*, and these studies have shown that this pattern formation is controlled by the hierarchical interaction between specific set of genes: maternal-effect genes, gap genes, pair-rule genes, segment polarity genes and *Homeotic selector (Hox) genes*. These hierarchal interactions give rise to a segmented larva. *Drosophila* embryogenesis has two distinct segmental registers: the first register, functioning early during embryogenesis, is the fourteen parasegments which later during development is transformed to give rise to the second register, the larval/adult segments that are composed of the posterior of one parasegment and the anterior of the next (**Figure 4.A**; Nüsslein-Volhard & Wieschaus, 1980; Martinez-Arias & Lawrence, 1984). Anterior-posterior pattern formation is initiated by signals provided to the developing egg from the maternal genome during oogenesis. These signals are messenger RNA molecules transcribed from the maternal-effect genes of the maternal germ-line genome. These RNA signals are important for determining the polarity of the developing embryo. The mRNA of the maternal-effect gene *bicoid (bcd)* defines the anterior end of the embryo while the mRNA of *nanos (nos)* gene defines the posterior end. The maternal-effect proteins regulate the expression of the first zygotically expressed genes, known as gap genes. The gap genes are expressed in broad domains along the A-P axis, and divide the embryo into broad regions. Gap proteins regulate the transcription of the pair-rule genes. The pair-rule genes are expressed in seven stripes of cells. Two pair-rule genes are very important for segmentation: *fushi tarazu (ftz)* and *even-skipped (eve*). The pair-rule gene *ftz* is involved in the establishment of even-numbered parasegments whereas *eve* defines the odd numbered parasegments (Maier *et*

Figure 4. *Drosophila* segments and parasegments. Two pair-rule genes are very important for segmentation. **(A)** During the cellular blastoderm stage, *ftz* establishes the even numbered parasegments; whereas, *eve* defines the odd numbered parasegments, and in doing so, *ftz* and *eve* form the first register, the fourteen parasegments, which later during development are transformed to give rise to the second segmental register the larval/adult segments that are composed of the posterior of one parasegment and the anterior of the next. **(B)** Once segments are established, *Hox* genes give identity to each segment, three head segments; Mandibular, Maxillary and Labial, the three thoracic segments; T1, T2 and T3. The eight abdominal segments (A1-A8). Reprinted by permission from Developmental Biology, by Gilbert, Scott F., 8th edition, Chapter 9, Sinauer Associates Inc.; 2006.

(A)


Segments
Ma Mx Lb T1 T2 T3 A1 A2 A3 A4 A5 A6 A7 A8 A9
Compartments
P A P A P A P A P A P A P A P A P A P A P A P A P A P A
Parasegments
1 2 3 4 5 6 7 8 9 10 11 12 13 14
ftz+

(B)


Head
Prothorax
Mesothorax
Metathorax
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
Abdominal segments

al., 1990), and in doing so, set up the parasegmental boundaries. The protein products of pair-rule genes regulate the transcription of the segment polarity genes, which are responsible for establishing the A-P axis of each segment of the embryo (Lawrence *et al*., 1987; Carroll *et al*., 1988). Once segments are established, all segmentation genes (gap genes, pair-rule genes, segment polarity genes) interact to regulate the *Homeotic (Hox)* genes that give identity to each segment (**Figure 4.B**; Mcginnis & Krumlauf 1992). For example, the genes of the *Antennapedia Complex (ANT-C)* control the formation of the three head segments; Mandibular, Maxillary and Labial, and the three thoracic segments; T1, T2 and T3 (Struhl, 1982). The segmental identities of the remaining eight abdominal segments (A1-A8) are controlled by the genes of the *Bithorax Complex (BX-C)* (Lewis, 1978; Martinez-Arias & Lawrence, 1984).

1.5 *Fushi tarazu*

Fushi tarazu (ftz) is one of the best studied genes in *D. melanogaster*. The *ftz* gene was originally isolated using positional cloning (Wiener *et al*., 1984). The expression patterns of *FTZ* mRNA and FTZ protein have been investigated extensively (Hafen *et al*., 1984; Krause *et al*., 1988). FTZ is expressed in two different phases of *D. melanogaster* development. First, FTZ is expressed in seven stripes, during the syncytial and cellular blastoderm and gastrulation stages, where FTZ plays a role in regulating segmentation (Krause *et al*., 1988). The seven stripes of FTZ expressed at the cellular blastoderm stage correlate with the cell of the future even-numbered parasegments. The seven FTZ expression stripes that define the even-number parasegments are interspersed with seven stripes of EVE expression that define the complementary odd-numbered parasegments. The second stage of expression is later during neurogenesis during the germband extension stage of embryogenesis where *ftz* is expressed in specific neurons (aCC, pCC, RP1 and RP2) (Doe *et al*., 1988; Carroll & Scott, 1985).

1.6 Fushi tarazu known protein domains

The *ftz* gene of *D. melanogaster* is a *Hox*-derived gene located within *ANT-C*, on the right arm of the third chromosome (Wakimoto & Kaufman, 1981; Scott *et al*., 1983). FTZ has three known protein domains: The HD, the FTZ-F1 binding site, and the PEST

degradation sequence. The HD, which is a conserved DNA binding domain encoded by the 180 bp homeobox sequence, interacts with a specific DNA binding site (Laughon & Scott, 1984). The HD is composed of 60 amino acids (aa) (McGinnis *et al*., 1984) which fold into a compact domain consisting of three alpha helixes connected by short loops, with two of the helixes forming a helix-turn-helix. This type of structure found in many other DNA binding domains (Laughon & Scott, 1984; Schier & Gehring, 1992). The first two helices are parallel, while the third helix is perpendicular to the axes of the first two helices. The third helix is the one that makes specific amino acid base contacts with DNA. HD-containing proteins have various roles, which include cellular differentiation and maintenance of pluripotency (McGinnis *et al*., 1984). The FTZ-F1 domain was first identified as binding to the zebra element of the *ftz* promoter; this *cis*-acting DNA sequence is found upstream of the *ftz* transcriptional start site (Hiromi *et al*. 1985). The zebra elements are involved in the regulation of *ftz* expression in the pair-rule periodicity in the embryo formation (Ueda *et al*, 1990). FTZ-F1 is an orphan nuclear receptor that is a DNA-binding transcription factor, which is expressed throughout development (Yu *et al*., 1997). In addition to binding to zebra elements, FTZ-F1 also binds to the FTZ protein via the FTZ-F1 binding site (Ueda *et al*, 1990; Guichet *et al*., 1997; Yu *et al*., 1997; Schwartz *et al.,* 2011). The formation of a FTZ/FTZ-F1 complex is required for segmentation, and is the reason why segmentation is determined by a FTZ HD-independent activity (Fitzpatrick *et al*., 1992). Lastly, the PEST degradation sequence is located between amino acids 207 to 218 in FTZ. The PEST region is rich in proline (P), glutamic acid (E), serine (S) and threonine (T), and plays a role in maintaining the stability of FTZ and also acts as a signal for its degradation (Kellerman *et al*., 1990).

The *D. melanogaster ftz* and *Hox* genes have evolved from a common ancestral gene which encodes a homeodomain (HD)-containing protein. During the evolution of *D. melanogaster*, *ftz* has acquired a non-homeotic function in segmentation (Alonso *et al*., 2001; Heffer *et al*., 2013). This is believed to have occurred due to loss of the HOX-specific interaction motif (YPWM), which is a common HOX interaction motif mediating the interaction with the HOX cofactor Extradenticle (EXD) (Heffer *et al*., 2013), and the gain of a new motif (LXXLL) that mediates interaction with another cofactor, FTZ-F1 (**Figure 5;** Yussa *et al*., 2001; Schwartz *et al*., 2001; Löhr & Pick, 2005). FTZ-F1 is

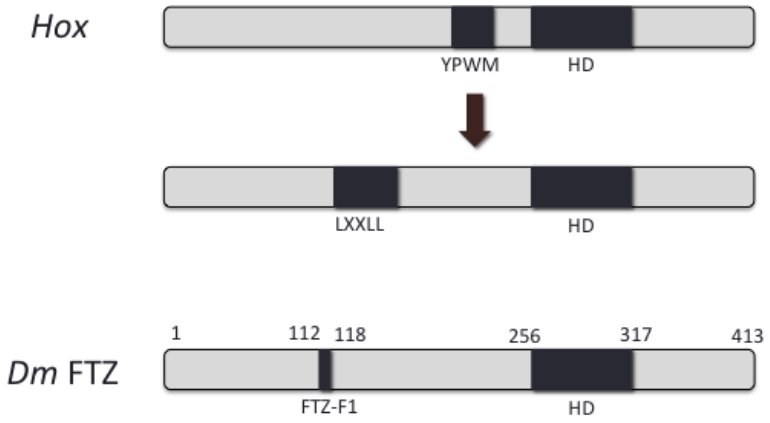


Figure 5. Evolution of *ftz* in *D. melanogaster*. The gene *ftz* arose from a *Hox* gene that during evolution lost its homeotic function by losing the YPWM motif, and gained a segmentation function by gaining the LXXLL motif. The FTZ protein in *D. melanogaster* has two major domains: FTZ-F1 binding site that is required for *ftz* segmentation activity, and a DNA-binding HD that is required for CNS development.

required with FTZ for segmentation, as previous studies have shown that embryos lacking FTZ-F1 expression had a ftz phenotype (**Figure 6**; Guichet *et al*., 1997; Yu *et al*., 1997). The FTZ protein has two distinct activities differentially required during development. The two FTZ activities are the HD-dependent and HD-independent FTZ activities. The HD-independent FTZ activity is required for FTZ function in segmentation, and regulating the expression of key segmentation genes such as increasing *engrailed (en)* and repressing *wingless (wg)* expression, respectively. The expression of *en* and *wg* in adjacent cells sets up the parasegmental boundary. The HD-dependent FTZ activity is proposed to be required for the accumulation of a high level of FTZ expression through early autoactivation of *ftz* via *ftz* enhancer (Schier & Gehring, 1992; Hyduk & Percival-Smith, 1996). Recently, HD-dependent FTZ activity has been investigated in more detail in the Central Nervous System (CNS). Studies of *ftz* have shown that the expression of the pair rule gene *eve* in RP2 neurons in the developing CNS requires the FTZ HD-dependent activity as these neurons grow abnormally in embryos that lack the FTZ HD-dependent activity (Doe *et al*., 1988; Heffer *et al*., 2013).

1.7 Rationale and objectives

Genetic analyses have shown that both the HD-dependent and HD-independent FTZ activities are somehow required for segmentation, but that the HD-independent FTZ activity, in specific situations, is sufficient for segmentation (Fitzpatrick *et al*., 1992). To reconcile the requirement of the HD dependent FTZ activity and the HD-independent FTZ activity for segmentation, a model was proposed by Hyduk and Percival-Smith (1996) (**Figure 7)**, in which the two FTZ activities operate at different times during *D. melanogaster* development. During the first stage, the FTZ HD-dependent transcriptional activation occurs in the cellular blastoderm embryo, where the FTZ HD binds directly to *ftz* enhancer activating high levels of FTZ expression through autoactivation. Later, during gastrulation, the FTZ HD-independent transcriptional activity is required to activate *ftz* enhancer, express EN and to establish a FTZ dependent cuticle (Hyduk & Percival-Smith, 1996). My project aimed at testing this model further by showing intragenic complementation of two engineered *ftz* alleles. My goal is to establish an expression system for FTZ by modifying the *ftz* locus using the CRISPR/Cas9 system.

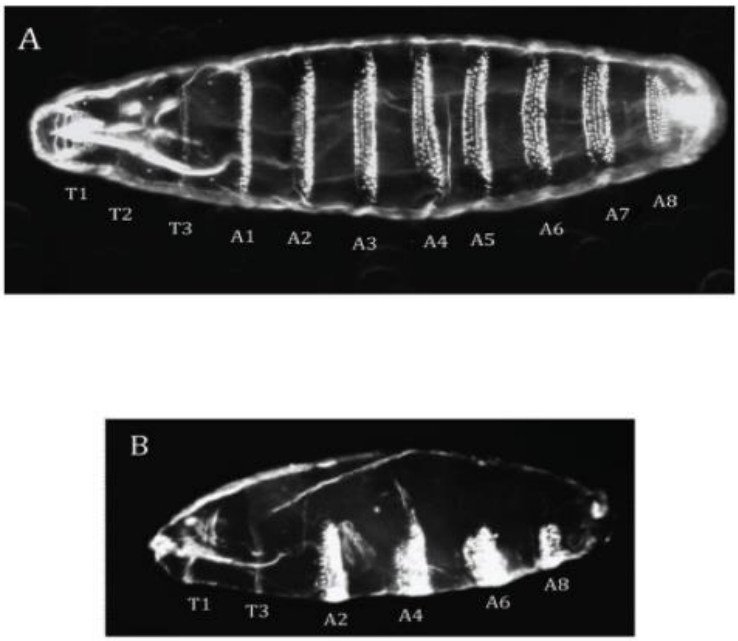


Figure 6. *ftz* phenotypes in *D. melanogaster* larval cuticle. (A) A wild type larva established all segments (T1, T2, T3, A1, A2, A3, A4, A5, A6, A7 and A8). The larval cuticle derived from *ftz* expression is the even-numbered parasegments (denticle belts of T2, A1, A3, A5 and A7), and the larval cuticle derived from *eve* expression is the odd-numbered parasegments (denticle belts of T1, T3, A2, A4, A6 and A8). **(B)** *ftz* mutant phenotype where the embryo failed to form the even-numbered parasegments, the larva developed from cuticle derived from the odd-numbered parasegments (T1, T3, A2, A4, A6 and A8) Nüsslein-Volhard & Wieschaus, 1980; Martinez-Arias & Lawrence, 1984). Reprinted by permission from Elyse Burlingham, (Burlingham, 2012)

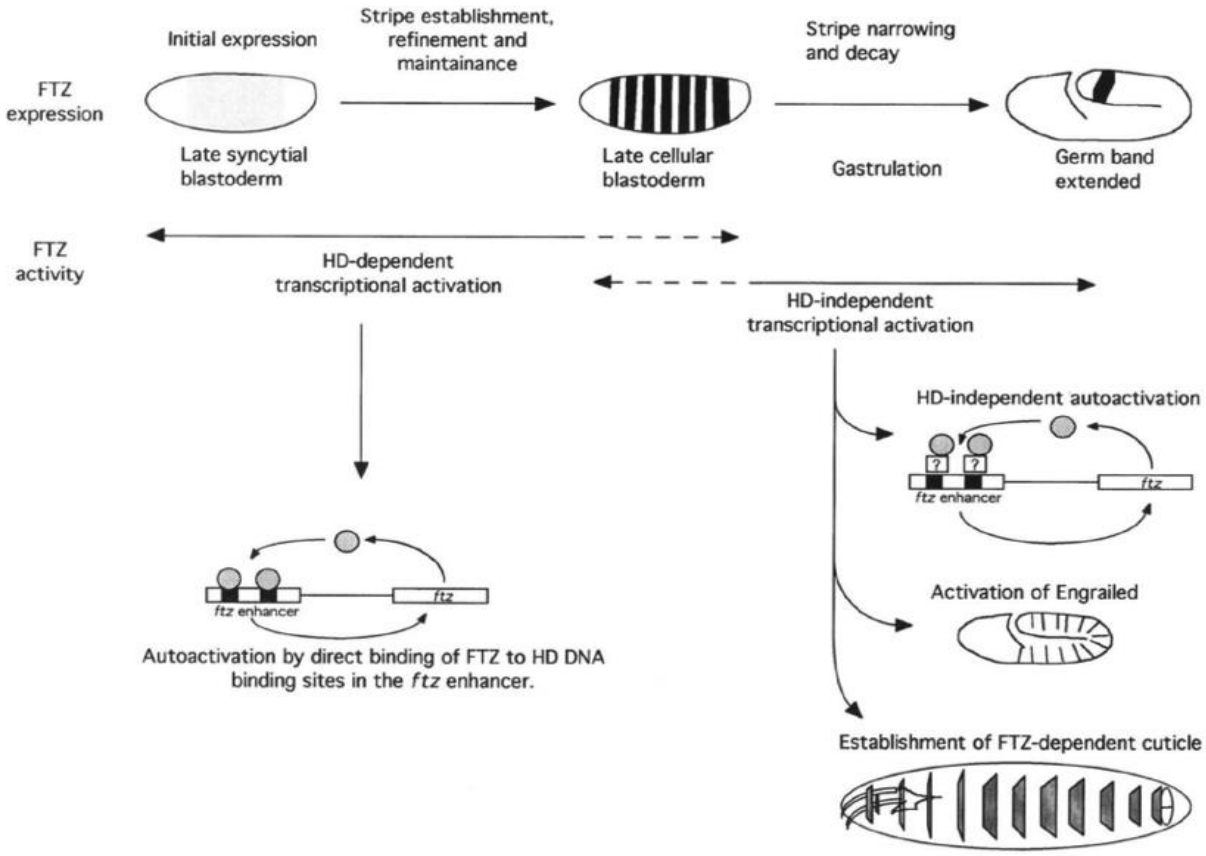


Figure 7. The model proposed by Hyduk and Percival-Smith (1996). The two FTZ activities operate at different times during *D. melanogaster* development. First to operate is the FTZ HD-dependent transcriptional activation in the cellular blastoderm embryo, where the FTZ HD binds directly to *ftz* enhancer activating high levels of FTZ expression through autoactivation. Later, during gastrulation, the FTZ HD-independent transcriptional activity is required to activate *ftz* enhancer, express EN and to establish FTZ dependent cuticle. Figure reproduced with permission of GENETICS SOCIETY OF AMERICA [ETC.] in the format Republish in a thesis/dissertation via Copyright Clearance Center.

First, I will modify the *ftz* locus such that the DNA will encode for three FTZ proteins: a full length- wild type FTZ (FTZ^{FL}), a FTZ polypeptide with a complete deletion of the HD ($FTZ^{\Delta HD}$), and a FTZ polypeptide with a deletion of FTZ-F1 binding site ($FTZ^{\Delta FTZ\text{-}F1}$) (**Figure 8**). Based on the model proposed by Hyduk and Percival-Smith, I expect ftz^{FL} larvae to develop normally with all even numbered parasegments, because this locus expresses both HD-dependent and HD-independent FTZ activities. Larvae expressing $ftz^{\Delta HD}$ and $ftz^{\Delta FTZ\text{-}F1}$ mutated genes will exhibit a ftz phenotype, because the $ftz^{\Delta HD}$ allele can not express sufficient FTZ protein to establish segmentation, and the $ftz^{\Delta FTZ\text{-}F1}$ allele lacks the HD-independent activity required for segmentation. However, to test the model that FTZ has two activities, embryos that are hemizygous for the $ftz^{\Delta HD}$ and $ftz^{\Delta FTZ\text{-}F1}$ alleles should result in intragenic complementation, where enough HD- independent FTZ activity is present to generate the wild type phenotype (**Figure 9**).

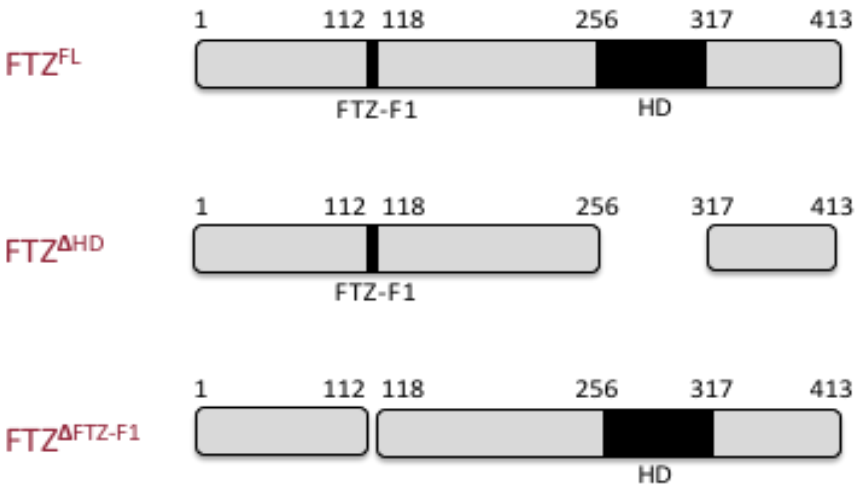


Figure 8. The structure of different FTZ proteins involved in this study. A full length FTZ (FTZ^{FL}), a FTZ polypeptide with a deletion of FTZ-HD ($FTZ^{\Delta HD}$), a FTZ polypeptide with a deletion of the FTZ-F1 binding site ($FTZ^{\Delta FTZ\text{-}F1}$).

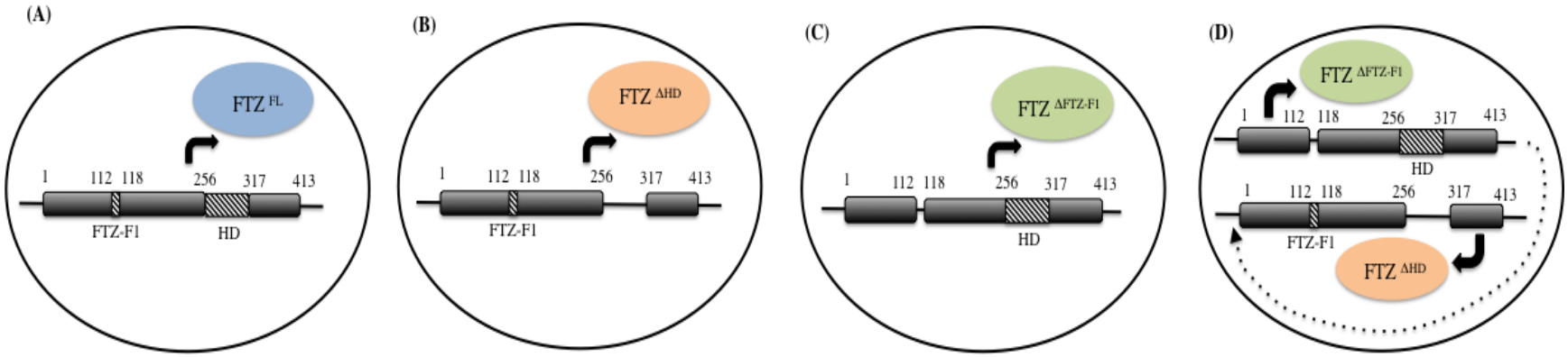


Figure 9. Intragenic complementation to test FTZ-HD requirement in segmentation. (A) Embryos expressing the *ftz*^{FL} gene will develop normally since the protein product of *ftz*^{FL} allele has both HD-dependent and independent FTZ activities. **(B)** The HD-dependent FTZ activity is responsible for the accumulation of high-levels of FTZ via the *ftz* enhancer in the late cellular blastoderm stage. Therefore, *ftz*$^{\Delta HD}$ embryos express a low-level of FTZ HD-independent activity and fail to form the even numbered parasegments. The abnormal CNS development is due to the inability of FTZ HD-dependent activity to regulate EVE expression in RP2 neurons. **(C)** Since FTZ/FTZ-F1 interaction is required for segmentation, *ftz*$^{\Delta FTZ\text{-}F1}$ embryos will show a ftz phenotype. **(D)** The intragenic complementation between *ftz*$^{\Delta HD}$ and *ftz*$^{\Delta FTZ\text{-}F1}$ alleles will result in wild-type embryos. The protein product of *ftz*$^{\Delta FTZ\text{-}F1}$ allele has a FTZ HD-dependent activity, which activates the high-levels of FTZ expression from *ftz*$^{\Delta HD}$ allele. The allele that lacks the HD will then continue to activate EN, repress WG and establish the even numbered parasegments.

2 MATERIALS AND METHODS

2.1 Construction of chiRNAs for CRISPR

Two chiRNAs were designed to recognize a specific sequence near the 3' donor and 5' acceptor splicing sites of the *ftz* intron between *ftz* exon 1 and exon 2. CRISPR chiRNA requires 20 nt homology to recognize the complementary target sequence in the genome followed by the PAM sequence (NGG) (**Figure 10**). The 5' phosphorylated primers (ordered from Invitrogen) encoding 20 nt complementary to the targets in *ftz* were designed (**APPENDIX 1**). The primers were annealed, and inserted into the cohesive ends generated by *BbsI* in pU6-*BbsI*-chiRNA vector (Gratz *et al*. 2013). Ligation of the vector and annealed fragments was carried out at 18°C using T4 DNA ligase (New England Biolabs) and the ligated mixture was transformed into subcloning-competent DH5α cells (Invitrogen). Ampicillin (Amp)-resistant colonies were selected on LB+100 µg/ml Amp plates. A Mini Plasmid Kit (Geneaid) was used to isolate the plasmid DNA. Restriction digest with *BbsI* verified that the desired chiRNA-encoding plasmids were generated (due to loss of *BbsI* restriction site following ligation). The constructs were sent for sequencing analysis at the DNA Sequencing Facility at Robarts Research Institute, London, Ontario, Canada for further confirmation. Finally, a QIAfilter Plasmid Midi Kit (Qiagen) was used to isolate the plasmid at high concentrations for microinjection.

2.2 Construction of repair vector for CRISPR

The repair vector was designed to contain a 5' and a 3' homology sequence homologous to *ftz*, separated by a *yellow*$^{+}$ *(y*$^{+}$*)* body colour marker gene flanked by two inverted *attP* docking sites for Recombinase-mediated cassette exchange (RMCE). The repair vector was constructed by the ordered ligation of four DNA fragments, the 5' and 3' sequences of *ftz* exons flanked by *attP* sequence and which contained *BsaI* restriction sites, introduced during PCR amplification from *Drosophila* genomic DNA. Primers were used to amplify the *yellow* gene from *NotI*-digested MiMIC plasmid (GenBank: GU370067; Venken *et al*., 2011) and designed to add *BsaI* sites to each end. The 5', 3' sequences of

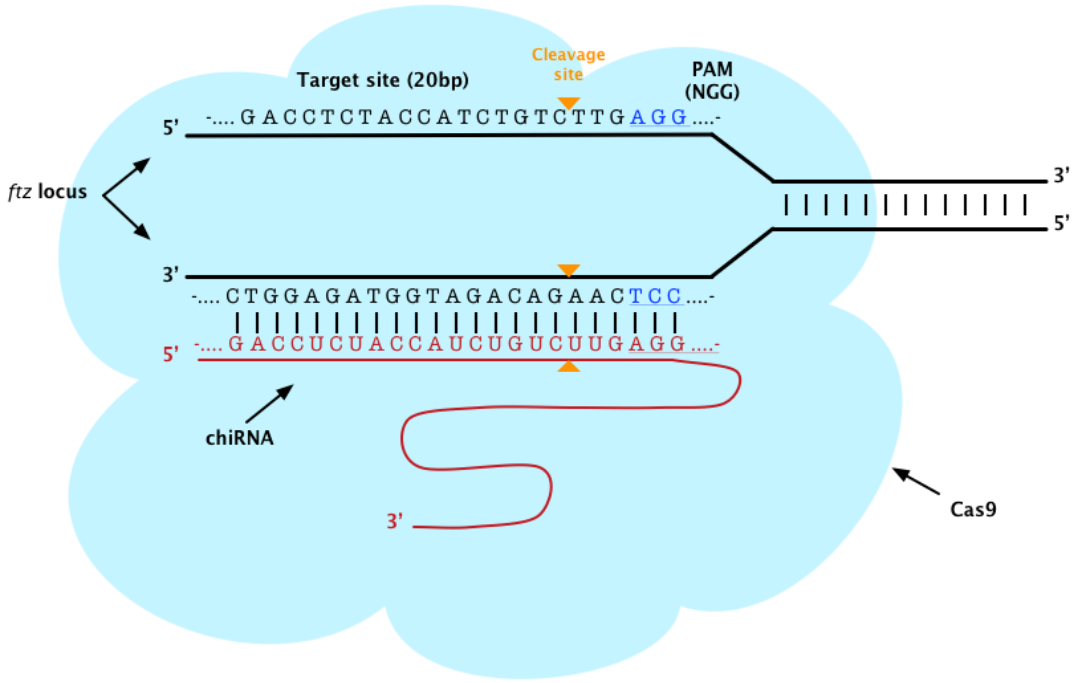


Figure 10. The site targeted in *ftz* by the 5' chiRNA/Cas9 riboprotein. The efficient target recognition of CRISPR chiRNA to induce DSBs requires 20 nt to recognize its complementary target sequence in the genome (black letters) followed by PAM sequence NGG (blue underlined letters).

ftz and y^+ (ordered from Invitrogen; **APPENDIX 2**) were generated by PCR from the appropriate template using a High Fidelity Platinum *Taq* Polymerase (Invitrogen). The DNA fragments were purified using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Canada Inc.). The fragments were digested with *BsaI* (New England Biolabs) to generate cohesive ends to ligate into the pFUS_A vector. The pFUS_A was also digested with the restriction endonuclease, *BsaI* and the 5' phosphates removed with calf intestinal phosphatase. All fragments were ligated together in a single reaction using T4 DNA ligase (New England Biolabs) (**Figure 11**). Subcloning efficiency competent cells DH5α (Invitrogen) were transformed with the ligation mixture and selected on LB+100µg/ml Spectinomycin (Spec) plates that were spread with 40µl 8% Xgal and 40µl 200µM IPTG spread prior to plating. Digestion of pFUS_A with *BsaI* removes the *LacZ* gene, and therefore, white-colored Spec resistant colonies were screened for. White colonies were re-streaked on LB+100µg/ml Spec plates for verification, followed by colony PCR using primers that were designed to amplify the *yellow* marker gene (**APPENDIX 3**). The PCR product was run on a 1% agarose gel to verify the presence of the *yellow* gene fragment. A Mini Plasmid Kit (Geneaid) was used to isolate plasmid DNA from a white-colored single colony, the structure of the repair vector was verified by restriction enzyme analysis. Finally, a QIAfilter Plasmid Midi Kit (Qiagen) was used to isolate the plasmid at high concentrations for microinjection purposes.

2.3 *In vitro* transcription of *ftz* and *Fst* chiRNAs for CRISPR

Two chiRNAs were designed for targeting the *ftz* and *Frost* (*Fst)* genes. The forward primer of the chiRNAs was designed to contain *ftz* or *Fst* sequences and the T7 RNA polymerase promoter sequence. The first two bases of the 20 base genome target sequence always start with GG, which is required for T7 RNA polymerase, and followed by the PAM (NGG) sequence:
(GAAATTAATACGACTCACTATAGGN_{18}GTTTTAGAGCTAGAAATAGC), where GGN_{18} is either *ftz* or *Fst* sequence. One common reverse primer was designed to encode the remainder of the chiRNA
(AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTAT

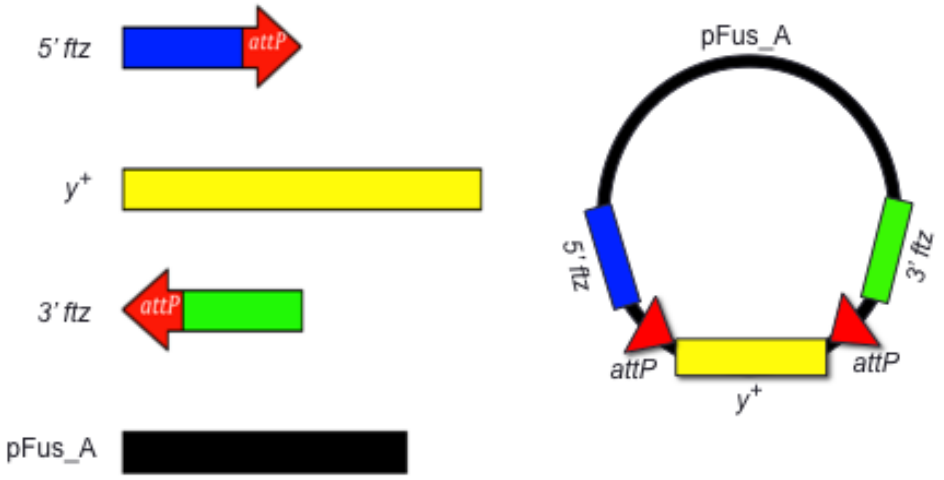


Figure 11. The structure of the repair vector for CRISPR. The repair vector contains the 5' and 3' sequences of *ftz* exons, separated by a y^+ gene flanked by two *attP* docking sites. Primers were designed to add *BsaI* sites to each end. The 5'and 3' sequences of *ftz* and y^+ were generated by PCR technology each with the appropriate template using a High Fidelity Platinum *Taq* Polymerase. The fragments were then digested with *BsaI* to generate unique cohesive ends to ligate with the pFUS_A vector, which was also digested with the restriction endonuclease, *BsaI*. All fragments were ligated together in a single reaction.

TTTAACTTGCTATTTCTAGCTCTAAAAC) (Bassett *et al*, 2013). The two primers (ordered from Invitrogen) hybridized to one another and a DNA fragment was generated using PCR (**Figure 12**; **APPENDIX 4**). The DNA fragment was purified with QIAquick PCR Purification Kit (Qiagen). For *in vitro* transcription (*IVT*), 0.5 µg of PCR product was used for 50µl total volume reaction using Megascript T7 Kit (Ambion) and incubated overnight. ChiRNAs were purified by phenol chloroform extraction and ethanol precipitation. To avoid RNAse in solutions, MilliQ water, 3M sodium acetate pH=5.2 and PBS were DEPC treated. Cas9 mRNA was obtained from Invitrogen.

2.4 Design of *ftz* constructs for RMCE

Four *ftz* constructs were designed for RMCE to create modified *ftz* loci *in vivo*. The ΦC31 integrase catalyzes the integration between the *attP* sites at the *ftz* $^{attP\ y+\ attP}$ locus, and the *attB* sites in the plasmids that contain DNA constructs encoding four FTZ proteins (FTZ^{FL} , $FTZ^{\Delta HD}$, $FTZ^{\Delta FTZ\text{-}F1}$ and FTZ^{TT}) involved in this study (**Figure 13)**. The *ftz* constructs were obtained from four ectopic expression constructs (obtained from the Percival-Smith Lab) by restriction digestion with *NotI*. The digests were separated by 2% agarose gel electrophoresis, and the *ftz* DNA fragments isolated using Gel/PCR DNA fragments Extraction Kit (Geneaid). The isolated fragments were ligated to *NotI*-digested pBS-SK plasmid containing inverted *attB* sites, previously generated by Laura Garofalo (University of Western Ontario, Canada). Ligation was carried out at 18°C using T4 DNA ligase (New England Biolabs) and the ligated fragment was transformed into subcloning efficiency competent cells DH5α (Invitrogen). Ampicillin resistant colonies were selected on LB+100 µg/ml Amp plates. Mini Plasmid Kit (Geneaid) was used to isolate plasmid DNA from a single colony and verified by restriction enzyme analysis. The constructs were sent for sequencing analysis at the DNA Sequencing Facility at Robarts Research Institute, London, Ontario, Canada for further confirmation. Finally, a QIAfilter Plasmid Midi Kit was used to isolate the plasmid at high concentrations for microinjection purposes.

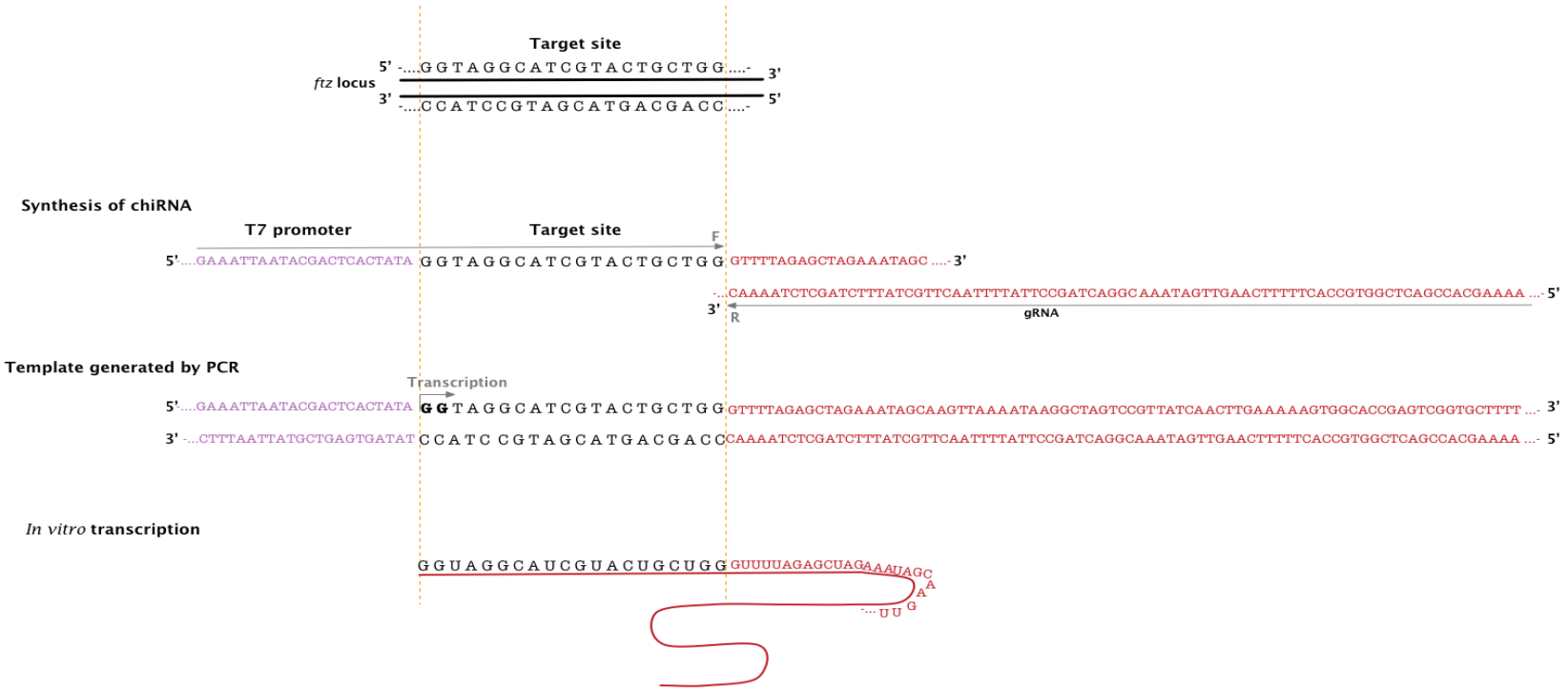


Figure 12. The site targeted in *ftz* by the synthesized 3' chiRNA. The first two bases of the genome target sequence always start with diguanine GG, which is required for precise initiation of the T7 promoter, and followed by the PAM NGG sequence. The two primers hybridize to one another and the DNA fragment generated by PCR used for *in vitro* transcription. The synthesized chiRNA directs Cas9 to recognize and cleave both DNA strands upstream PAM sequence NGG.

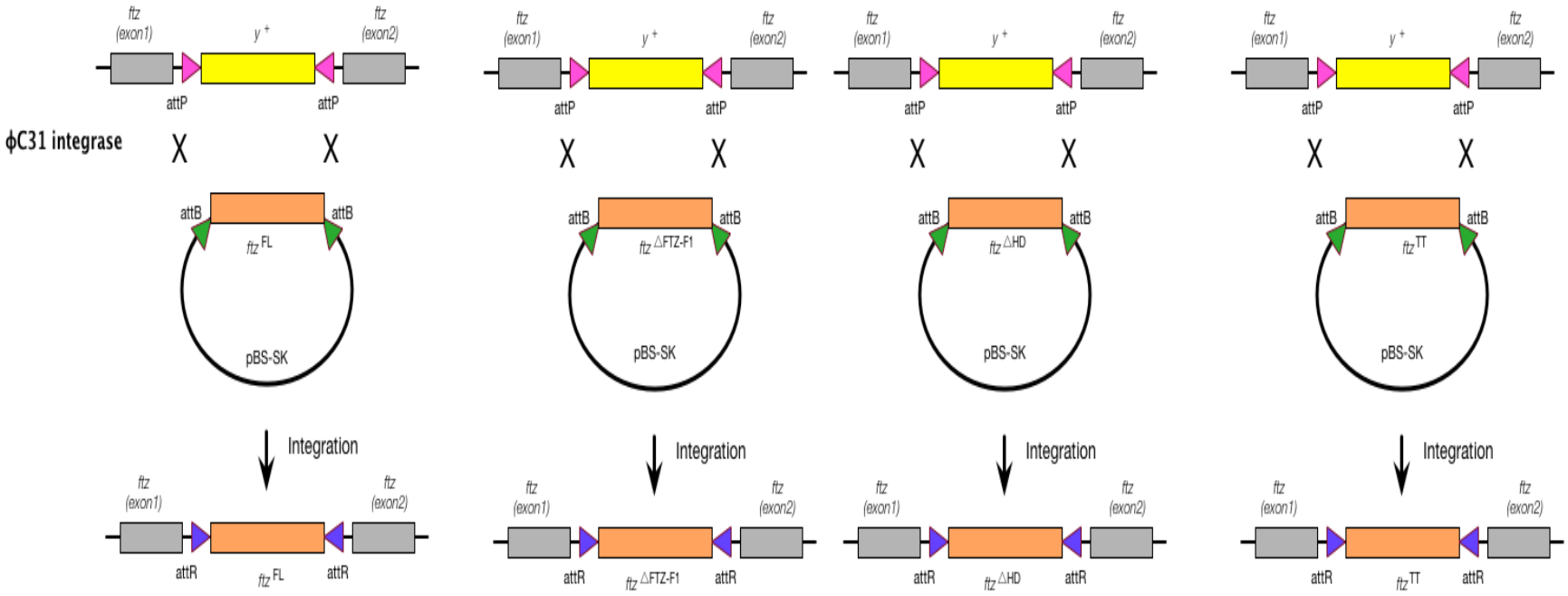


Figure 13. Schematic of RMCE via ΦC31 integrase. The exchange of the pre-existing cassette *ftz* $^{attP\ y+\ attP}$ with the new cassettes: *ftz* $^{attB\ FL\ attB}$, *ftz* $^{attB\ \Delta FTZ\text{-}F1\ attB}$, *ftz* $^{attB\ \Delta HD\ attB}$ *and ftz* $^{attB\ FTZ\ TT\ attB}$,is mediated by RMCE following injection of the ΦC31 integrase and pBS-SK plasmids that contain *ftz* DNA flanking *attB* sites. After recombination between *attP* and *attB* sites, the cassette is integrated between *attR* sites to establish *ftz* fly lines: *ftz* $^{attR\ FL\ attR}$, *ftz* $^{attR\ \Delta FTZ\text{-}F1\ attR}$, *ftz* $^{attR\ \Delta HD\ attR}$ *and ftz* $^{attR\ FTZ\ TT\ attR}$.

2.5 Fly strains

The three fly strains used in this study were: *y w*, act-cas9 flies *y1 M{Act5C-Cas9.P}ZH-2A w** (stock number 54590) and nos-cas9 flies with genotype *y1 M{nos-Cas9.P}ZH-2A w** (stock number 54591). The fly stocks were obtained from the Bloomington *Drosophila* Stock Centre.

2.6 Microinjection of embryos and screening for successful germ-line transformants

Eggs were collected every 30 min after egg laying (AEL) on apple juice plates smeared with yeast paste to stimulate oogenesis. To dechorionate the embryos, 3% bleach was poured on the plate for 1 min. The embryos were washed off into a mesh basket and rinsed with tap water. Using a dissecting needle, the embryos were lined up on the edge of an agar strip in a specific anterior-posterior orientation. The aligned embryos were picked up on double-sided tape adhered to a glass microscope slide. Embryos were dried under a hair dryer for 3.45-4.45 min (depending on the room temperature and humidity), and then covered with halocarbon oil. The glass slide was placed under the microscope in order to inject embryos at the posterior end with a glass needle that was filled with the appropriate injection mixture. The injected embryos expressing Cas9 (*act*-Cas9, *nos*-Cas9), or not expressing Cas9 (*y w*), were kept at 18°C for 48 h, then moved to 25°C to hatch into larva and then an adult fly. The injected embryos that developed into adult flies (G0) were crossed with 3-4 virgin *y w* flies of the opposite sex to verify a successful germ-line transmission of the targeted modification, and the F1 progeny of each cross (from 50-100 flies) were carefully screened under a microscope for the desired marker phenotype *(y^+ or w^+).*

DNA Injection mixture:

For *y w* flies:

500ng/µl Cas9

250ng/µl Each chiRNA

100ng/µl Repair template

10% Glycerol

1X PBS solution

For Cas9 flies:

250ng/µl Each chiRNA

500ng/µl Repair template

10% Glycerol

1X PBS solution

P-Element Injection mixture:

400ng/µl P-Element

200ng/µl Helper plasmid (Δ2-3*wc*)

<u>RNA Injection mixture:</u>

For *y w* flies:

100ng/µl Cas9 mRNA

500ng/µl Each chiRNA

300ng/µl Repair template

10% Glycerol

1X PBS solution

For Cas9 flies:

500ng/µl Each chiRNA

300ng/µl Repair template

10% Glycerol

1X PBS solution

3 RESULTS

The aim of the study was to create a model to test for the interaction of FTZ HD-dependent and HD-independent activities. The overall goal was to generate site-specific mutant *ftz* alleles and then test these alleles for intragenic complementation. The creation of these alleles was attempted in a two-step protocol. First, CRISPR was used to induce DSBs and repaired with HR to establish a mutant *ftz* locus containing *attP* sites. Second, the *attP* sites would be used to introduce engineered *ftz* alleles into the *ftz* locus via the RMCE technique (Groth *et al*., 2004).

3.1 Design of constructs for CRISPR/Cas9-mediated HR

3.1.1 ChiRNAs for CRISPR

In the CRISPR system, a riboprotein consisting of chiRNA and the Cas9 protein makes site-specific DSBs in DNA. In my studies, I used two methods to generate chiRNAs. The first method was to clone the target sequence into the unique *BbsI* site of pU6-*BbsI*-chiRNA plasmid, which after injection into the *Drosophila* syncytial blastoderm embryo is transcribed to give the chiRNA. The sites targeted in *ftz* by these chiRNAs are indicated in (**Figure 14.A**). In the second method I injected the mRNA directly into syncytial blastoderm embryos. The synthetic chiRNAs were transcribed *in vitro* by T7 RNA polymerase, which starts the transcription with diguanine (GG). For that reason, the target sequence must start with GG for initiation of transcription at the T7 promoter (**Figure 14.B**). The two chiRNAs plasmids for *ftz*, and the two *in vitro* transcribed chiRNAs for each *ftz* and *Frost (Fst),* a positive control, were designed and analyzed by BLAST to minimize the chance of off-target effects. The synthesized chiRNAs direct Cas9 to recognize and cleave both DNA strands upstream of the PAM sequence NGG of the 5’ end and 3’ end of *ftz* or *Fst* exons, stimulating the cellular DNA repair mechanism HDR to occur.

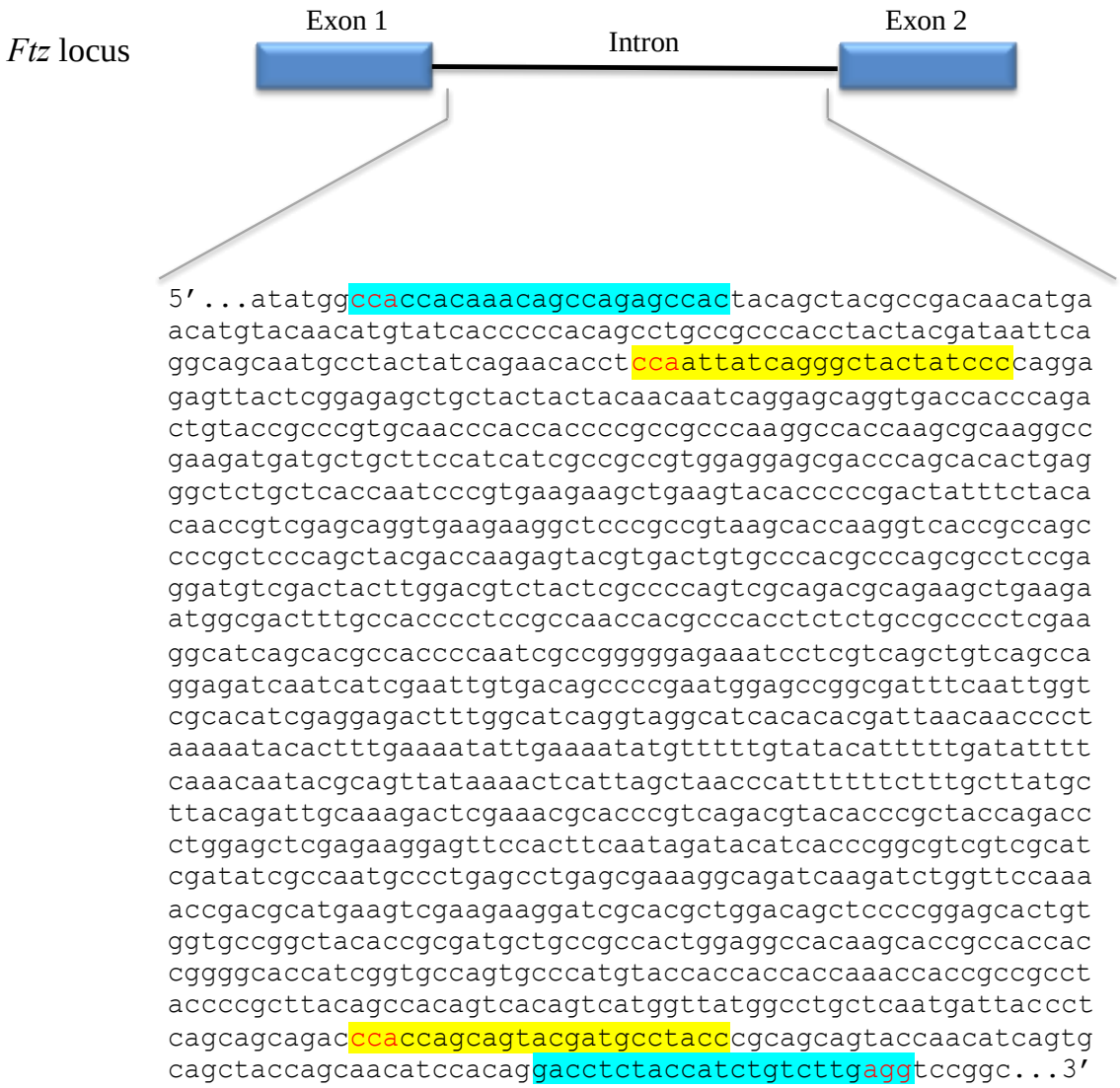

Ftz locus
Exon 1
Intron
Exon 2
5′...atatggccaccacaaacagccagagccactacagctacgccgacaacatga
acatgtacaacatgtatcacccccacagcctgccgcccacctactacgataattca
ggcagcaatgcctactatcagaacacctccaattatcagggctactatcccagga
gagttactcggagagctgctactactacaacaatcaggagcaggtgaccacccaga
ctgtaccgcccgtgcaacccaccaccccgccgcccaaggccaccaagcgcaaggcc
gaagatgatgctgcttccatcatcgccgccgtggaggagcgacccagcacactgag
ggctctgctcaccaatcccgtgaagaagctgaagtacacccccgactatttctaca
caaccgtcgagcaggtgaagaaggctcccgccgtaagcaccaaggtcaccgccagc
cccgctcccagctacgaccaagagtacgtgactgtgcccacgcccagcgcctccga
ggatgtcgactacttggacgtctactcgccccagtcgcagacgcagaagctgaaga
atggcgactttgccacccctccgccaaccacgcccacctctctgccgcccctcgaa
ggcatcagcacgccaccccaatcgccgggggagaaatcctcgtcagctgtcagcca
ggagatcaatcatcgaattgtgacagccccgaatggagccggcgatttcaattggt
cgcacatcgaggagactttggcatcaggtaggcatcacacacgattaacaacccct
aaaaatacactttgaaaatattgaaaatatgtttttgtatacattttgatatttt
caaacaatacgcagttataaaactcattagctaacccatttttttctttgcttatgc
ttacagattgcaaagactcgaaacgcacccgtcagacgtacacccgctaccagacc
ctggagctcgagaaggagttccacttcaatagatacatcacccggcgtcgtcgcat
cgatatcgccaatgccctgagcctgagcgaaaggcagatcaagatctggttccaaa
accgacgcatgaagtcgaagaaggatcgcacgctggacagctccccggagcactgt
ggtgccggctacaccgcgatgctgccgccactggaggccacaagcaccgccaccac
cggggcaccatcggtgccagtgcccatgtaccaccaccaccaaaccaccgccgcct
accccgcttacagccacagtcacagtcatggttatggcctgctcaatgattaccct
cagcagcagaccccaccagcagtacgatgcctaccgcagcagtaccaacatcagtg
cagctaccagcaacatccacaggacctctaccatctgtcttgaggtccggc...3′
(A)
5′...ccaccacaaacagccagagccac...3′!
5′...gacctctaccatctgtcttgagg...3′
(B)
5′...ccaattatcagggctactatccc...3′
5′...ccaccagcagtacgatgcctacc...3′

Figure 14. The sites targeted in *ftz* by the 5' and 3' chiRNAs. The intron region between the two *ftz* exons with the sequences chosen for design of CRISPR chiRNAs to direct cleavage of both DNA strands by Cas9 nuclease are indicated in blue and yellow. **(A)** The targeted cut site of chiRNAs expressed from plasmid DNA (purple arrow). The chiRNA required 20 nt to recognize its complementary target sequence in the genome followed by PAM sequence NGG (highlighted in blue; PAM in red letters). **(B)** The targeted cut site of chiRNAs directly injected as RNA (purple arrow). The chosen sequence was selected following the principle: GGN_{18} found on the sense or anti-sense strand of the targeted gene (highlighted in yellow).

3.1.2 Repair Template for CRISPR

The repair template promotes HDR after the generation of DSBs at a gene locus by CRISPR/Cas9 system (Carroll & Beumer, 2014). The repair template constructed contains 5' and 3' sequences to *ftz* exons (homology arms), y^+ as a body marker flanked by two inverted *attP* ϕC31 recombination sites. Thus, the marker was used to identify transformants flies (*ftz* $^{attP\ y+\ attP}$), if the repair template was inserted between *ftz* exons by HR replacing *ftz* coding sequence. The double-stranded DNA (dsDNA) was assembled into the pFUS_A vector using an ordered assembly strategy. Restriction digests were used to confirm the structure of the repair template (**Figure 15**).

3.2 Screens for germ-line transformants

During injection of a syncytial blastoderm embryo at the posterior end, pole cells take up the repair template and express the chiRNA riboprotein. I screened for insertion of the y^+ gene as an indication that HR directed repair of a DSB had occurred. Many methods have been used to express the chiRNA/Cas9 riboprotein in syncytial blastoderm embryos. First, the chiRNA was transcribed from a plasmid with a U6 promoter or the chiRNA was transcribed *in vitro* and injected directly into the embryo. Second, the Cas9 protein was either translated from a mRNA transcribed from an injected plasmid, expressed from a transgene containing Cas9 expressed from an *actin* or *nos* promoter inserted into the genome, or translated from a Cas9 mRNA which was injected directly into the embryo. In order to establish a germ-line transformant *ftz* flies via CRISPR/Cas9 system, injection was performed into two fly strains. Flies not expressing Cas9 (*y w*) which required three CRISPR components to be injected into syncytial blastoderm embryos: the Cas9 and chiRNAs-encoding plasmids (for CRISPR DNA injection), or Cas9 and chiRNA mRNA (for CRISPR RNA injection) along with the *ftz* repair template. *y w* flies embryos were injected with CRISPR DNA components which resulted in 78 fertile flies but no transformants with y^+ body colour were obtained when F1 progeny were screened (**Table 1**). CRISPR RNA components were also injected into *y w* embryos of which 80 flies were fertile but again no transformants were obtained (**Table 2**). The second fly strain used was Cas9 transgenic flies, expressing the Cas9 nuclease in the germ-line. In this strain, only two CRISPR components are required to be injected. For all injections the repair

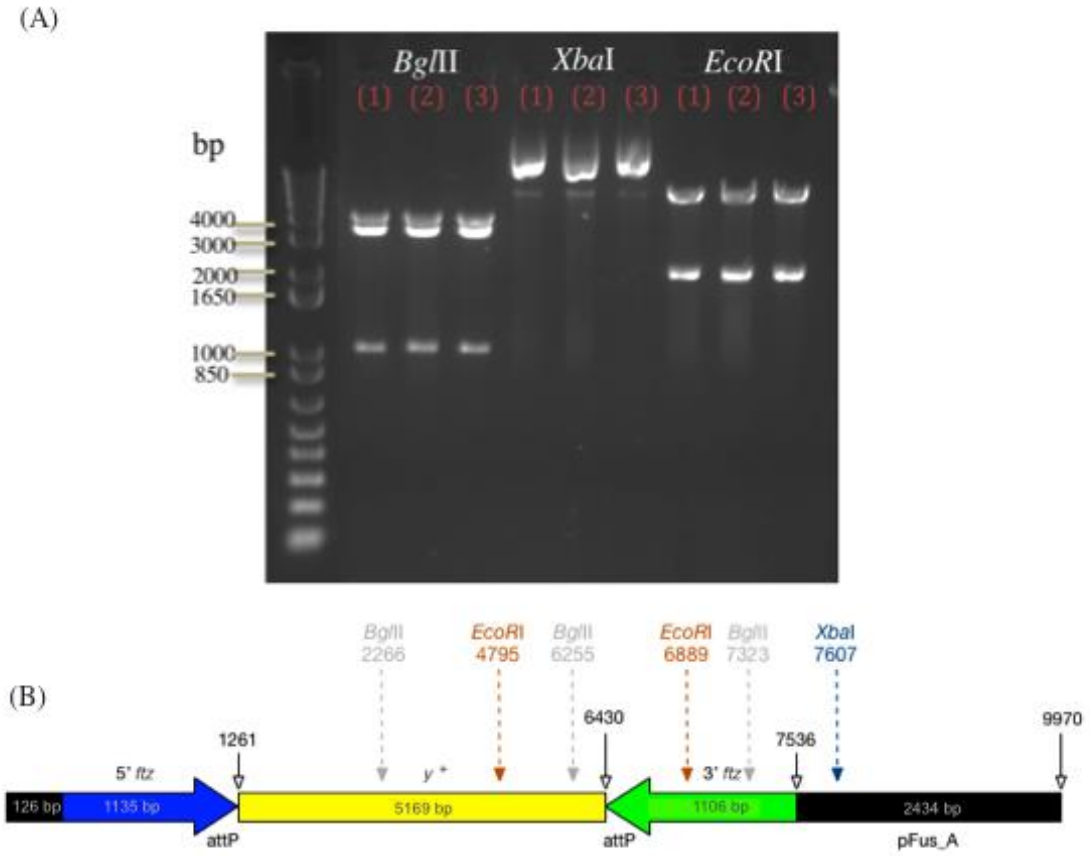


Figure 15. Restriction enzyme analysis of the repair template. (A) Restriction enzyme digests confirmed the orientation of the structured repair template (isolated from three single white colonies). Resolution of the digest products by 1% agarose gel electrophoresis showed an expected pattern of cleavage using the restriction enzymes *BglII* (4913, 3989, 1068 bp), *XbaI* (9970) and *EcoRI* (7876, 2094). **(B)** Schematic indicates where the restriction enzymes *BglII* , *XbaI* and *EcoRI* make the cuts in the repair template.

Table 1. Screens for germ-line transformants after CRISPR DNA injection.

Fly strain	DNA injection	Total of survivors	Fertile	Sterile	Transformant fly
y w	*ftz*	107	78	29	0
nos-Cas9 or *act*-Cas9	*ftz*	122	92	30	0
	Total=	229	170	59	0

Table 2. Screens for germ-line transformants after CRISPR RNA injection.

Fly strain	RNA injection	Total of survivors	Fertile	Sterile	Transformant fly
y w	*ftz*	133	80	53	0
nos-Cas9 or *act*-Cas9	*ftz*	116	86	30	0
	Total=	249	166	83	0

DNA template was injected. In one set of injections chiRNA encoding plasmids were injected and out of 92 fertile flies no transformants were identified (**Table 1**). In a second set of injections *in vitro* transcribed chiRNA was injected and out of 86 fertile flies no transformants were identified (**Table 2**). When preparing the RNA, it is important to avoid RNAse contamination. Thus, 2µl of each CRISPR component were analysed by 2% agarose gel electrophoresis before injection (**Figure 16**). The gel showed RNA degradation in the presence of two of *ftz* repair templates, therefore the RNAse-free template was used in injection.

CRISPR DNA injections of *Frost* (*Fst*) have shown an efficiency rate of 10% in *Drosophila* (unpublished data of Dr. Anthony Percival-Smith). *Frost* is a gene that plays a role in *Drosophila* thermal tolerance (Colinet *et al*., 2010). To test RNA injection efficiency, CRISPR RNA injection was tested to modify *Fst* locus in Cas9-expressing or not expressing flies. The chiRNAs and *Fst* repair template with/without the Cas9 mRNA were injected into syncytial blastoderm embryos. However, none of the screened F1 progeny yielded any transformant progeny with the w^+ marker (**Table 3**). The *Fst* RNA injection mixture was also tested prior and post injection to ensure integrity (**Figure 17**). To test my injection efficiency further, *y w* embryos at the syncytial blastoderm stage were injected with two components: a P-element containing plasmid and a helper plasmid transposase source (Δ2-3*wc*). One transformant fly w^+ was observed after screening through F1 progeny (**Table 3**), suggesting that the injection procedure being followed works, albeit with low efficiency.

3.3 Survival and sterility for the various CRISPR approaches

The low survival rates have been observed in *Drosophila* CRISPR studies (Bassett *et al*. 2013; Gratz *et al*., 2014). The injected CRISPR components could be toxic to some degree, which might have resulted in a low survival rate in the range of 2.3-14% post-

Table 3. Screens for germ-line transformants after P-element and CRISPR injections used as a control.

Fly strain	Injection	Total of survivors	Viable	Sterile	Transformant fly
y w	P-element	106	70	36	1
	CRISPR *(Fst)*	30	23	7	0
nos-Cas9 or *act*-Cas9	CRISPR *(Fst)*	10	8	2	0
	Total=	146	101	45	1

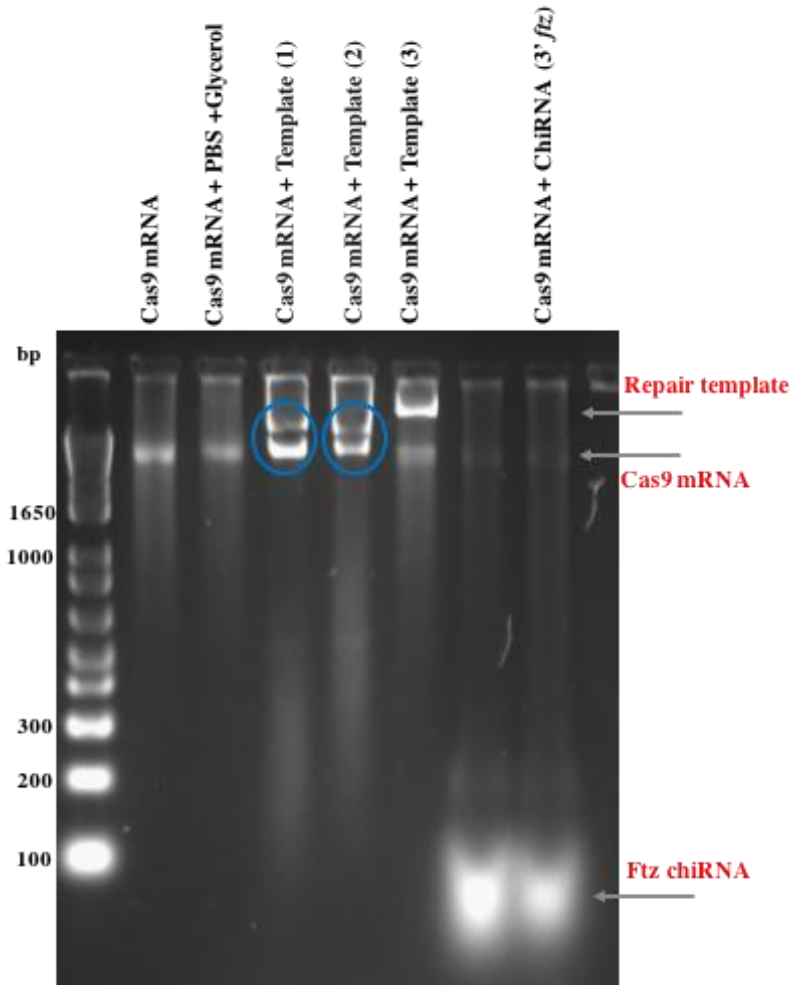


Figure 16. CRISPR RNA stability for *ftz* injection. CRISPR components used in RNA injection mixture were analysed by 2% agarose gel electrophoresis before injection to test RNA integrity. The gel showed that Cas9 mRNA was degraded in the presence of *ftz* repair templates (1) and (2) (blue circles). Therefore, *ftz* repair template (3) was used for CRISPR RNA injection.

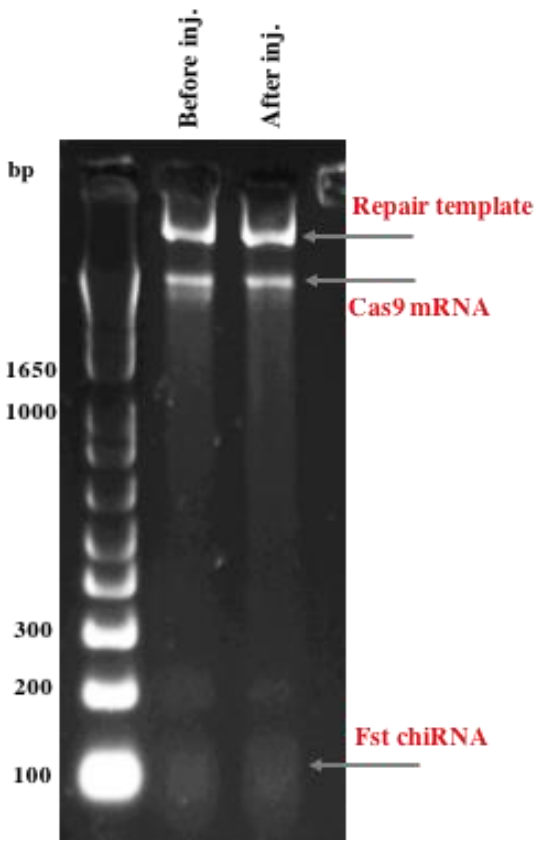


Figure 17. CRISPR RNA stability for *Fst* injection. *Fst* Injection mixture was analysed by 2% agarose gel electrophoresis before and after injection. The gel showed that all CRISPR components were still present after 4 hours of injection.

injection reported in (**Table 4** & **Table 5**). In addition, it has been reported that 5.6-78.4% of the injected survivor flies were sterile which was also found in this study (**Table 4** & **Table 5**; Bassett *et al.* 2013; Gratz *et al.,* 2013; Sebo *et al.*, 2014; Yu *et al.,* 2013; Ren *et al.,* 2014).

Table 4. The average survival rate and sterility rate of CRISPR DNA injection.

Fly strain	DNA Injection	Number of injected embryos	Number of survivors	Average of survival rate	Number of sterile survivors	Average of sterility rate
y w	*ftz* double chiRNAs (5' and 3')	852	63	8.4%	29	27.1%
	ftz single chiRNA (5' or 3')	464	44			
nos Cas9- or *act*-Cas9	*ftz* double chiRNAs (5' and 3')	3368	93	2.3%	30	24.6%
	ftz single chiRNA (5' or 3')	1600	29			

Table 5. The average survival rate and sterility rate of CRISPR RNA injection.

Fly strain	RNA Injection	Number of injected embryos	Number of survivors	Average of survival rate	Number of sterile survivors	Average of sterility rate
y w	*ftz* double chiRNAs (5' and 3')	1028	56	5.7%	53	39.8%
	ftz single chiRNA (5' or 3')	1300	77			
	Fst double chiRNAs (5' and 3')	214	30	14.0%	7	23.3%
nos Cas9- or *act*-Cas9	*ftz* double chiRNAs (5' and 3')	2074	114	3.1%	30	25.9%
	ftz single chiRNA (5' or 3')	272	2			
	Fst double chiRNAs (5' and 3')	246	10	4.1%	2	20.0%

4 Discussion

4.1 Limitation of CRISPR/Cas9 technique

CRISPR/Cas9 system is a relatively recent genome editing technique that has only been employed for the past three years to manipulate not only the *Drosophila* genome, but also the genomes of many other model and non-model organisms. CRISPR/Cas9 is a highly selective method to induce site-specific breaks in double-stranded DNA. CRISPR DSBs introduce small insertion and deletion (indels) by NHEJ repair mechanism at the site of the DSB, or allow specific genomic alteration by HDR pathway when an exogenous repair template is supplied (Bassett *et al*., 2013; Gratz *et al*., 2013; Kondo & Ueda, 2013; Sebo *et al*., 2014; Yu *et al*., 2013; Port *et al*., 2014). The germ-line transmission rate reported in a previous study when injection plasmids encoding Cas9 and chiRNA protein into *Drosophila* embryos to induce DSBs followed by NHEJ-mediated repair was 5.9% for the induction of indels (Gratz *et al*., 2013). However, a higher mutagenesis rate of around 80% was observed when injecting CRISPR RNA (Cas9 mRNA and chiRNA) into syncytial blastoderm *Drosophila* embryos (Bassett *et al*., 2013; Yu *et al*., 2013). The difference of mutagenesis efficiency between CRISPR DNA and RNA injections may be due to the higher expression levels of direct injection of Cas9 and chiRNA mRNA, compared to their expression from an injected plasmids DNA (Bassett & Liu, 2014). CRISPR studies also use an effective, but time-consuming method (Bassett & Liu, 2014) to achieve genetic modification by crossing two transgenic flies: one expressing Cas9 using the *nanos* promoter, and another line expressing *chiRNA* driven through the *U6* promoter. This method achieved the highest mutagenesis with 90% of flies yielding a mutant offspring (Kondo & Ueda, 2013; Bassett & Liu, 2014). However, an alternative to this method with 12-75% efficiency, is to inject Cas9-expressing flies with chiRNA encoding plasmids (Ren *et al*., 2013; Sebo *et al*., 2014).

CRISPR/Cas9 can be used to create precise genome modifications. The DSBs stimulate a HDR pathway in the presence of an exogenous template that shares a 20 nt homology

with the target DNA on either side of the break site. CRISPR/Cas9 potential to facilitate this integration is reported to be lower than induced mutations by NHEJ repair (Gratz *et al*., 2013; Gratz *et al*., 2014; Port *et al*., 2014). The injection of the three CRISPR components: Cas9 and chiRNA encoding DNA plasmids along with a repair template had yielded an integration efficiency of 0.3% (Gratz *et al*., 2013). Cas9-expressing flies showed an increase in integration between 0-11% (Gratz *et al*., 2014). This can be explained by fewer CRISPR components being injected, considering 11-38% of the offsprings integrated the exogenous template (Port *et al*., 2014) when introduced into Cas9 and chiRNA-expressing transgenic flies.

The ability of CRISPR/Cas9 system to create DSBs at a specific target in the genome is solely dependent on the 20 nt homology of chiRNA, which guides the Cas9 nuclease to the target sequence. However, a 20 nt homology to the target sequence might not be good enough for proper targeting of Cas9 to the desired locus. A 20 nt homology length might increase the chances of creating off-target DSBs (Bassett & Liu, 2014). In this study, the failure to modify *ftz* loci by CRISPR/Cas9 system using HDR, could be explained by the low rate of integration observed in previous studies mentioned above. As a control for my injection technique, I was successful in establishing a germ-line transformant fly by the P-element mediated transformation technique, albeit at a low frequency.

Furthermore, the stability of chiRNA *in vivo* can affect the efficiency of CRISPR/Cas9 system. If chiRNAs are unstable due to some *in vivo* degradation mechanism, it would fail to direct the Cas9 to the target sequence. Accessibility to chromatin can be another factor that can interfere with the CRISPR/Ca9 system. Epigenetic mechanisms like methylation have been known to protect a sequence from being mutated (Gowher *et al*., 2000; Takayama *et al.,* 2014). Epigenetic silencing might be the case for *Hox* and derived *Hox* genes, which are crucial for the development of an organism. Thus, it remains to be determined whether *Hox* genes can at all be modified.

4.2 Alternative strategies

To establish a germ-line transformant *ftz* fly, I attempted to inject syncytial blastoderm of *y w* and Cas9-expressing flies with both CRISPR DNA and RNA, but in all cases, no germ-line transformants were obtained (**Table 1** & **Table 2**). An alternative experiment is to inject the repair template into fly embryos that express both Cas9 and chiRNA from transgenes. This would minimize the number of CRISPR components required to be injected, thus increasing the efficiency of the CRISPR system to create and repair DSBs (Port *et al*., 2014).

There are no reports of CRISPR/Cas9-mediated editing of *Hox* genes in the literature. It has been shown that genes which could not be modified by CRISPR/Cas9 system, were successfully edited by TALENs (Treen *et al*. 2013; Sasaki *et al*. 2014). If CRISPR fails to edit/modify the *Hox* genes, which are indispensable for the proper development of the organism, this failure of CRISPR suggests that there might exist an inherent epigenetic mechanism, for instance, methylation, which protects these evolutionarily conserved genes from being mutated.

4.3 Future / Expected results

If a modified *Drosophila* with a manipulable *ftz* locus is generated and *ftz* constructs are reintroduced with RMCE, the initial goals of this project can then be pursued. The *ftz* locus that expresses wild type FTZ^{FL} should develop normally with all even numbered parasegments, as this locus expresses FTZ with both HD-dependent and HD-independent FTZ activities. *ftz* $^{attR\ ftz\Delta HD\ attR}$ larvae will exhibit a ftz phenotype because of an insufficient accumulation of FTZ protein due to the absence of the early transcriptional autoactivation of *ftz* via the *ftz* enhancer, which is HD-dependent. Subsequently, FTZ does not accumulate to a high level, such that the HD-independent activity of FTZ cannot rescue the formation of the even numbered parasegments. Also, embryos should have an abnormal nervous system, because the HD-dependent FTZ activity is required for nervous system development (Heffer *et al.*, 2013). *ftz* $^{attR\ ftz\Delta FTZ\text{-}F1\ attR}$ larvae will also have

a ftz phenotype due to the inability of FTZ $^{\Delta FTZF1}$ to regulate EN and WG expression, since the FTZ segmentation function requires the FTZ-F1 interaction, via the LXXLL motif (Schwartz *et al.*, 2001). Furthermore, the nervous system should develop normally, since it requires the HD-dependent FTZ activity. To test the hypothesis that FTZ has two activities, we expect that establishment of flies that are hemizygous for the *ftz* $^{attR\ ftz\Delta HD\ attR}$ and *ftz* $^{attR\ ftz\Delta FTZ\text{-}F1\ attR}$ alleles should result in intragenic complementation, where a complementation occurs between two *ftz* mutant alleles, to give the wild type phenotype. This is because the *ftz* $^{attR\ ftz\Delta FTZ\text{-}F1\ attR}$ allele, which has a HD-dependent FTZ activity, will be able to activate high levels of FTZ expression from the *ftz* $^{attR\ ftz\Delta HD\ attR}$ allele. The *ftz* $^{attR\ ftz\Delta HD\ attR}$ allele that encodes the HD-independent FTZ activity will activate the expression of EN, repress the expression of WG and establish the even numbered parasegments (**Figure 9**). Future studies using alternative genome editing strategies will hopefully allow the creation of novel models to test FTZ HD-dependent and HD-independent functions.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. & George, R. A. (2000). The genome sequence of *Drosophila melanogaster*. Science, 287, 2185-2195.

Alonso, C. R., Maxton-Kuechenmeister, J., & Akam, M. (2001). Evolution of FTZ protein function in insects. Current Biology, 11, 1473-1478.

Banga, S. S., & Boyd, J. B. (1992). Oligonucleotide-directed site-specific mutagenesis in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences, 89, 1735-1739.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A. & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science, 315, 1709-1712.

Bassett, A. R., & Liu, J. L. (2014). CRISPR/Cas9 and genome editing in *Drosophila*. Journal of Genetics and Genomics, 41, 7-19.

Bassett, A. R., Tibbit, C., Ponting, C. P., & Liu, J. L. (2013). Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. Cell Reports, 4, 220-228.

Bateman, J. R., Lee, A. M. & Wu, C. T. (2006). Site-specific transformation of *Drosophila* via ϕC31 integrase-mediated cassette exchange. Genetics, 173, 769-777.

Beall, E. L., & Rio, D. C. (1998). Transposase makes critical contacts with, and is stimulated by, single- stranded DNA at the P element termini *in vitro*. The EMBO Journal, 17, 2122-2136.

Bhaya, D., Davison, M., & Barrangou, R. (2011). CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annual Review of Genetics, 45, 273-297.

Bibikova, M., Golic, M., Golic, K. G., & Carroll, D. (2002). Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics, 161, 1169-1175.

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., & Basler, K. (2007). An optimized transgenesis system for *Drosophila* using germ-line-specific φC31 integrases. Proceedings of the National Academy of Sciences, 104, 3312-3317.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development,118, 401-415.

Burlingham, E. S. (2012). Characterization of a Negative Regulatory Doman in Fushi tarazu, a Pair-rule Protein in *Drosophila melanogaster*. London, Ont.: Department of Biology, University of Western Ontario.

Capecchi, M. R. (1989). Altering the genome by homologous recombination. Science, 244, 1288-1292.

Carlson, D. F., Fahrenkrug, S. C., & Hackett, P. B. (2012). Targeting DNA with fingers and TALENs. Molecular therapy-Nucleic acids, 1, e3. doi:10.1038/mtna.2011.5

Carroll, D., & Beumer, K. J. (2014). Genome engineering with TALENs and ZFNs: repair pathways and donor design. Methods, 69, 137-141.

Carroll, S. B., Laughon, A., & Thalley, B. S. (1988). Expression, function, and regulation of the hairy segmentation protein in the *Drosophila* embryo. Genes & Development, 2, 883-890.

Carroll, S. B., & Scott, M. P. (1985). Localization of the *fushi tarazu* protein during *Drosophila* embryogenesis. Cell, 43, 47-57.

Cho, S. W., Kim, S., Kim, J. M., & Kim, J. S. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nature Biotechnology, 31, 230-232.

Colinet, H., Lee, S. F., & Hoffmann, A. (2010). Functional characterization of the *Frost* gene in *Drosophila melanogaster*: importance for recovery from chill coma. PLoS One, 5, e10925. doi:10.1371/journal.pone.0010925

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science, 339, 819-823.

DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., & Church, G. M. (2013). Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Research, 41, 4336-4343..

Doe, C. Q., Hiromi, Y., Gehring, W. J., & Goodman, C. S. (1988). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. Science, 239, 170-175.

Fitzpatrick, V. D., Percival-Smith, A., Ingles, C. J., & Krause, H. M. (1992). Homeodomain-independent activity of the FUSHI TARAZU polypeptide in *Drosophila*. Nature, 356, 610-612.

Friedland, A. E., Tzur, Y. B., Esvelt, K. M., Colaiácovo, M. P., Church, G. M., & Calarco, J. A. (2013). Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. Nature Methods, 10, 741-743.

Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology, 31, 397-405.

Gilbert S.F. (2006). Developmental Biology 8th ed. Sunderland, MA: Sinauer Associates.

Gowher, H., Leismann, O., & Jeltsch, A. (2000). DNA of *Drosophila melanogaster* contains 5- methylcytosine. The EMBO Journal, 19, 6918-6923.

Gratz, S. J., Cummings, A. M., Nguyen, J. N., Hamm, D. C., Donohue, L. K., Harrison, M. M., Wildonger, J. & O'Connor-Giles, K. M. (2013). Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. Genetics, 194, 1029-1035.

Gratz, S. J., Ukken, F. P., Rubinstein, C. D., Thiede, G., Donohue, L. K., Cummings, A. M., & O'Connor-Giles, K. M. (2014). Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. Genetics, 196, 961-971.

Groth, A. C., Fish, M., Nusse, R., & Calos, M. P. (2004). Construction of transgenic *Drosophila* by using the site-specific integrase from phage φC31. Genetics, 166, 1775-1782.

Guichet, A., Copeland, J. W., Erdélyi, M., Hlousek, D., Závorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H.M. & Ephrussi, A. (1997). The nuclear receptor homologue FTZ-F1 and the homeodomain protein FTZ are mutually dependent cofactors. Nature, 385, 548-552

Hafen, E., Kuroiwa, A., & Gehring, W. J. (1984). Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. Cell, 37, 833-841.

Heffer, A., Xiang, J., & Pick, L. (2013). Variation and constraint in *Hox* gene evolution. Proceedings of the National Academy of Sciences, 110, 2211-2216.

Hiromi, Y., Kuroiwa, A., & Gehring, W. J. (1985). Control elements of the *Drosophila* segmentation gene *fushi tarazu*. Cell, 43, 603-613.

Horn, C., & Handler, A. M. (2005). Site-specific genomic targeting in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America, 102, 12483-12488.

Huang, J., Zhou, W., Dong, W., Watson, A. M., & Hong, Y. (2009). Directed, efficient, and versatile modifications of the *Drosophila* genome by genomic engineering. Proceedings of the National Academy of Sciences, 106, 8284-8289.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R.T., Yeh, J.J. & Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature Biotechnology, 31, 227-229.

Hyduk, D., & Percival-Smith, A. (1996). Genetic Characterization of the Homeodomain-Independent Activity of the *Drosophila fushi taruzu* Gene Product. Genetics, 142, 481-492.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in

Escherichia coli, and identification of the gene product. Journal of Bacteriology, 169, 5429-5433.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science, 337, 816-821.

Kellerman, K. A., Mattson, D. M., & Duncan, I. (1990). Mutations affecting the stability of the FUSHI TARAZU protein of *Drosophila*. Genes & Development, 4, 1936-1950.

Kondo, S., & Ueda, R. (2013). Highly improved gene targeting by germline-specific Cas9 expression in *Drosophila*. Genetics, 195, 715-721.

Konev, A. Y., Yan, C. M., Acevedo, D., Kennedy, C., Ward, E., Lim, A., Tickoo, S. & Karpen, G. H. (2003). Genetics of P-element transposition into *Drosophila melanogaster* centric heterochromatin. Genetics, 165, 2039-2053.

Krause, H. M., Klemenz, R., & Gehring, W. J. (1988). Expression, modification, and localization of the *fushi tarazu* protein in *Drosophila* embryos. Genes & Development, 2, 1021-1036.

Laughon, A., & Scott, M. P. (1984). Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA-binding proteins. Nature, 310, 25-31.

Lawrence, P. A., Johnston, P., Macdonald, P., & Struhl, G. (1987). Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes. Nature, 328, 440-442.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. In Genes, Development and Cancer. Springer US, 1978. 205-217.

Liu, J., Li, C., Yu, Z., Huang, P., Wu, H., Wei, C., Zhu, N., Shen, Y., Chen, Y., Zhang, B. & Deng, W. M. (2012). Efficient and specific modifications of the *Drosophila* genome by means of an easy TALEN strategy. Journal of Genetics and Genomics, 39, 209-215.

Löhr, U., & Pick, L. (2005). Cofactor-interaction motifs and the cooption of a homeotic Hox protein into the segmentation pathway of *Drosophila melanogaster*. Current Biology, 15, 643-649.

Maier, D., Preiss, A., & Powell, J. R. (1990). Regulation of the segmentation gene *fushi tarazu* has been functionally conserved in *Drosophila*. The EMBO Journal, 9, 3957.

Majumdar, S., & Rio, D. C. (2015). P transposable elements in *Drosophila* and other eukaryotic organisms. Microbiology Spectrum, 3,doi:10.1128/microbiolspec.MDNA3-0004-2014.

Martinez-Arias, A., & Lawrence, P. A. (1984). Parasegments and compartments in the *Drosophila* embryo. Nature, 313, 639-642.

McGinnis, W., & Krumlauf, R. (1992). *Homeobox* genes and axial patterning. Cell, 68, 283-302.

McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A., & Gehring, W. J. (1984). A homologous protein-coding sequence in *Drosophila homeotic* genes and its conservation in other metazoans. Cell, 37, 403-408.

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature, 287, 795-801.

Port, F., Chen, H. M., Lee, T., & Bullock, S. L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. Proceedings of the National Academy of Sciences, 111, E2967-E2976.

Ren, X., Sun, J., Housden, B. E., Hu, Y., Roesel, C., Lin, S., Liu, L.P., Yang, Z., Mao, D., Sun, L. & Wu, Q. (2013). Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. Proceedings of the National Academy of Sciences, 110, 19012-19017.

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., Yang, S.J., Qiao, H.H., Wang, X., Hu, Q. & Deng, P. (2014). Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*. Cell Reports, 9, 1151-1162.

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

Rubin, G. M. (1988). *Drosophila melanogaster* as an experimental organism. Science, *240*, 1453-1459.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nature Biotechnology, *32*, 347-355.

Sasaki, H., Yoshida, K., Hozumi, A., & Sasakura, Y. (2014). CRISPR/Cas9- mediated gene knockout in the ascidian Ciona intestinalis. Development, Growth & Differentiation, 56, 499-510.

Schier, A. F., & Gehring, W. J. (1992). Direct homeodomain–DNA interaction in the autoregulation of the *fushi tarazu* gene. Nature, 356, 804-807.

Schwartz, C. J., Sampson, H. M., Hlousek, D., Percival- Smith, A., Copeland, J. W., Simmonds, A. J., & Krause, H. M. (2001). FTZ- Factor1 and FUSHI TARAZU interact via conserved nuclear receptor and coactivator motifs. The EMBO Journal, 20, 510-519.

Scott, M. P., Weiner, A. J., Hazelrigg, T. I., Polisky, B. A., Pirrotta, V., Scalenghe, F., & Kaufman, T. C. (1983). The molecular organization of the *Antennapedia* locus of *Drosophila*. Cell, 35, 763-776.

Sebo, Z. L., Lee, H. B., Peng, Y., & Guo, Y. (2014). A simplified and efficient germline-specific CRISPR/Cas9 system for *Drosophila* genomic engineering. Fly, 8, 52-57.

Siegal, M. L., & Hartl, D. L. (1996). Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*.Genetics, *144*, 715-726.

Spradling, A. C., & Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science, 218, 341-347.

Struhl, G. (1982). Genes controlling segmental specification in the *Drosophila* thorax. Proceedings of the National Academy of Sciences, 79, 7380-7384.

Takayama, S., Dhahbi, J., Roberts, A., Mao, G., Heo, S. J., Pachter, L., Martin, D.I. & Boffelli, D. (2014). Genome methylation in *D. melanogaster* is found at specific short motifs and is independent of DNMT2 activity. Genome Research, 24, 821-830.

Treen, N., Yoshida, K., Sakuma, T., Sasaki, H., Kawai, N., Yamamoto, T., & Sasakura, Y. (2014). Tissue-specific and ubiquitous gene knockouts by TALEN electroporation provide new approaches to investigating gene function in Ciona. Development, 141, 481-487.

Ueda, H., Sonoda, S., Brown, J. L., Scott, M. P., & Wu, C. (1990). A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. Genes & Development, 4, 624-635.

Venken, K. J., & Bellen, H. J. (2007). Transgenesis upgrades for *Drosophila melanogaster*. Development, 134, 3571-3584.

Venken, K. J., Schulze, K. L., Haelterman, N. A., Pan, H., He, Y., Evans-Holm, M., Carlson, J.W., Levis, R.W., Spradling, A.C., Hoskins, R.A. & Bellen, H. J. (2011). MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. Nature Methods, 8, 737-743.

Wakimoto, B. T., & Kaufman, T. C. (1981). Analysis of larval segmentation in lethal genotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. Developmental Biology, 81, 51-64.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell, 153, 910-918.

Weiner, A. J., Scott, M. P., & Kaufman, T. C. (1984). A molecular analysis of *fushi tarazu*, a gene in *Drosophila melanogaster* that encodes a product affecting embryonic segment number and cell fate. Cell, 37, 843-851.

Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N., & Pick, L. (1997). The nuclear hormone receptor FTZ-F1 is a cofactor for the *Drosophila* homeodomain protein FTZ. Nature, 385, 552-555.

Yu, Z., Ren, M., Wang, Z., Zhang, B., Rong, Y. S., Jiao, R., & Gao, G. (2013). Highly efficient genome modifications mediated by CRISPR/Cas9 in *Drosophila*. Genetics, 195, 289-291.

Yussa, M., Löhr, U., Su, K., & Pick, L. (2001). The nuclear receptor FTZ-F1 and homeodomain protein FTZ interact through evolutionarily conserved protein domains. Mechanisms of Development, 107, 39-53.

Appendices

List of Primers

Appendix 1. Primers used for cloning *ftz* chiRNAs into pU6_*BbsI*_chiRNA plasmid

Primer	Sequence
5' chiRNA-F	5'-...CTTCGACCTCTACCATCTGTCTTG…-3'
5' chiRNA-R	5'-...AAACCAAGACAGATGGTAGAGGTC…-3'
3' chiRNA-F	5'-...CTTCGTGGCTCTGGCTGTTTGTGG…-3'
3' chiRNA-R	5'-...AAACCCACAAACAGCCAGAGCCAC…-3'

Appendix 2. Primers used for constructing the repair template

Primer	Sequence	PCR Template
5' *ftz*-F	5'-...CAGCTAGGTCTCGCTATAGCATCCATAGACAACCTACTTAAA…-3'	*y w* (Genomic DNA)
5' *ftz*-R	5'-...CAGCTAGGTCTCCCATGCCCCCAACTGAGAGAA CTCAAAGGTTACCCCAGTTGGGGCGGATGTGTATTGCTAGATTTC…-3'	
y^+-F	5'-...CAGCTAGGTCTCCCATGCGACTATTAAATGATTATCGCC…-3'	MiMIC (Venken *et al.*, 2011)
y^+-R	5'-...CAGCTAGGTCTCGGTCCTCGACCTGCAGGTCAACGGATC…-3'	
3' *ftz*-F	5'-...CAGCTAGGTCTCGGGACCCCCCAACTGAGAGAA CTCAAAGGTTACCCCAGTTGGGGGGTCCGGCGATGCTCAGTTAC…-3'	*y w* (Genomic DNA)
3' *ftz*-R	5'-...CAGCTAGGTCTCCCGCCCCAAAATGTGACATTTTCTCTGGCG…-3'	

Appendix 3. Primers used for colony PCR screening of the correct repair template

Primer	Sequence
Screen 1-F	5'-...CTGGCAGTTCCCTACTCTCG…-3'
Screen 1-R	5'-...GGTAAATCAGCGGGCTGCGTTCG…-3'
Screen 2-F	5'-...CAGGGAAAGTTCAACTTAATCGC…-3'
Screen 2-R	5'-...CTGTCCTGGCTGGTCTAGACGTC…-3'
Screen 3-F	Same as (Screen 2-F)
Screen 3-R	5'-...GAGCCGCCACCAATTGGACC…-3'

Appendix 4. Primers used to generate chiRNAs template for *IVT*

Primer	Sequence
T7-*ftz* chiRNA1-F	5'-...GAAATTAATACGACTCACTATAGGGATAGTAGCCCTGATAAT GTTTTAGAGCTAGAAATAGC…-3'
T7-*ftz* chiRNA2-F	5'-...GAAATTAATACGACTCACTATAGGTAGGCATCGTACTGCTGG GTTTTAGAGCTAGAAATAGC…-3'
T7-*Fst* chiRNA1-F	5'-...GAAATTAATACGACTCACTATAGGCGGTTGGTTCGGAAATTT GTTTTAGAGCTAGAAATAGC…-3'
T7-*Fst* chiRNA2-F	5'-...GAAATTAATACGACTCACTATAGGAGCCCCAACCGAACCTCC GTTTTAGAGCTAGAAATAGC…-3'
Common primer-R	5'-...AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATTTCTAGCTCTAAAAC…-3'

Copyright Permissions for Figures

Appendix 5. Permission to use (figure 1 + figure 3)

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Mar 15, 2016

This is a License Agreement between Alaa Briek ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3830050376329
License date	Mar 15, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Biotechnology
Licensed content title	CRISPR-Cas systems for editing, regulating and targeting genomes
Licensed content author	Jeffry D Sander, J Keith Joung
Licensed content date	Mar 2, 2014
Volume number	32
Issue number	4
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
High-res required	no
Figures	Figure 1:Nuclease-induced genome editing, Figure2: Naturally occurring and engineered CRISPR-Cas9 systems
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	A genetic test for two Activities of fushi tarazu protein in Drosophila melanogaster
Expected completion date	Apr 2016
Estimated size (number of pages)	60
Total	0.00 CAD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer*, the following credit line

applies.
Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.
Special Terms:
v1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Appendix 6. Permission to use (figure 4)

SINAUER ASSOCIATES, Inc. • Publishers • P.O. Box 407, 23 Plumtree Rd. • Sunderland, MA 01375
Telephone: (413) 549-4300
Fax: (413) 549-1118
Email: permissions@sinauer.com

PERMISSIONS AGREEMENT

March 22, 2016

Permission granted to:
Alaa Briek

Material to be reproduced:
Gilbert: *Developmental Biology, Eighth Edition*
Figure 9.7, page 259 and Figure 9.27 A, page 276

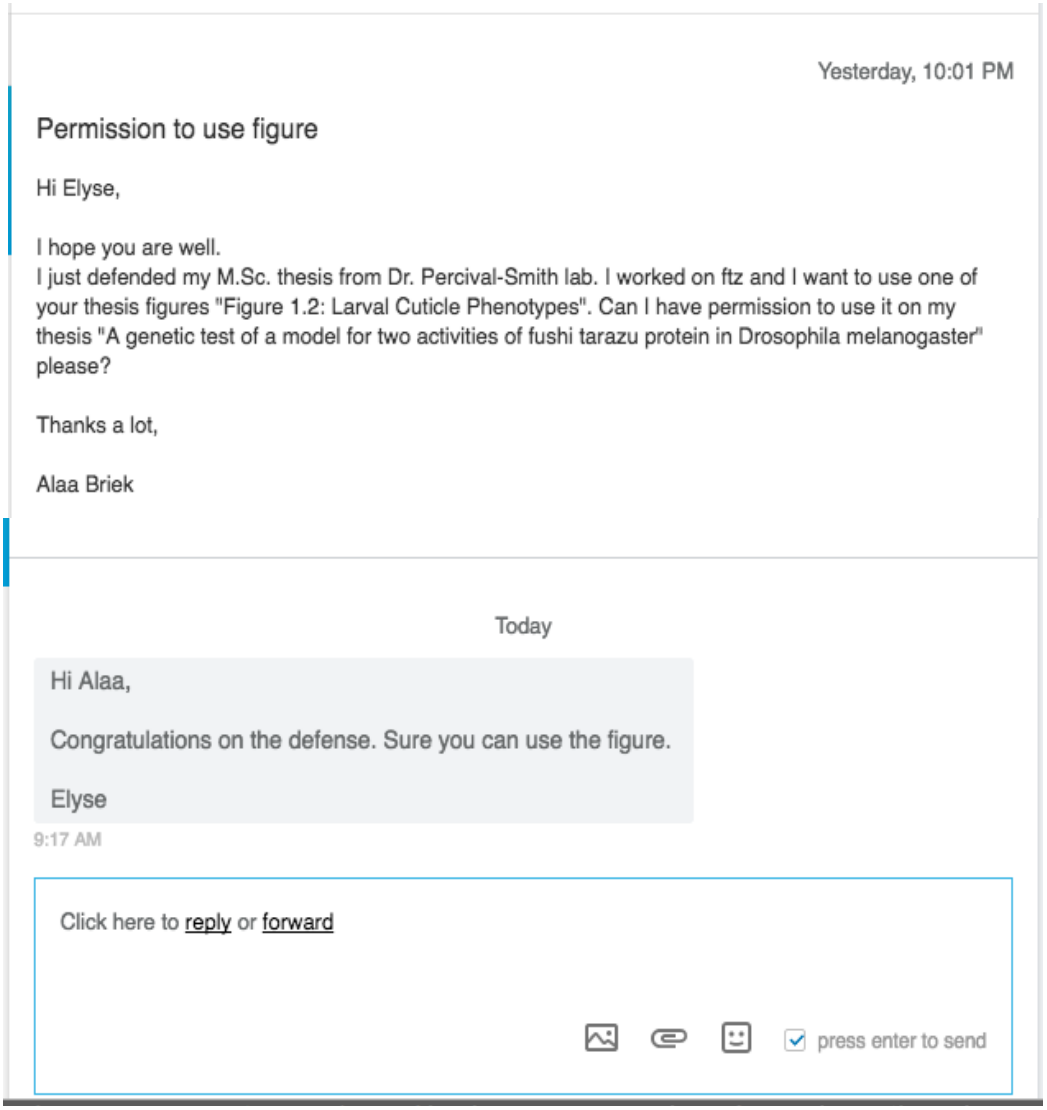
To be reproduced in the work:
Alaa Briek's M.Sc. Thesis entitled "A genetic test for two activities of fushi tarazu protein in Drosophila melanogaster" to be published by the University of Western Ontario

Sinauer Associates owns copyright to the material described above and hereby grants permission for the one-time use of the material as specified, and for nonexclusive world rights provided that full and appropriate credit is given to the original source and that the work is for NON-COMMERCIAL use only. Please request permission for further use in subsequent editions, translations, or revisions of the work.

March 22, 2016
Date

Sherri L. Ellsworth
Permissions Coordinator

Appendix 7. Permission to use (figure 6)



Yesterday, 10:01 PM

Permission to use figure

Hi Elyse,

I hope you are well.
I just defended my M.Sc. thesis from Dr. Percival-Smith lab. I worked on ftz and I want to use one of your thesis figures "Figure 1.2: Larval Cuticle Phenotypes". Can I have permission to use it on my thesis "A genetic test of a model for two activities of fushi tarazu protein in Drosophila melanogaster" please?

Thanks a lot,

Alaa Briek

Today

Hi Alaa,

Congratulations on the defense. Sure you can use the figure.

Elyse

9:17 AM

Click here to reply or forward

press enter to send

Appendix 8. Permission to use (figure 7)

Genetics Society of America LICENSE TERMS AND CONDITIONS

Apr 25, 2016

This is a License Agreement between Alaa Briek ("You") and Genetics Society of America ("Genetics Society of America") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Genetics Society of America, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3838961404312
License date	Mar 30, 2016
Licensed content publisher	Genetics Society of America
Licensed content title	Genetics
Licensed content date	Jan 1, 1916
Type of Use	Thesis/Dissertation
Requestor type	Academic institution
Format	Print, Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	1
Title or numeric reference of the portion(s)	Figure 8
Title of the article or chapter the portion is from	Genetic Characterization of the Homeodomain-Independent Activity of the Drosophila fushi tarazu Gene Product
Editor of portion(s)	NA
Author of portion(s)	NA
Volume of serial or monograph.	NA
Page range of the portion	490
Publication date of portion	1996/02/01
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no

The lifetime unit quantity of new product	Up to 999
Made available in the following markets	education
The requesting person/organization is:	Alaa Briek- University of Western Ontario
Order reference number	None
Author/Editor	Alaa Briek
The standard identifier	NA
Title	A GENETIC TEST OF A MODEL FOR TWO ACTIVITES OF FUSHI TARAZU PROTEIN IN DROSOPHILA MELANOGASTER
Publisher	The university of Western Ontario
Expected publication date	Apr 2016
Estimated size (pages)	60
Total (may include CCC user fee)	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS

The following terms are individual to this publisher:

None

Other Terms and Conditions:

STANDARD TERMS AND CONDITIONS

1. Description of Service; Defined Terms. This Republication License enables the User to obtain licenses for republication of one or more copyrighted works as described in detail on the relevant Order Confirmation (the "Work(s)"). Copyright Clearance Center, Inc. ("CCC") grants licenses through the Service on behalf of the rightsholder identified on the Order Confirmation (the "Rightsholder"). "Republication", as used herein, generally means the inclusion of a Work, in whole or in part, in a new work or works, also as described on the Order Confirmation. "User", as used herein, means the person or entity making such republication.

2. The terms set forth in the relevant Order Confirmation, and any terms set by the Rightsholder with respect to a particular Work, govern the terms of use of Works in connection with the Service. By using the Service, the person transacting for a republication license on behalf of the User represents and warrants that he/she/it (a) has been duly authorized by the User to accept, and hereby does accept, all such terms and conditions on behalf of User, and (b) shall inform User of all such terms and conditions. In the event such person is a "freelancer" or other third party independent of User and CCC, such party shall be deemed jointly a "User" for purposes of these terms and conditions. In any event, User shall be deemed to have accepted and agreed to all such terms and conditions if User republishes the Work in any fashion.

3. Scope of License; Limitations and Obligations.

3.1 All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The license created by the exchange of an Order Confirmation (and/or any invoice) and payment by User of the full amount set forth on that document includes only those rights expressly set forth in the Order Confirmation and in these terms and conditions, and conveys no other rights in the Work(s) to User. All rights not expressly granted are hereby reserved.

3.2 General Payment Terms: You may pay by credit card or through an account with us payable at the end of the month. If you and we agree that you may establish a standing account with CCC, then the following terms apply: Remit Payment to: Copyright Clearance Center, Dept 001, P.O. Box 843006, Boston, MA 02284-3006. Payments Due: Invoices are payable upon their delivery to you (or upon our notice to you that they are available to you for downloading). After 30 days, outstanding amounts will be subject to a service charge of 1-1/2% per month or, if less, the maximum rate allowed by applicable law. Unless otherwise specifically set forth in the Order Confirmation or in a separate written agreement signed by CCC, invoices are due and payable on "net 30" terms. While User may exercise the rights licensed immediately upon issuance of the Order Confirmation, the license is automatically revoked and is null and void, as if it had never been issued, if complete payment for the license is not received on a timely basis either from User directly or through a payment agent, such as a credit card company.

3.3 Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) is "one-time" (including the editions and product family specified in the license), (ii) is non-exclusive and non-transferable and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Order Confirmation or invoice and/or in these terms and conditions. Upon completion of the licensed use, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period).

3.4 In the event that the material for which a republication license is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) which are identified in such material as having been used by permission, User is responsible for identifying, and seeking separate licenses (under this Service or otherwise) for, any of such third party materials; without a separate license, such third party materials may not be used.

3.5 Use of proper copyright notice for a Work is required as a condition of any license granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Republished with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc. " Such notice must be provided in a reasonably legible font size and must be placed either immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

3.6 User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

4. Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and

expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

5. Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors and assigns.

6. Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

7. Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the license set forth in the Order Confirmation and/or these terms and conditions, shall be a material breach of the license created by the Order Confirmation and these terms and conditions. Any breach not cured within 30 days of written notice thereof shall result in immediate termination of such license without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

8. **Miscellaneous.**

8.1 User acknowledges that CCC may, from time to time, make changes or additions to the Service or to these terms and conditions, and CCC reserves the right to send notice to the User by electronic mail or otherwise for the purposes of notifying User of such changes or additions; provided that any such changes or additions shall not apply to permissions already secured and paid for.

8.2 Use of User-related information collected through the Service is governed by CCC's privacy policy, available online here:
http://www.copyright.com/content/cc3/en/tools/footer/privacypolicy.html.

8.3 The licensing transaction described in the Order Confirmation is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the license created by the Order Confirmation and these terms and conditions or any rights granted hereunder; provided, however, that User may assign

such license in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in the new material which includes the Work(s) licensed under this Service.

8.4 No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the licensing transaction described in the Order Confirmation, which terms are in any way inconsistent with any terms set forth in the Order Confirmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

v 1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Curriculum Vitae

Name:	Alaa Briek
Post-secondary Education and Degrees:	King Abdul-Aziz University Jeddah, Saudi Arabia 2004-2008 B.Sc. The University of Western Ontario London, Ontario, Canada 2013-2016 M.Sc.
Honours and Awards:	King Abdullah Scholarship 2010-2015
Related Work Experience	Intern Al-Burj Laboratories Attended the following labs: Microbiology, Parasitology, Hematology and Chemistry and Performed: Blood, urine and stool tests 2008 Researcher The University of Ottawa Worked in the "Human Ovarian Cancer Biology and Chemoresistance" 2011