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

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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

even-skipped (gene)

EXD  

Extradenticle (protein)

Fst  

Frost

ftz  

fushi tarazu (gene)

FTZ –F1  

Fushi tarazu-factor 1

ftz\textsuperscript{FL}  

full length ftz (gene)

ftz\textsuperscript{ΔHD}  

ftz with a complete deletion of the HD (gene)

ftz\textsuperscript{ΔFTZ-F1}  

ftz with a deletion of FTZ-F1 binding site (gene)

FTZ\textsuperscript{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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PAM</td>
<td>protospacer adjacent motif</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RMCE</td>
<td>recombinase-mediated cassette exchange</td>
</tr>
<tr>
<td>TALE/TALEN</td>
<td>transcription activator-like effector / nuclease</td>
</tr>
<tr>
<td>tracrRNA</td>
<td>trans-activating CRISPR RNA</td>
</tr>
<tr>
<td>w⁺</td>
<td>miniwhite⁺ (gene)</td>
</tr>
<tr>
<td>wg</td>
<td>wingless (gene)</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>y⁺</td>
<td>yellow⁺ (gene)</td>
</tr>
<tr>
<td>ZFN</td>
<td>zinc-finger nuclease</td>
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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 (protosspacer) 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 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 \textit{Drosophila melanogaster}

The development of the fertilized \textit{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 \textit{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 \textit{Homeotic selector (Hox) genes}. These hierarchical interactions give rise to a segmented larva. \textit{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 \textit{bicoid (bcd)} defines the anterior end of the embryo while the mRNA of \textit{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: \textit{fushi tarazu (ftz)} and \textit{even-skipped (eve)}. The pair-rule gene \textit{ftz} is involved in the establishment of even-numbered parasegments whereas \textit{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.
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 (dentine belts of T2, A1, A3, A5 and A7), and the larval cuticle derived from *eve* expression is the odd-numbered parasegments (dentine 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\textsuperscript{FL}), a FTZ polypeptide with a complete deletion of the HD (FTZ\textsuperscript{ΔHD}), and a FTZ polypeptide with a deletion of FTZ-F1 binding site (FTZ\textsuperscript{ΔFTZ-F1}) (Figure 8). Based on the model proposed by Hyduk and Percival-Smith, I expect *ftz*\textsuperscript{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*\textsuperscript{ΔHD} and *ftz*\textsuperscript{ΔFTZ-F1} mutated genes will exhibit a *ftz* phenotype, because the *ftz*\textsuperscript{ΔHD} allele can not express sufficient FTZ protein to establish segmentation, and the *ftz*\textsuperscript{ΔFTZ-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*\textsuperscript{ΔHD} and *ftz*\textsuperscript{ΔFTZ-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 (FTZFL), a FTZ polypeptide with a deletion of FTZ-HD (FTZΔHD), a FTZ polypeptide with a deletion of the FTZ-F1 binding site (FTZΔFTZ-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, $ftz^{ΔHD}$ 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, $ftz^{ΔFTZ-F1}$ embryos will show a $ftz$ phenotype. (D) The intragenic complementation between $ftz^{ΔHD}$ and $ftz^{ΔFTZ-F1}$ alleles will result in wild-type embryos. The protein product of $ftz^{ΔFTZ-F1}$ allele has a FTZ HD-dependent activity, which activates the high-levels of FTZ expression from $ftz^{ΔHD}$ 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:

(GAAATTAATACGACTCATA[TAGGN]₁₈GT[TTTAGAGCT][AGAAATAGC]),

where GGN₁₈ is either ftz or Fst sequence. One common reverse primer was designed to encode the remainder of the chiRNA

(AAAAGCACCACACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTAT
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.
TTTAACCTGCTATTTCTAGCTCTAAAAC (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 ФC31 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 ΔHD, FTZ ΔFTZ 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 \textit{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 \textit{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 $ftz^{\text{attP}} y^+ \text{attP}$ with the new cassettes: $ftz^{\text{attB FL attB}}$, $ftz^{\text{attB ΔFTZ-F1 attB}}$, $ftz^{\text{attB ΔHD attB}}$ and $ftz^{\text{attB FTZ TT attB}}$, 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: $ftz^{\text{attR FL attR}}$, $ftz^{\text{attR ΔFTZ-F1 attR}}$, $ftz^{\text{attR ΔHD attR}}$ and $ftz^{\text{attR FTZ TT attR}}$. 
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 (Δ2-3wc)

RNA Injection mixture:

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.
**A**

5′...\textcolor{cyan}{ccaccacaacagccagccac}...3′!

5′...\textcolor{cyan}{gacctctaccatctgtttaggg}...3′

**B**

5′...\textcolor{cyan}{ccaattatcagggactacttcc}...3′

5′...\textcolor{cyan}{ccaccagcagtaagctcgctacc}...3′

(A) 5′...\textcolor{cyan}{ccaccacaacagccagccac}...3′!

(B) 5′...\textcolor{cyan}{ccaccagcagtaagctcgctacc}...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.

<table>
<thead>
<tr>
<th>Fly strain</th>
<th>DNA injection</th>
<th>Total of survivors</th>
<th>Fertile</th>
<th>Sterile</th>
<th>Transformant fly</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w</td>
<td>ftz</td>
<td>107</td>
<td>78</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>nos-Cas9 or act-Cas9</td>
<td>ftz</td>
<td>122</td>
<td>92</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total=</strong></td>
<td><strong>229</strong></td>
<td><strong>170</strong></td>
<td><strong>59</strong></td>
<td><strong>0</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>
Table 2. Screens for germ-line transformants after CRISPR RNA injection.

<table>
<thead>
<tr>
<th>Fly strain</th>
<th>RNA injection</th>
<th>Total of survivors</th>
<th>Fertile</th>
<th>Sterile</th>
<th>Transformant fly</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w</td>
<td>ftz</td>
<td>133</td>
<td>80</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td>nos-Cas9 or act-Cas9</td>
<td>ftz</td>
<td>116</td>
<td>86</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total=</strong></td>
<td><strong>249</strong></td>
<td><strong>166</strong></td>
<td><strong>83</strong></td>
<td><strong>0</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>
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, 2 µl 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 (Δ2-3wc). 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.

<table>
<thead>
<tr>
<th>Fly strain</th>
<th>Injection</th>
<th>Total of survivors</th>
<th>Viable</th>
<th>Sterile</th>
<th>Transformant fly</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w</td>
<td>P-element</td>
<td>106</td>
<td>70</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CRISPR (Fst)</td>
<td>30</td>
<td>23</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>nos-Cas9 or act-Cas9</td>
<td>CRISPR (Fst)</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total=</td>
<td>146</td>
<td>101</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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.

<table>
<thead>
<tr>
<th>Fly strain</th>
<th>DNA Injection</th>
<th>Number of injected embryos</th>
<th>Number of survivors</th>
<th>Average of survival rate</th>
<th>Number of sterile survivors</th>
<th>Average of sterility rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>yw</td>
<td>ftz double chiRNAs (5' and 3')</td>
<td>852</td>
<td>63</td>
<td>8.4%</td>
<td>29</td>
<td>27.1%</td>
</tr>
<tr>
<td></td>
<td>ftz single chiRNA (5' or 3')</td>
<td>464</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nos Cas9-</td>
<td>ftz double chiRNAs (5' and 3')</td>
<td>3368</td>
<td>93</td>
<td>2.3%</td>
<td>30</td>
<td>24.6%</td>
</tr>
<tr>
<td>or act-Cas9</td>
<td>ftz single chiRNA (5' or 3')</td>
<td>1600</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. The average survival rate and sterility rate of CRISPR RNA injection.

<table>
<thead>
<tr>
<th>Fly strain</th>
<th>RNA Injection</th>
<th>Number of injected embryos</th>
<th>Number of survivors</th>
<th>Average of survival rate</th>
<th>Number of sterile survivors</th>
<th>Average of sterility rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>yw</em></td>
<td><em>fitz</em> double chiRNAs (5' and 3')</td>
<td>1028</td>
<td>56</td>
<td>5.7%</td>
<td>53</td>
<td>39.8%</td>
</tr>
<tr>
<td></td>
<td><em>fitz</em> single chiRNA (5' or 3')</td>
<td>1300</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fst</em></td>
<td><em>Fst</em> double chiRNAs (5' and 3')</td>
<td>214</td>
<td>30</td>
<td>14.0%</td>
<td>7</td>
<td>23.3%</td>
</tr>
<tr>
<td><em>nos</em> Cas9- or <em>act-Cas9</em></td>
<td><em>fitz</em> double chiRNAs (5' and 3')</td>
<td>2074</td>
<td>114</td>
<td>3.1%</td>
<td>30</td>
<td>25.9%</td>
</tr>
<tr>
<td></td>
<td><em>fitz</em> single chiRNA (5' or 3')</td>
<td>272</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Fst</em> double chiRNAs (5' and 3')</td>
<td>246</td>
<td>10</td>
<td>4.1%</td>
<td>2</td>
<td>20.0%</td>
</tr>
</tbody>
</table>
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 FTZFL should develop normally with all even numbered parasegments, as this locus expresses FTZ with both HD-dependent and HD-independent FTZ activities. ftzattR ftzΔHD 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). ftzattR ftzΔFTZ-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$^{attR ftz\Delta FTZ-F1 attR}$ 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$^{attR ftz\Delta FTZ-F1 attR}$ 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


Appendices

List of Primers

**Appendix 1. Primers used for cloning ftz chiRNAs into pU6\_BbsI\_chiRNA plasmid**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' chiRNA-F</td>
<td>5'-...CTTCGACCTCTACCATCTGTCTTG…-3'</td>
</tr>
<tr>
<td>5' chiRNA-R</td>
<td>5'-....AAACCAAGACAGATGGTAGAGGTC…-3'</td>
</tr>
<tr>
<td>3' chiRNA-F</td>
<td>5'-...CTTCGTGGCTCTGGCTTTGTGG…-3'</td>
</tr>
<tr>
<td>3' chiRNA-R</td>
<td>5'-....AAACCCACAAACAGCCAGCCAC…-3'</td>
</tr>
</tbody>
</table>
### Appendix 2. Primers used for constructing the repair template

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' ftz-F</td>
<td>5'...CAGCTAGGTCTCGCTATAGCATCCATAGACAACCTACTTTAA...-3'</td>
<td>y w</td>
</tr>
</tbody>
</table>
| 5' ftz-R | 5'...CAGCTAGGTCTCCCATGCCCCAACTGAGAGA  
CTCAAAGGGTTACCCAGTTGGGGCGCATGTGTATTGGCTAGATTT...-3' | y w                        | (Genomic DNA)               |
| y+F      | 5'...CAGCTAGGTCTCCCATGCGACTATTAAATGATTATCGCC...-3'                      | MiMIC                      | (Venken et al., 2011)       |
| y+R      | 5'...CAGCTAGGTCTCGGTCTCGACCTGCAGGTCAACGGATC...-3'                      | y w                        | (Genomic DNA)               |
| 3' ftz-F | 5'...CAGCTAGGTCTCGGGACCCCCCAACTGAGAGA  
CTCAAAGGGTTACCCAGTTGGGGGCTCGGCGATGCTCAGTTAC...-3' | y w                        | (Genomic DNA)               |
| 3' ftz-R | 5'...CAGCTAGGTCTCCGCCCCAAAATGACATTTTCTCTGGCG...-3'                      | y w                        | (Genomic DNA)               |
### Appendix 3. Primers used for colony PCR screening of the correct repair template

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screen 1-F</td>
<td>5'...CTGGCAGTTCCCTACTCTCG…-3'</td>
</tr>
<tr>
<td>Screen 1-R</td>
<td>5'...GGTAAATCAGCGGGCTGCGTTG…-3'</td>
</tr>
<tr>
<td>Screen 2-F</td>
<td>5'...CAGGGAAAGTTCAACTTAAATCG…-3'</td>
</tr>
<tr>
<td>Screen 2-R</td>
<td>5'...CTGTCCTGGCTGGTCTAGACGTC…-3'</td>
</tr>
<tr>
<td>Screen 3-F</td>
<td>Same as (Screen 2-F)</td>
</tr>
<tr>
<td>Screen 3-R</td>
<td>5'...GAGCCGCCACCAATTGGACC…-3'</td>
</tr>
</tbody>
</table>
Appendix 4. Primers used to generate chiRNAs template for IVT

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>T7-ftp chiRNA1-F</td>
<td>5’-...GAAATTAATACGACTCACTATAGGGATAGTAGCCCTGATAAT</td>
</tr>
<tr>
<td></td>
<td>GTTTTAGAGCTAGAAATAGC…-3’</td>
</tr>
<tr>
<td>T7-ftp chiRNA2-F</td>
<td>5’-...GAAATTAATACGACTCACTATAGGGATAGGCATCGTACTGCTGG</td>
</tr>
<tr>
<td></td>
<td>GTTTTAGAGCTAGAAATAGC…-3’</td>
</tr>
<tr>
<td>T7-Fst chiRNA1-F</td>
<td>5’-...GAAATTAATACGACTCACTATAGGGCTGGGTTCGGAATTT</td>
</tr>
<tr>
<td></td>
<td>GTTTTAGAGCTAGAAATAGC…-3’</td>
</tr>
<tr>
<td>T7-Fst chiRNA2-F</td>
<td>5’-...GAAATTAATACGACTCACTATAGGGCTGGGTTCGGAACCTCC</td>
</tr>
<tr>
<td></td>
<td>GTTTTAGAGCTAGAAATAGC…-3’</td>
</tr>
<tr>
<td>Common primer-R</td>
<td>5’-...AAAAGCACCCTCGGTGCCACTTTTTCAGTTTAGATAACCGACTAGCCTTTTTTAA</td>
</tr>
<tr>
<td></td>
<td>CTTGCTATTTTCTAGCTCTAAAAC…-3’</td>
</tr>
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