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Investigating HOX Protein Requirement for Tarsus Determination in Drosophila melanogaster

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

INVESTIGATING HOX PROTEIN REQUIREMENT FOR TARSUS DETERMINATION IN *DROSOPHILA MELANOGASTER*

(Thesis format: Monograph)

by

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of the requirements for the degree of
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Abstract

Generally, all bilaterans examined have similar conservation of HOX protein structure, function, expression, and requirement. However, at the level of being the same, it is unknown whether the HOX protein, Antennapedia, is required for tarsus determination in *Drosophila melanogaster* as in *Tribolium casteneum*, or whether the requirement of HOX proteins in determination of body parts diverges in insects. I proposed to use a heat shock-inducible nanobody (*UAS- NSlmb-vhhGFP4* driven by *hsp-GAL4*) activated during the third larval stage in all cells to degrade thoracically expressed HOX proteins (Sex combs reduced, Antennapedia, and Ultrabithorax) tagged with green fluorescence protein (GFP) derivatives; GFP, YFP, CFP, or 17 amino acid epitope of GFP. Due to difficulties in establishing CRISPR mediated homologous recombination, only the initial steps have been completed, but the system is now established to determine whether HOX requirement for tarsus determination is conserved in insects.

Keywords

Drosophila melanogaster, Homeotic selector (*Hox*) genes, *Sex combs reduced*, *Antennapedia*, *Ultrabithorax*, thoracic segmental identity, green fluorescence protein (GFP), tarsus determination, CRISPR/Cas9, nanobody, V_HH, NSlmb-vhhGFP4

Co-Authorship Statement

I performed all experimental procedures and drafted the manuscript. Laura Garofalo contributed her results to one injection experiment: injection of flies with P element or *Frost.* My supervisor, Dr. Anthony Percival-Smith, made intellectual contributions to experimental design, assisted in construction of the repair vectors, and provided editorial comments.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

aa	amino acid
abdA	*abdominal-A*
AbdB	*Abdominal-B*
act	*actin5c promoter*
ANT-C	*Antennapedia* Complex
Antp	*Antennapedia*
A-P	anterior to posterior
bp	base pair
BX-C	*Bithorax* Complex
Cas	CRISPR-associated
CDR	complementarity determining region
CFP	cyan fluorescence protein
chiRNA	chimeric RNA
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Dfd	*Deformed*
DSB	double stranded break
dsDNA	double-stranded DNA
Fab	antigen-binding region of an antibody
FBP	F-box protein
Fc	constant region of an antibody
Fst	*Frost*
GFP	green fluorescence protein
HOM-C	*Homeotic* Complex
HR	homologous recombination
hsp	*heat-shock promoter*
IgG	immunoglobulins
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pairs

lab	*labial*
LB	Luria Bertani
lf	loss-of-function
Mb	mega base pairs
NHEJ	non-homologous end joining
nos	*nanos promoter*
nt	nucleotide
PAM	protospacer adjacent motif
pb	*proboscipedia*
PBS	phosphate buffered saline
Ptl	*prothoraxless*
RVD	repeat variable di-residues
Scr	*Sex combs reduced*
Slmb	Supernumerary limbs
SRSR	short regularly spaced repeats
ssDNA	single-stranded DNA
TALE/TALEN	transcription activator-like effector / nuclease
tracrRNA	*trans*-activating CRISPR RNA
ts	temperature sensitive
UAS	upstream activation sequence
Ubx	*Ultrabithorax*
V_HH	single heavy chain variable domain (nanobody)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP	yellow fluorescence protein
ZFN	zinc-finger nuclease

N.B. SI units are not listed

1 Introduction

1.1 Advantages of *Drosophila* as a Model Organism

Drosophila melanogaster, the common fruit fly, was first documented as being used in 1901 by William Castle's laboratory at Harvard. However, Thomas Hunt Morgan is considered to be the "father" of *Drosophila* research (Kohler, 1994). Morgan began using *Drosophila* for the experimental study of evolution around 1906 and shortly thereafter he discovered the *white* gene (flies with this mutation have white eyes instead of red). This led to fruit flies making important contributions to the fields of medical and scientific research, including molecular biology, cell biology, developmental biology, and population biology (Stephenson & Metcalfe, 2013). Morgan won the Nobel Prize in Physiology or Medicine in 1933 *"for his discoveries concerning the role played by the chromosome in heredity"* and his students also went on to make key advances in genetics and helped to expand *Drosophila* as the leading genetic system. One of whom, Muller, won a Nobel Prize in Physiology or Medicine in 1946 "*for the discovery of the production of mutations by means of x-ray irradiation*" and is also credited with the development of balancer chromosomes. The past few decades has seen *Drosophila* become a predominant model used to understand how genes direct the development of an embryo from a single cell to a mature multicellular organism (Edward B. Lewis, Christiane Nüsslein-Volhard, and Eric F. Wieschaus won a Nobel Prize in Physiology or Medicine 1995 "*for their discoveries concerning the genetic control of early embryonic development")*. Many of the processes that direct development within the fly have been conserved through evolution and are strikingly similar to the processes that direct development in all organisms. The *Drosophila* genome sequence was first released in March 2000 and is freely available, along with annotations, via "Flybase", an online database dedicated to *Drosophila* (dos Santos et al., 2014). Currently, it is estimated that there are ~14,000 genes in *Drosophila* (Adams et al., 2000). The past century has seen a huge range of genetic tools become available for *Drosophila* that match or surpass those for any other multicellular organisms. There are vast assortments of strains containing endogenous mutations available from stock centres or the labs that generated them which are collated in Flybase.

Drosophila have a relatively short life cycle and are inexpensive and easy to keep. This means that genetic experiments that would take months or even years in vertebrate models, such as mice, *Xenopus*, or zebrafish, can be completed in a matter of weeks. Female flies can lay up to ~100 eggs per day for up to 20 days and it takes approximately 10 days at 25ºC for an embryo to develop into a fertile adult fly (Dahmann, 2008). Thus, it is relatively easy to generate large numbers of embryos (only ~500 µm in size) or flies for an experiment (Jennings, 2011). Furthermore, unlike other vertebrate models, *Drosophila* is not subject to animal licensing laws and there are generally very few restrictions on their use in the laboratory as there are minimal ethical and safety issues (Jennings, 2011). Additionally, individual flies are easily manipulated if necessary through safe anaesthetisation with carbon dioxide. Fly strains are maintained through living stocks (Jennings, 2011).

1.2 Genetic Tools Available in *Drosophila*

Prior to the 1940s, *Drosophila* was used as a major model organism in the field of genetics (Hartwell et al., 2004). However, after the establishment of recombinant technology with bacteria and virus models, *Drosophila* again became a prominent model organism for the study of developmental genetics (Hartwell et al., 2004). The renewed interest in *Drosophila* was because genes previously identified by mutant alleles that had been shown to be important in development or behavior could now be isolated and analyzed using recombinant technology (Hartwell et al., 2004). The isolation of *Drosophila* genes led to the discovery that similar genes were found in higher organisms (Hartwell et al., 2004).

1.2.1 Balancer Chromosomes

In *Drosophila*, crossing-over only occurs in females. The absence of crossing-over in males allows linkage relationships to be maintained by ensuring strict male inheritance (Hartwell et al., 2004). In females, only one or two crossing-over events typically occur on each major chromosome arm per meiosis. To help maintain linkage, "balancer chromosomes", which contain multiple, overlapping inversions, are used. Balancer chromosomes normally carry a dominant marker to allow researchers to follow the

balancer through crosses, as well as a recessive lethal mutation, such that flies homozygous for the balancer chromosome don't survive (Herman et al., 1976). This allows no recombinant chromosomes to be passed to the next generation, and therefore, balancer chromosomes and their homologs are inherited as intact single mendelian units ("An Introduction to Balancers," Bloomington *Drosophila* Stock Center website). Balancer chromosomes are essential in maintaining stocks of flies carrying lethal mutations and in many genetic manipulations.

1.2.2 Transgenesis: P Element

"Transgenesis" is a general term referring to a group of technologies that allows the reintroduction of DNA into the genome of an organism. P element mediated transformation was the first of these methods to be developed. Originally identified as a tool for mutagenesis, Rubin & Spradling (1982) recognized the ability to use P elements for transgenesis. The transposase enzyme causes the excision of a P element from its initial position in the host genome and the reinsertion of the element randomly at another position in the genome. To prevent uncontrolled transposition, the two parts of the P element, the transposase enzyme and the recognition sequences for transposase action, are separated on two separate plasmids, the P element plasmid and a helper plasmid (Karess & Rubin, 1984). This method has a wide variety of applications, including enhancer trapping, gene-tagging, targeted misexpression, RNA interference (RNAi) delivery, and homologous recombination/gene replacement.

1.2.3 *LoxP*/Cre

Advances in the field of transgenesis have improved fly transgenic methods. The P element method is limited by the small size of the DNA that can be integrated, and that the insertion site cannot be controlled. Methods using recombinases and integrases are integration site specific (Venken & Bellen, 2007). In one such method, the *loxP*/Cre system is composed of the recombinase, Cre, which recognizes the target site, *loxP*. When the genome has a single *loxP* site, Cre can target a transgene to this site that also has a *loxP* site. When a transgene is integrated in DNA and contains two flanking *loxP* sites, the Cre recombinase removes the transgene. However, when the Cre recombinase excises DNA, one of the *loxP* sites remains in the genome, referred to as a "scar". When

multiple, sequential recombination events are required, as is the case for this project, the *loxP* scar will interact with other *loxP* sequences in the genome causing chromosomal deletions and inversions (Delneri et al., 2000, 2003). A mutant *loxP, lox2272,* does not recombine with the standard *loxP* site but is still recognized by Cre recombinase to allow multiple, sequential recombination events (Lee & Saito, 1998; Missirlis et al., 2006).

1.2.4 GAL4/UAS

Transgenesis can also be used to induce ectopic misexpression of genes, that is, abnormal temporal expression or expression in cells or tissues outside where the gene is normally expressed. For example, fusing a *heat-shock promoter (hsp)* to a gene renders the expression of the gene to be temperature-dependent and ubiquitous (Bonner et al., 1984). An improved technique allows gene expression to be controlled both temporally and spatially using a yeast transcriptional activator *GAL4* (Brand & Perrimon, 1993). In the GAL4/UAS system, GAL4 expression induces a *GAL4* responsive promotor, or upstream activation sequence (*UAS*). Depending on the promoter used, GAL4 can be expressed in a specific pattern, at a specific time, (example using a *hsp*), or ubiquitously (example using the *armadillo* promoter). Genes that are downstream of a *UAS* sequence can only be activated by GAL4, and therefore, control of the expression of a gene of interest at almost any stage of development and in any tissue is possible (Ryder & Russell, 2003). To further refine the temporal expression of GAL4 activity, a temperature-sensitive (ts) $GAL80^{ts}$ is often used (Matsumoto et al., 1978). GAL80 protein binds to GAL4, repressing the activation of gene expression (Brand & Perrimon, 1993). Therefore, only at high temperatures does $GAL80^{ts}$ dissociate from GAL4 allowing gene expression.

1.2.5 RNAi

Contrary to ectopically expressing genes, is to silence gene expression. One method to accomplish this is with inducible RNA interference (RNAi). RNAi allows silencing of a gene without having to mutate the endogenous copy by injecting double-stranded RNA into an embryo which then acts as a degradation template for the host mRNA (Fire et al., 1998; Yang et al., 2000). However, induction of RNAi in *Drosophila* by injection has problems associated with it, including that the effect is not stably inherited and that silencing a gene early in development can hinder the analysis of its effects later in

development (Kennerdell & Carthew, 1998). In addition, RNAi is not very efficient and results in off-target effects (Qiu et al., 2005).

1.2.6 Transgenesis: Zinc-finger and Transcription Activator-like Effector Nucleases

The big disadvantage of P element or any transposon-mediated system of transgenesis, is that the DNA is incorporated randomly in the genome. The expression of the transgene is then susceptible to the chromatin environment around the insertion site, referred to as position effect. The use of site-specific recombinases allows the insertion of all constructs at the same position in the genome (Venken & Bellen, 2007). But perhaps the best approach in transgenesis is the ability to modify the endogenous locus through homologous recombination (HR). HR is a powerful tool for genome editing in which a double stranded break (DSB) in the endogenous DNA is repaired by an exogenous repair template resulting in an exchange of nucleotide (nt) sequence (Capecchi, 1989). DSBs stimulate cellular DNA repair mechanisms, including either HR when a repair template is supplied, or error-prone non-homologous end joining (NHEJ; Lieber, et al., 2003; West, 2003). The first method of creating DSBs in the *Drosophila* genome was purely genetic, using a yeast I-SceI endonuclease (Bellaiche et al., 1999; Rong & Golic, 2000). However, this method is very labour intensive and inefficient. Some improvements for creation of site-specific, targeted DSBs have since been discovered using nucleases like Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs are protein fusions between the FokI nuclease and three zinc-finger DNA-binding domains that introduce sequence specificity (Bibikova et al., 2003). TALENs are a protein fusion between the FokI nuclease and a transcription activator-like effector (TALE; Christian et al., 2010). These two methods, although useful, are labour-intensive and time-consuming.

1.2.7 Transgenesis: CRISPR

A simple and more efficient alternative has recently emerged to induce targeted DSBs: CRISPR (clustered regularly interspaced short palindromic repeats). In type II CRISPR systems, short sequences complementary to foreign DNA, CRISPR RNA (crRNA), and *trans*-activating CRISPR RNA (tracrRNA), direct sequence-specific cleavage of exogenous DNA through interaction with a CRISPR-associated nuclease (Cas9; Gaj et

al., 2013; Gratz et al., 2013; Figure 1.A). The Cas9 protein requires a 20 nt guide sequence complementary to the target site within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence, NGG, upstream of the crRNA binding region (Jinek et al., 2012). On average, this sequence (NGG) occurs every 8 bases in the DNA and recent reports suggest that this requirement may be relaxed to include NAG sequences, increasing the number of potential target sites (Mali et al., 2013a). Additionally, Cas9 nucleases may be engineered with altered PAM specificities (Kleinstiver et al., 2015). This allows the system to be used to target virtually any sequence for cleavage simply by redesigning the crRNA. The system has been simplified into a minimal two-component system – Cas9 and a chimeric RNA (chiRNA) that includes both the crRNA and the tracrRNA from *Streptococcus pyogenes* (Jinek et al., 2012; Figure 1.B). The CRISPR system has been used to accomplish efficient genome engineering in many organisms, including but not limited to *Drosophila* (Chang et al., 2013; Cho et al., 2013; Cong et al., 2013; DiCarlo et al., 2013; Ding et al., 2013; Friedland et al., 2013; Gratz et al., 2013; Guo & Li, 2015; Hwang et al., 2013; Jiang et al., 2013; Jinek et al., 2012; Mali et al., 2013b; Wang et al., 2013). Optimization to improve specificity and efficiency has seen development of transgenic flies expressing *Cas*9 and/or chiRNA. The germline-specific *nanos (nos)* promoter and the ubiquitous *actin5C (act)* promoter are two commonly used promoters to drive *Cas9* expression in *Drosophila* (Kondo & Ueda, 2013; Port et al., 2014; Ren et al., 2013).

1.3 CRISPR: A History

CRISPRs are the most efficient method of genome editing to date. A series of repeats were first discovered in the *Escherichia coli* genome in 1987 (Ishino et al., 1987). They were originally termed Short Regularly Spaced Repeats (SRSRs) and in 2000, these repeats were found to exist in the genomes of other archaea and bacteria (Mojica & Díez-Villaseñor, 2000). CRISPR loci have been found in 80% archaeal genomes and about 40% of bacterial genomes sequenced to date ("CRISPRs Database," 2014). In 2002, SRSRs were renamed CRISPRs when it was established that the three major components of a CRISPR locus are: *cas* genes (encode a nuclease), a leader sequence, and a repeat-spacer array (Jansen et al., 2002). In 2005, three independent research groups indicated

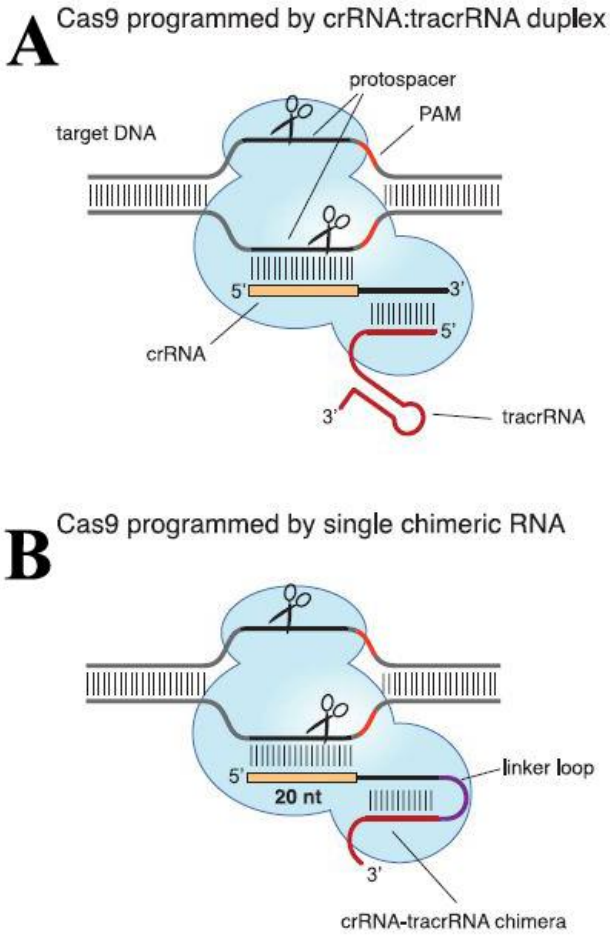

Figure 1. CRISPR System.

(A) In the type II CRISPR system, transcripts from the CRISPR repeat arrays are processed into crRNAs, each harboring a variable sequence homologous to that of the invading DNA, known as the "protospacer" sequence, and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA, known as the tracrRNA, and these two RNAs complex with the Cas9 nuclease. The crRNA directs Cas9 to cleave complementary target-DNA sequences, if they are adjacent to short sequences known as protospacer adjacent motifs (PAMs). (B) The system was further simplified to a two-component system that utilizes a chiRNA, or guide RNA, which is a fusion of the crRNA and tracrRNA, and Cas9. The chiRNA uses 20 nt of sequence that corresponds to the target DNA sequence (Jinek et al., 2012. Reprinted with permission from AAAS). N.B. Copyright image permissions are provided in Appendix C for all figures/images.

that the CRISPR/Cas system has a role in bacterial adaptive immunity and worked analogously to the eukaryotic system of RNAi, with the spacers serving as a template for RNA molecules (Bolotin et al., 2005; Makarova et al., 2006; Mojica et al., 2005; Pourcel et al., 2005). Barrangou et al. (2007) showed that the resistance specificity was due to the spacer DNA sequence coinciding to phage DNA sequence. The study of CRISPR/Cas relationships identified 3 major systems, Type I, Type II, and Type III, which all have three stages: adaptation, expression and interference (Figure 2; Makarova et al., 2011). The adaptation stage involves the recognition of invading DNA and then incorporation of some of the invading DNA sequence, called spacers, into the CRISPR array by Cas proteins (Makarova et al., 2011). The CRISPR array is then transcribed in the expression stage to form a long pre-CRISPR RNA (pre-crRNA) that is processed and assembled into one of three crRNA/Cas riboproteins (Makarova et al., 2011). The Type I, Type II, or Type III crRNA/Cas riboproteins interfere with invading DNA, cleaving it at the target sites (Makarova et al., 2011). The Type II system, which uses the nuclease, Cas9, was used by researchers in 2012 to edit the genome of human tissue culture cells (Jinek et al., 2012). In type II CRISPR systems, short sequences complementary to foreign DNA, crRNA, and tracrRNA, direct sequence-specific cleavage of exogenous DNA through interaction with a Cas9 nuclease (Jinek et al., 2012; Figure 1.A). Jinek et al. (2012) then further simplified the type II system to be a minimal two-component system – Cas9 and a chimeric RNA (chiRNA) that is a fusion of the crRNA with the tracrRNA from *Streptococcus pyogenes* (Figure 1.B). Since then, the CRISPR system has been exploited and used efficiently in many organisms and cells, including, yeast (DiCarlo et al., 2013), zebrafish (Chang et al., 2013; Hwang et al., 2013), mice (Wang et al., 2013), nematodes (Friedland et al., 2013), plants (Jiang et al., 2013), monkey embryos (Guo & Li, 2015), human stem cells (Cho et al., 2013; Cong et al., 2013; Ding et al., 2013; Jinek et al., 2012; Mali et al., 2013b) and *Drosophila* (Gratz et al., 2013). More importantly, CRISPR has interesting applications in medicine, for example curing genetic disease. Mice were cured of a liver disorder (Yin et al., 2014). Libraries of tens of thousands of guide RNA (chiRNA) are already available for human genes (Pennisi, 2013) and the CRISPR systems have continued to be optimized.

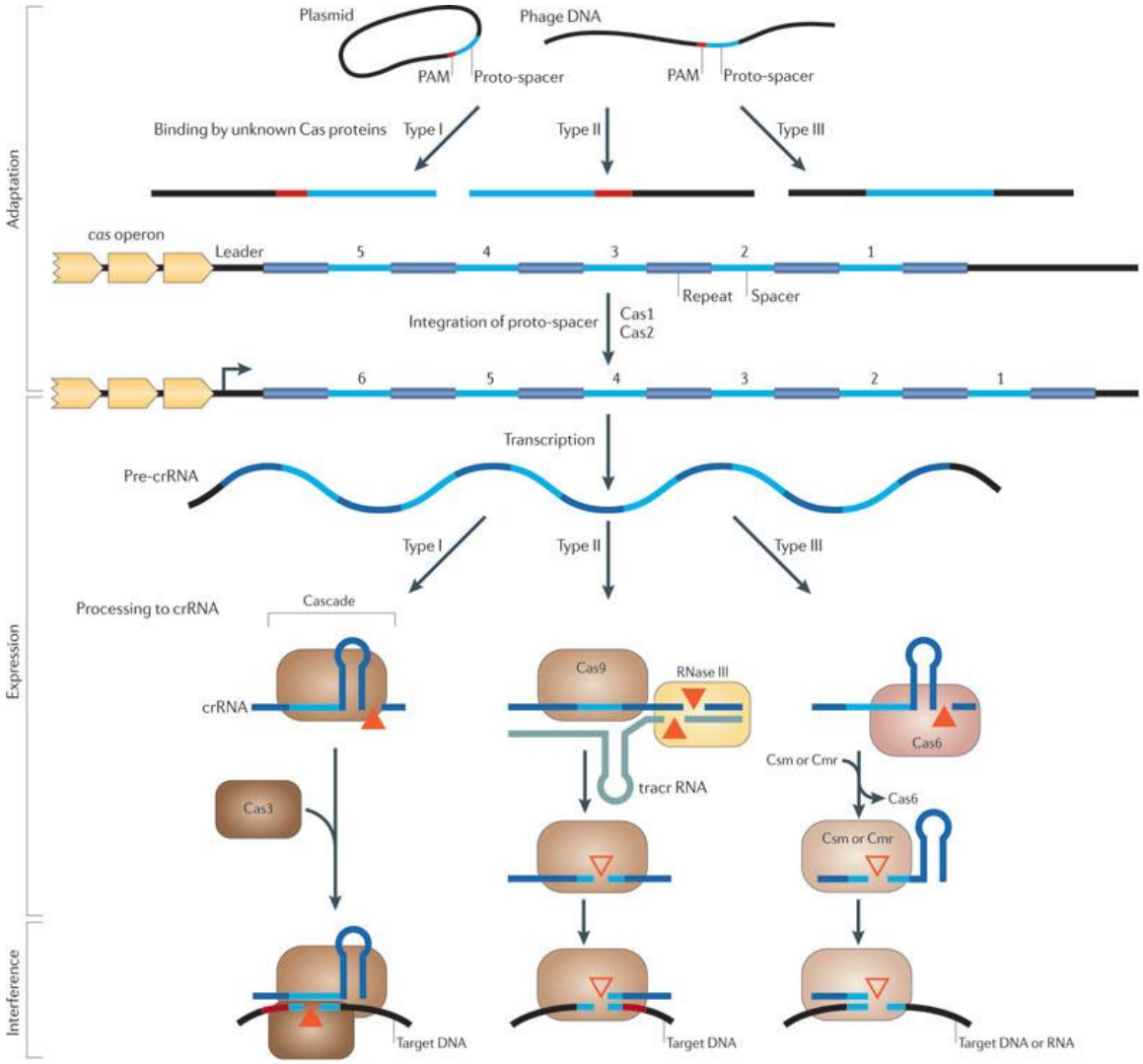

Figure 2. The Three Stages of CRISPR–Cas Action.

CRISPR–Cas systems act in three stages: adaptation, expression and interference. Adaptation begins by recognition of invading DNA by Cas1 and Cas2 and integration of short sequences in a CRISPR array. Expression involves transcription of the CRISPR array into a long pre-crRNA. CrRNA processing and interference (nucleic acid cleavage) is different in each of the three CRISPR systems, Type I, II, and III (Makarova et al., 2011)

1.4 Development of *Drosophila*

Fruit flies like to lay their eggs near the surface of fermenting (ripening) foods or other organic materials. *Drosophila*, like butterflies and moths, are holometabolous insects, which mean that they undergo a four stage life cycle; egg, larva, pupa, and adult fly (Figure 3). At 25ºC, the fertilized embryo develops in the egg for around one day before hatching as a larva. The initiation of *Drosophila* embryogenesis is characterized by a syncytial blastoderm, where nuclei divide in a multinucleate common cytoplasm for around 2 hours. During this syncytial blastoderm stage, a subset of nuclei cellularizes gaining a plasma membrane. These cells are the pole cells and are the progenitors of the germ line, the sperm and ova (Campos-Ortega & Hartenstein, 1997). After a few more nuclear divisions, the rest of the nuclei cellularize to form the cellular blastoderm. The cellular blastoderm then undergoes gastrulation, giving rise to the three germ layers. For the next 6 hours, germ band elongation takes place, in which the ventral epidermis lengthens and mitotic divisions begin to occur. The neuroblasts are determined, the embryo becomes segmented, and the cephalic furrow begins formation. Subsequently, the head features start to develop and the neuroblast complete their formation. Germ band retraction begins at ~9.5 hours post-fertilization which involves ventral closure and segment formation within the embryo. The central and peripheral nervous systems become differentiated, followed by dorsal closure of the midgut and epidermis. Head involution begins, and dorsal closure is completed. Next, the ventral nerve cord shortens and air fills the tracheal tree. Embryogenesis concludes with the hatching of a first instar larva at 21-22 hours after fertilization. Over the next five days, the larva eats and grows, going through two molts, to become a second and third instar larva, until it pupates. While a pupa, it undergoes metamorphosis into the adult fly over the course of four days. Most of the embryonic and larval tissue is destroyed during metamorphosis, and the adult tissues (e.g. wing, leg, eye) develop from groups of cells known as imaginal discs. Imaginal discs are derived from cells set aside during embryogenesis that form the adult-specific structures. During the larval stages and metamorphosis, the imaginal cells proliferate to form differentiated discs of cells. When metamorphosis is complete, the adult-specific identities are formed and a mature adult fly ecloses.

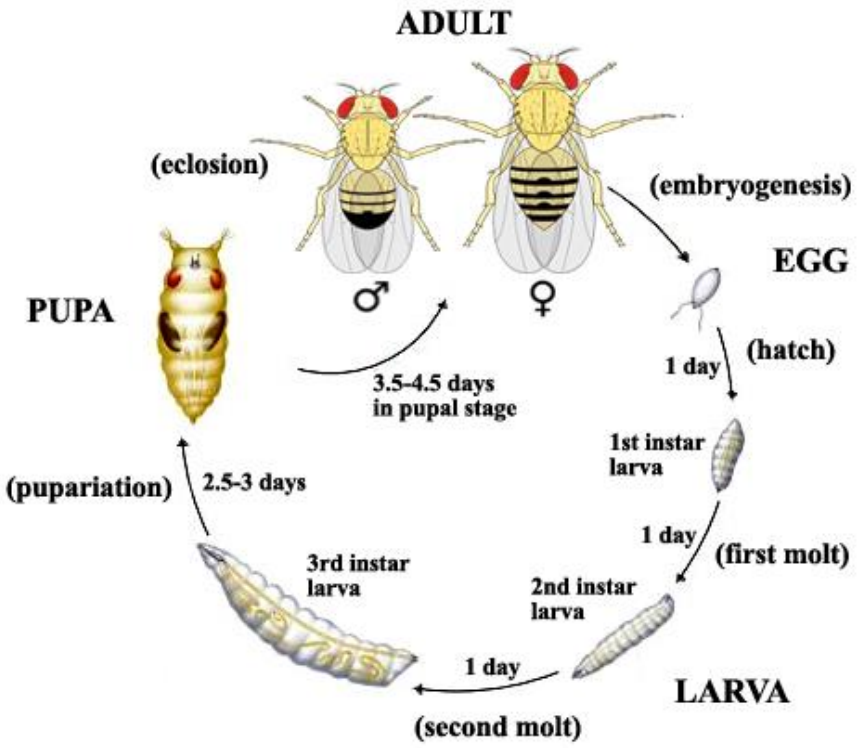

Figure 3. Schematic of the Stages of the *Drosophila* Life Cycle.

After fertilization and the initiation of embryogenesis, an embryo hatches as a first instar larva before it molts twice to form a second and third instar larva. The third instar larva pupariates into a pupa and then ecloses as a mature adult fly. This figure was adapted from Hartwell et al. (2004).

1.5 The *Drosophila* Genome

The *Drosophila* genome has been well characterized and was one of the first eukaryotic genomes to be sequenced, closely following yeast (*Saccharomyces cerevisiae)* and the nematode (*Caenorhabditis elegans)* (Adams et al., 2000; The C. elegans Sequencing Consortium, 1998; Goffeau et al., 1996). The DNA of *Drosophila* is broken up into six units: three autosomes (chromosome 2, 3, and 4), two sex chromosomes (X and Y; Figure 4) as well as the mitochondrial genome. Out of ~180 Mb, 120 Mb is euchromatin, which is mainly located on the two large autosomes (2 and 3) and the X chromosome (Adams et al., 2000). The small fourth chromosome contains only ~1 Mb of euchromatin (Adams et al., 2000). The genomic DNA sequence of *Drosophila* is combined with all the known knowledge of *Drosophila* genetics and function in a publicly available database, called FlyBase (dos Santos et al., 2014). Although "*Drosophila*" is typically used to refer to *Drosophila melanogaster*, and indeed this is the case throughout this work, there are actually over 1500 different species in the *Drosophila* genus of the Drosophilidae family, of which 12 have been sequenced (Bächli, TaxoDros website; *Drosophila* 12 Genomes Consortium, 2007).

1.6 Gene Expression in *Drosophila*

The development of the larval anterior to posterior (A-P), or head to tail, axis has been the subject of intensive research. The process of the segmentation and organization of the body plan resulting in the formation of distinct developmental identities is proposed to be controlled by a regulatory hierarchy of five sets of genes: maternal effect genes, gap genes, pair rule genes, segment polarity genes, and homeotic genes (Figure 5). Most of these genes encode transcription factors. Segmentation begins before the egg is laid during oogenesis of the mother. In the ovary of the mother, specific genes are transcribed and their transcripts are transported to the egg and function after fertilization to pattern the embryo and specify cell states (St Johnston & Nüsslein-Volhard, 1992). For example, *Bicoid* RNA is localized in the cytoplasm at the anterior pole of the egg and is translated after fertilization to produce an A-P concentration gradient of Bicoid protein. A mother homozygous for a *bicoid* mutation produces embryos in which the head and thoracic

segments are missing (Driever & Nüsslein-Volhard, 1988). Maternal effect proteins

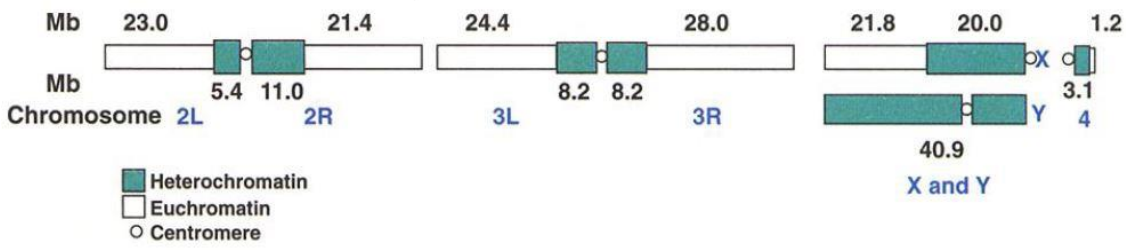

Figure 4. Mitotic Chromosomes of *Drosophila*, Showing Euchromatic Regions, Heterochromatic Regions, and Centromeres.

Arms of the autosomes are designated 2L, 2R, 3L, 3R, and 4. Length is in megabases (Mb). The heterochromatic block of the X chromosome is polymorphic among stocks and varies from one-third to one-half of the length of the mitotic chromosome. The Y chromosome is nearly entirely heterochromatic (Adams et al., 2000. Reprinted with permission from AAAS).

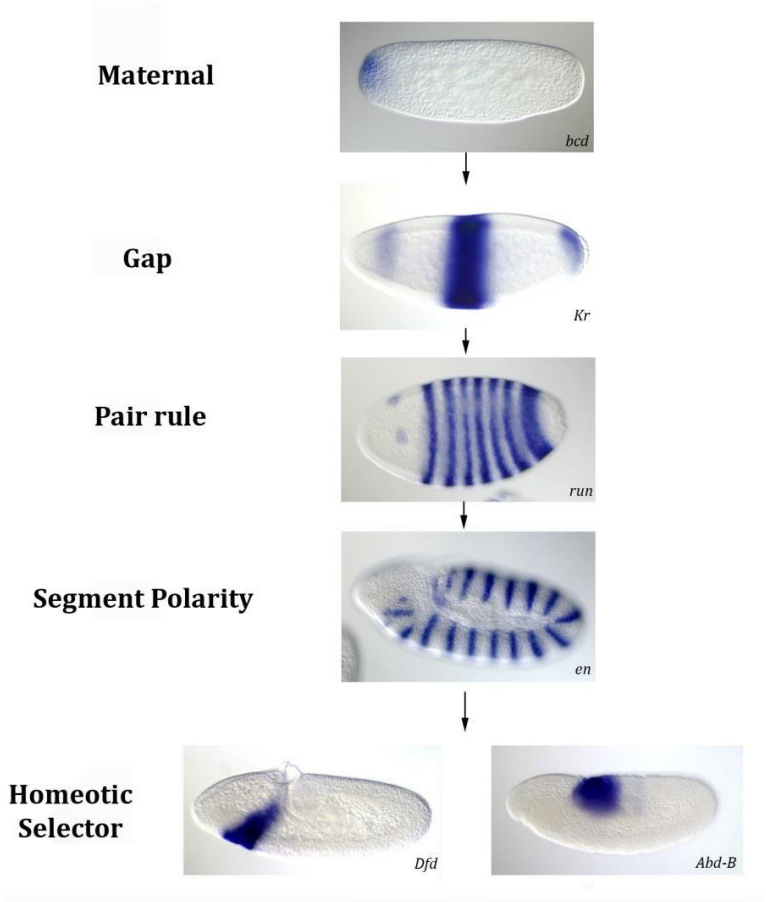

Figure 5. Segmentation Genes are Expressed as a Hierarchy to Regulate the Pattern of Development Along the A-P axis of a Developing *Drosophila* Embryo.

These are *in situ* hybridizations to mRNA of representative genes from each class of segmentation gene in the segmental hierarchy. The protein products of genes expressed earlier in the hierarchy regulate the expression of genes further down in the hierarchy to segment the developing embryo and determine segmental identities. The expression patterns of the Maternal coordinate gene, *bicoid* (*bcd*), Gap gene, *Krüppel* (*Kr*), Pair rule gene, *runt* (*run*), Segmental polarity gene, *engrailed* (*en*), and Homeotic selector genes, *Deformed* (*Dfd*) and *Abdominal-B* (*Abd-B*) are shown (Tomancak et al., 2002, 2007).

regulate the expression of the second class of segmentation genes, the gap genes. Gap genes are amongst the first zygotically expressed genes and are transcribed in spatially restricted expression domains along the A-P axis of the embryo and include: *huckebein, tailless, giant, hunchback, Krüppel,* and *knirps* (St Johnston & Nüsslein-Volhard, 1992). Gap loss-of-function (lf) mutations result in the loss of multiple, contiguous segments. The gap proteins regulate the expression of the third class of segmentation genes, the pair rule genes. Pair rule genes are expressed in a pattern of seven repetitive stripes (Rivera-Pomar & Jäckle, 1996). In homozygous pair rule mutants, every other segment is deleted (Jürgens et al., 1984; Wakimoto & Kaufman, 1981). The expression pattern of pair rule genes establishes 14 parasegments. Subsequently, the pair rule proteins regulate the expression of segment polarity genes. Segment polarity genes establish the polarity within a segment. Segment polarity mutations cause posterior cells to form anterior structures, often producing a mirror image of the anterior region (Nüsslein-Volhard & Wieschaus, 1980). The expression of the segment polarity genes, *wingless* and *engrailed*, define the parasegmental boundaries.

After the body is segmented by the first four classes of genes, the expression of the fifth class, *Hox* genes, determines the unique structure of individual body segments, referred to as segmental identity. *Hox* genes are important regulatory genes that are expressed in spatially restricted domains along the A-P axis of the embryo by the segmentation genes (Ingham & Martinez Arias, 1992). For example, the *Hox* gene, *Dfd* is expressed in the maxillary segment near the anterior end of the embryo, while the *Hox* gene, *Abd-B* is expressed in the most posterior end of the embryo in abdominal segments 8 and 9 (Figure 5). The phenotype of lf and gain-of-function alleles in *Hox* genes are the homeotic transformations of one segment into the likeness of another (Bateson, 1894; Bridges, 1915).

1.7 The Homeotic Selector (or *Hox*) Genes

The order of the Homeotic, or *Hox* genes along the chromosome coincides with the relative position of their expression pattern along the A-P axis of a developing embryo. For example, in *Drosophila*, the left most gene in the complex is the *labial (lab)* gene,

which is expressed most anteriorly in the embryo, and the right most gene, *AbdB*, is expressed most posteriorly in the embryo. All of the intervening genes along the complex, *proboscipedia (pb), Dfd, Sex combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx),* and *abdominal-A (abdA),* are expressed appropriately along the A-P pattern in the embryo according to their position in the complex (Figure 6). This pattern of expression is referred to as collinearity. The collective composition of the *Hox* gene complex is referred to as the homeotic complex (*HOM-C*). The *HOM-C* complex is broken into two in *Drosophila*, the *Antennapedia* and the Bithorax complexes (*ANT-C* and *BX-C)*.

The *Hox* genes contain a homeobox that encodes a DNA-binding motif, called a homeodomain. The HOX proteins are transcription factors and the homeodomain recognizes and binds to specific sequences of DNA (Levine & Hoey, 1988). *Hox* mutant alleles result in homeosis, in which the morphology of a body region is transformed to that of a different region (Bateson, 1894). Therefore, *Hox* genes act as binary switches, where loss of function produces one transformation in the region where the gene is active and gain of function produces the opposite transformation where the gene is normally inactive. For example, the antenna and leg are homologous appendages and mutations can cause the transformation of one into the other (Postlethwait & Schneiderman, 1971; Struhl, 1982). The appendages are homologous in that the different parts correlate to similar positions along the proximal-distal axis (Figure 7). The first antennal segment is homologous to the leg coxa, where they join to the body of the fly. The second antennal segment is homologous to the trochanter of the leg. The third antennal segment is homologous to the femur, tibia, and first tarsal segment of the leg and the arista and its base are homologous to the distal tarsal segments including the tarsal claw (Postlethwait & Schneiderman, 1971).

One of the major findings in developmental biology is that *Hox* genes are conserved at four levels: structure, expression, requirement, and function. The structure of individual *Hox* genes is conserved as well as the structure of the *Hox* gene complexes (Graham et al., 1989). The collinear pattern of expression of *Hox* genes is conserved (Graham et al., 1989). The products of the *Hox* genes are required in a similar manner for the

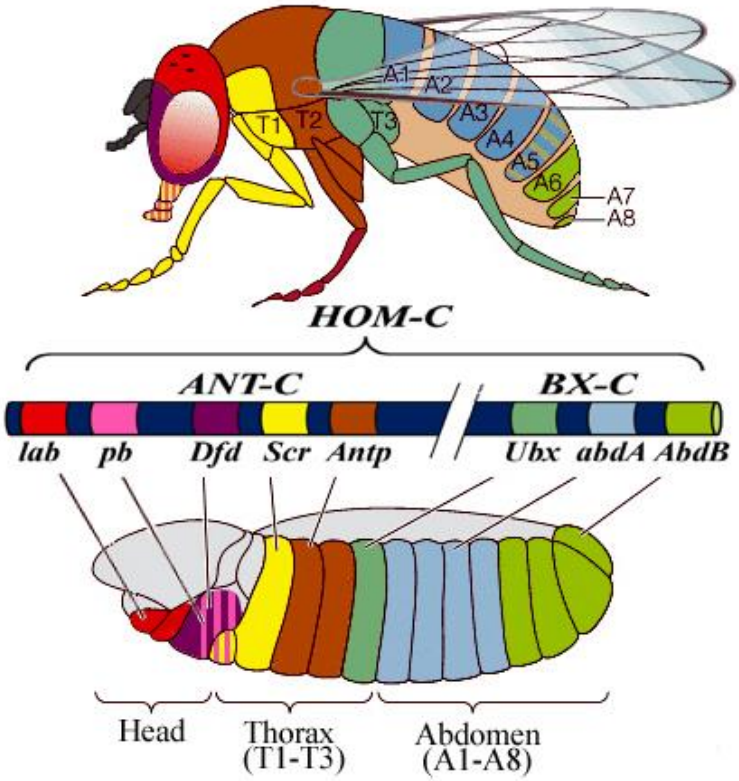

Figure 6. *Hox* Genes in *Drosophila*.

The order in which the *Hox* genes appear A-P in the complex on the chromosome correspond to the domain of expression along the A-P axis of the embryo and the segmental identity of A-P body parts in the adult fly. Abbreviations: *HOM-C, Homeotic* complex; *ANT-C, Antennapedia* complex; *BX-C, Bithorax* complex; *lab, labial; pb, proboscipedia; Dfd, Deformed; Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; abdA, abdominal-A; AbdB, Abdominal-B.* This figure was adapted from Sadava et al. (2008).

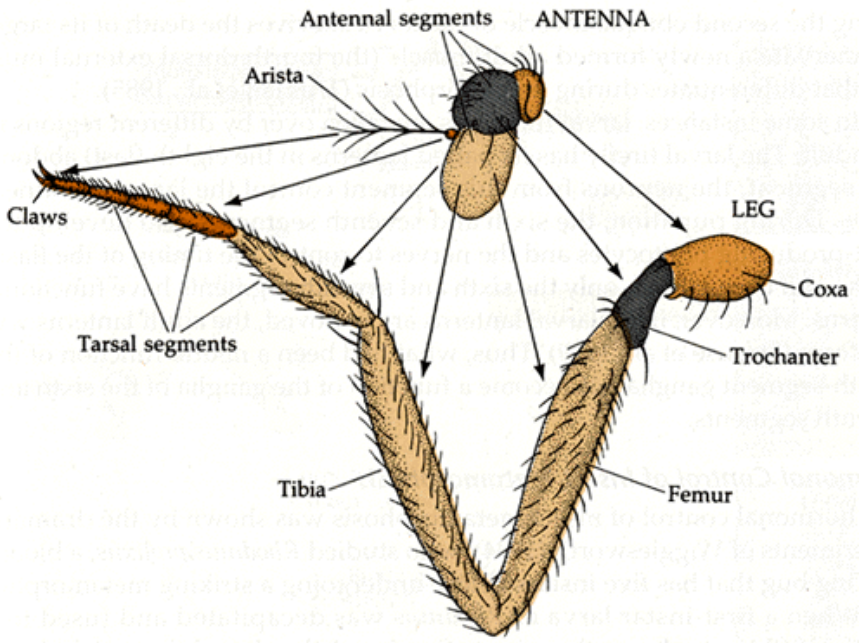

Figure 7. Homologous Appendages: Insect Leg and Antenna.

The first antennal segment is homologous to the leg coxa, where they join to the body of the fly. The second antennal segment is homologous to the trochanter of the leg. The third antennal segment is homologous to the femur, tibia, and first tarsal segment of the leg and the arista and its base are homologous to the distal tarsal segments including the tarsal claw (Gilbert, 2013 modified from Postlethwait & Schneiderman, 1971).

development of body plans of bilaterally symmetric organisms. Finally, a *Hox* gene of one organism can functionally replace that of another organism. These levels of *Hox* conservation are generally well established at a level of similarity; however, this work examines conservation of requirement in insects at a more detailed level of whether they are the same.

1.8 The Homeotic Transcription Factors, *Scr, Antp,* and *Ubx*

This project attempts to examine the requirement of a specific set of *Hox* genes potentially involved in tarsus determination. These *Hox* genes are, *Sex combs reduced (Scr), Antennapedia (Antp),* and *Ultrabithorax (Ubx),* which have an integral role in thorax development (Struhl, 1982). *Scr* activity is required for the prothorax (the first thoracic segment, T1) and *Ubx* activity is required for the metathorax (third thoracic segment, T3; Struhl, 1982). When these two genes are absent, both segments develop like the mesothorax (the second thoracic segment, T2; Struhl, 1982). If *Ubx* is lost early in development, while *Scr* remains, then posterior portions of the mesothorax and the metathorax develop as in the prothorax (Kerridge & Morata, 1982; Morata & Kerridge, 1981). All three thoracic segments require *Antp* activity and when it is absent, part of the mesothorax is transformed into corresponding antennal structures (Figure 8; Struhl, 1981b, 1982). Similar transformations to antenna occur in the prothorax and metathorax when *Scr* and *Ubx* are absent as well (Struhl, 1982). When examining normal HOX protein expression patterns early on in development, both ANTP and SCR are expressed in all leg imaginal discs. Specifically, ANTP is expressed in the ectoderm and by the late 3rd larval stage , ANTP is not expressed in the tarsus primordium (Wirz et al., 1986), but is restricted to the proximal leg primordium to help determine proximal leg structures (Abu-Shaar & Mann, 1998; Casares & Mann, 2001; Emerald & Cohen, 2004). SCR is expressed in the mesoderm of all leg imaginal discs (Glicksman & Brower, 1988). UBX is expressed strongly in the ectoderm of the third thoracic leg, and weakly in the second thoracic leg (Brower, 1987).

The analysis of orthologs in other species that evolved from a common ancestor can be used to compare how gene's function and requirement has evolved. As well as being

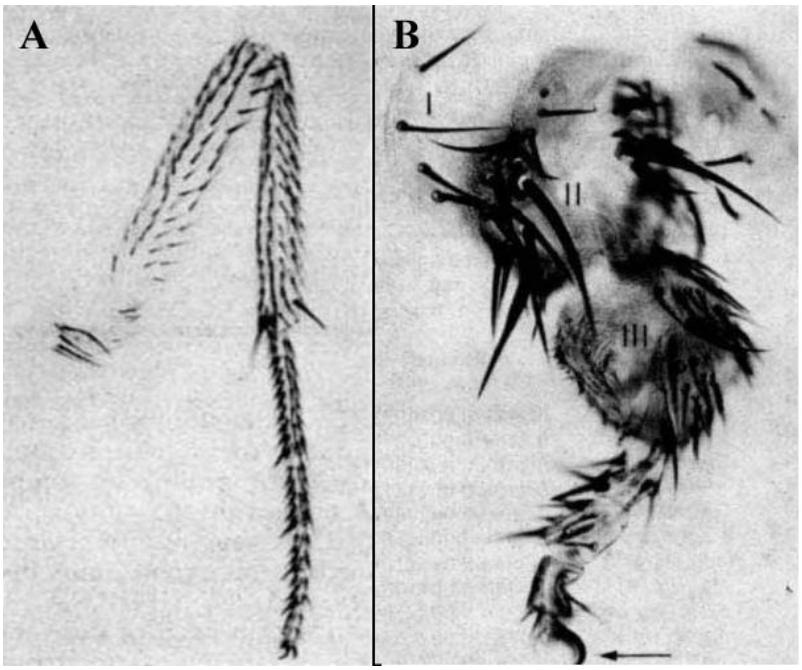

Figure 8. Phenotype of Somatic Clones Lacking the *Antp* Gene in the *Drosophila* Second Leg.

(A) Wild-type, second leg. (B) A blastoderm *Antp* clone covering the entire anterior compartment of the second leg, transforms it into corresponding portions of the antenna. Note the presence of structures characteristic of the first (I), second (II) and third (III) antennal segments. Note also that marked cells situated at the margins of the clone form normal leg tissue (in this case, a claw (arrow) and bristles characteristic of the distal leg). The untransformed posterior compartment lies beneath the transformed anterior compartment and hence is out of the plane of focus (Struhl, 1981b).

extensively studied in *Drosophila*, *Hox* genes have been studied at length in the closely related insect, *Tribolium casteneum*, red flour beetle, which both diverged from a common ancestor around 300 million years ago. In *Tribolium*, the *Scr* ortholog, *Cephalothorax*, is required for normal development of the first thoracic segment (T1) (Beeman et al., 1993), and the *Ubx* ortholog, *Ultrathorax*, is required for the normal development of T3 (Beeman et al., 1989), as found in *Drosophila* (Figure 9.A). However, the beetle *Antp* ortholog, *prothoraxless* (*ptl*) may not be required in the same manner as in *Drosophila*. In *Tribolium*, lf *ptl* mutant alleles result in transformations of all three thoracic legs to antennae (Figure 9.B&C; Beeman et al., 1993). In *Drosophila*, $Antp^{lf}$ alleles affect the development of the proximal leg only resulting in a leg to third antennal segment transformation and do not result in a tarsus to arista transformation of the distal leg as observed in *Tribolium* (Figure 8; Struhl, 1981). Unfortunately, this analysis of *Antp* function in adult *Drosophila* was performed using mosaic analysis where only small clones of $Antp^{lf}$ tissue were generated and analyzed in an otherwise wild type leg (Percival-Smith et al., 2005; Struhl, 1981b). In addition, tarsus determination is a non-cell autonomous process, which would not allow the identification of a tarsus to arista transformation in the leg of an *Antp* lf/+ mosaic, even if *Antp* is required for tarsus determination (Percival-Smith et al., 2005; Struhl, 1981). Furthermore, when the HOX proteins, SCR, ANTP, and UBX, as well as the other *Drosophila* HOX proteins excluding PB, are ectopically expressed in the antenna, an arista to tarsus transformation is induced, such that the HOX protein required for tarsus determination in *Drosophila* is unknown (Abbott & Kaufman, 1986; Burgess & Duncan, 1990; Casares et al., 1996; Emerald & Cohen, 2004; Gibson & Gehring, 1988; Gibson et al., 1990; Mann & Hogness, 1990; Percival-Smith et al., 2005; Percival-Smith et al., 1997; Schneuwly et al., 1987; Struhl, 1981b, 1982).

1.9 Nanobodies

A novel method has recently been developed to selectively target and knock down proteins. In 1993, it was discovered that the immune systems of *Camelidae* (i.e. camels and llamas) contain functional antibodies that are composed of heavy polypeptide chains only (Hamers-Casterman et al., 1993). Conventional antibodies (or immunoglobulins,

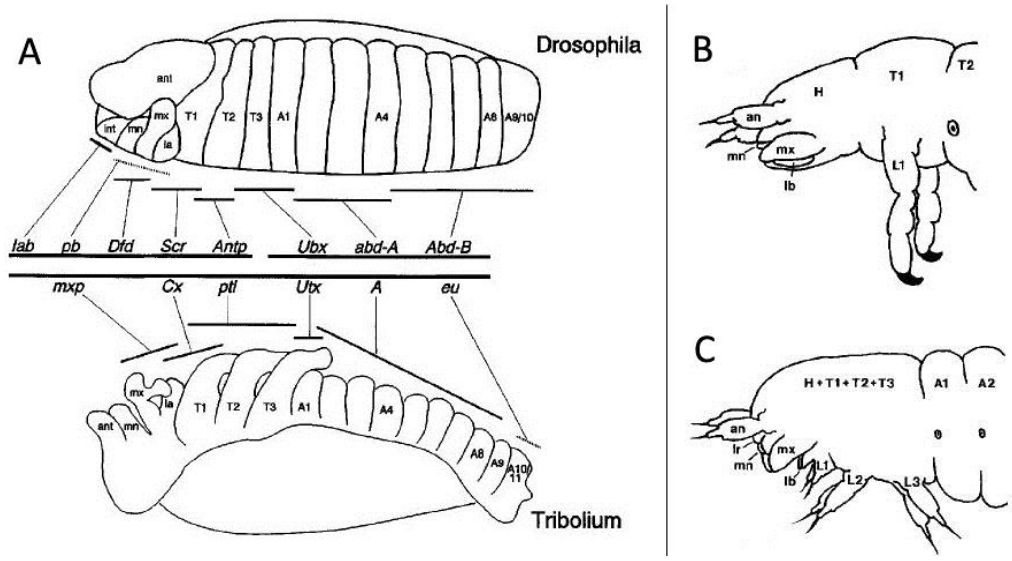

Figure 9. (Left) The *Hox* Genes in *Drosophila* and *Tribolium*. (Right) Terminal Phenotype of Lethal *ptl* lf Mutation in *Tribolium*.

(A) The regions of the A-P axis affected by lf mutations in *Drosophila* and *Tribolium* *Hox* genes are indicated by lines, with *pb* and *extra urogomphi* (*eu*) being dotted to indicate that they do not affect embryos. Segment abbreviations: ant, antennal; int, intercalary; mn, mandibular; mx, maxillary; la, labial; T, thoracic; A, abdominal. (B) Lateral view of head (H) through T2 of wild-type first instar larva. (C) Lateral view of head through A2 of a first instar ptl^{D6O} homozygote. Note transformation of all three pairs of thoracic legs into antennae. Abbreviations: lb, labial palps; an, antennae; mn, mandible; lr, labrum; mx, maxillary palps (Beeman et al., 1993).

IgG) are all comprised of a similar basic structural unit of two identical heavy polypeptide chains and two identical light polypeptide chains which are linked together by disulphide bridges to form two antigen-binding (Fab) regions that are linked to a constant (Fc) region via a flexible hinge (Figure 10; Woof & Burton, 2004) . The amino terminal variable domains of the two Fab regions of the antibody mediate the highly specific recognition of the antigen target (epitope). IgG of *Camelidae* species differ from conventional antibodies as antigen binding is achieved by a single heavy chain variable domain (V_HH; Figure 10). The V_HH domain represents the smallest (only 15 kDa) available intact antigen-binding fragment, referred to as a nanobody (Muyldermans, 2001). This is half the size of the smallest intact antigen-binding fragment that can be generated from conventional antibodies, and has the potential to allow access to epitopes that are not reached by conventional antibodies (Muyldermans, 2001). Interestingly, in 1995 a research team at the University of Miami found a similar type of heavy-chain only antibody in sharks and other cartilaginous fish (termed V_{NAR}; Greenberg et al., 1995). The V_HH is able to bind to targets with high affinity and specificity because of a long complementarity determining region 3 (CDR3) loop, which enlarges the surface area of the antigen binding site of the V_HH (Arbabi Ghahroudi et al., 1997). Compared to antigen-binding derivatives of conventional antibodies, the single domain nature of V_HH gives rise to several unique features and advantages. First, the antigen recognition is encoded by a single polypeptide in a single gene, which allows easy design of systems for the selection of antigens with a particular epitope. Second, V_HHs are naturally robust, highly soluble and stable. Their solubility is greatly improved over that of other antibodies due to substitutions of hydrophobic by hydrophilic residues within the V_HH folded domains (Muyldermans, 2001). Finally, the close homology of the V_HH fragment to human V_H fragments is of great diagnostic and therapeutic value (Muyldermans, 2001). Presently, mouse therapeutic antibodies can elicit an immune response in the human patient, whereas nanobodies are less likely to do so (Courtenay-Luck et al., 1986; Muyldermans, 2001).

To isolate a specific V_HH domain that recognizes GFP, an alpaca (*Lama pacos*) was immunized with purified green fluorescence protein (GFP) and a highly specific GFP-

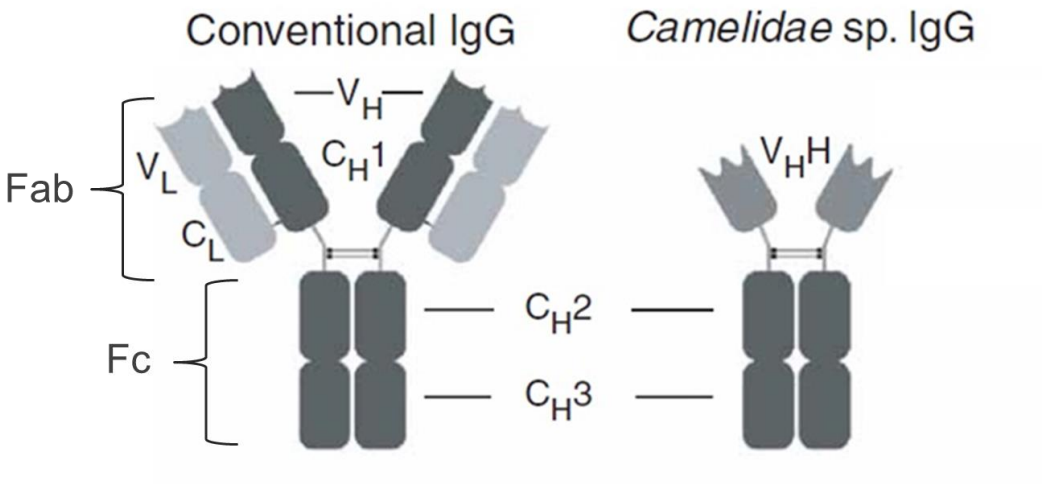

Figure 10. Conventional Immunoglobulins Versus *Camelidae* Species Immunoglobulins.

Conventional antibodies (or immunoglobulins, IgG) are composed of two identical heavy chains and two identical light chains, linked by disulphide bridges (Fab), which are connected by a flexible hinge to constant (Fc) domains. Light chains fold into a variable domain (V_L) and a constant domain (C_L), whereas heavy chains are composed of one variable domain (V_H) and multiple constant domains (C_H). The variable domains of both the heavy and light chains at the tip of the Fab form the antigen-binding sites. IgG of *Camelidae* species differ in antigen binding is achieved by a single heavy chain variable domain (V_HH or nanobody; Rothbauer et al., 2006).

binding antibody fragment, α-GFP V_HH (GFP-nanobody), was identified (Rothbauer et al., 2006). The GFP-nanobody has the ability to precipitate its antigen from cell extracts more efficiently than mono- and polyclonal anti-GFP antibodies (Rothbauer et al., 2008). Furthermore, GFP remains stably bound to the GFP-nanobody even under high salt conditions, high temperature conditions, and between the range of pH 4 to pH 11 (Rothbauer et al., 2008). The structure of the GFP:GFP-nanobody complex formation was determined using X-ray crystallography to determine the criteria of specificity for recognition of fluorescent proteins (Figure 11; Kubala et al., 2010). The GFP-nanobody can also bind to the yellow fluorescent protein (YFP), and a modified cyan fluorescent protein (CFP; Kubala et al., 2010). Therefore, GFP, YFP, and a modified CFP can be used in conjunction with the GFP-nanobody (Kubala et al., 2010). This is especially useful since GFP, CFP, and YFP fluorescence can be visualized separately when they are co-expressed. This technology has been used *in vitro*, *in vivo*, and *in planta* (Rothbauer et al., 2008, 2006; Schornack et al., 2009). Additionally, the 17 amino acids (ILGHKLEYNYNSHNVYI) spanning the epitope on GFP that is recognized by the GFP-nanobody is a sufficient, small target for binding (Grallert et al., 2013).

Caussinus et al. (2012) engineered a method to knock out GFP fusion proteins in any eukaryotic genetic system using the endogenous ubiquitin pathway. The ubiquitin pathway is an integral component of selective protein degradation in eukaryotes. Target proteins that acquire multiple ubiquitin molecules, which are covalently attached through a complex cascade of enzymes, are subsequently degraded by the proteasome (Ciechanover, 1998). Substrate specificity in the ubiquitin pathway is determined by F-box proteins (FBP). FBPs are modular with two distinct domains: An F-box domain that interacts with the ubiquitin pathway and a protein-protein interaction domain (such as WD40 repeats) on the C-terminus which binds to a specific substrate (Zhang et al., 2003). Supernumerary limbs (Slmb) is a *Drosophila* FBP involved in normal growth and patterning during development (Jiang & Struhl, 1998). A new FBP, NSlmb-vhhGFP4, was engineered by fusing the F-box domain contained in the N-terminal part of Slmb to the GFP-nanobody (vhhGFP4), which permits target proteins fused to GFP (or relevant derivatives) to be specifically degraded through the proteasome upon expression

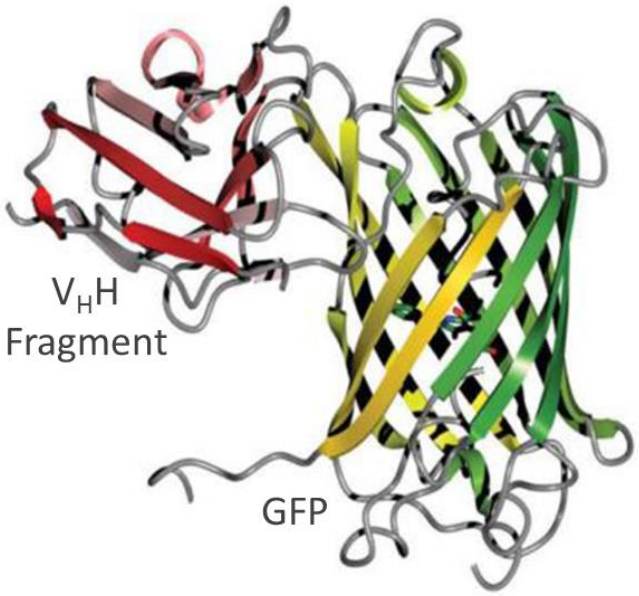

Figure 11. Structure of the Complex Formed When the GFP-nanobody is Bound to GFP.

Ribbon diagram (based on X-ray crystallography) showing perpendicular views of the GFP:GFP-nanobody complex. GFP is shown in green to gold and the GFP-nanobody (V_HH Fragment) is shown in red to pink (N- to C-terminal; Kubala et al., 2010).

(Figure 12; Caussinus et al., 2012). Additionally, since the target protein is fused to a fluorescence protein, the removal of the target protein can be easily monitored. To determine the kinetics of target protein degradation by NSlmb-vhhGFP4, live imaging of *Drosophila* embryos was used to monitor the decrease in nuclear fluorescence of a target protein signal relative to the appearance of another nuclear fluorescence that served as the NSlmb-vhhGFP4 expression reporter (Figure 13; Caussinus et al., 2012). The accumulated target protein begins to disappear from the embryos less than 30 min after expression of NSlmb-vhhGFP4 and is reduced to 10% of its maximum intensity in less than 3 hours (Caussinus et al., 2012). Transgenic *Drosophila* lines in which targeted NSlmb-vhhGFP4 expression can be achieved with the UAS-GAL4 system, have already been generated (Caussinus et al., 2012). This technique allows the dissection of morphogenetic forces in a cell- and tissue-specific manner and allows fast tissue-specific knockouts which were previously not feasible in post-mitotic cells (Caussinus et al., 2012).

1.10 Goal

My goal is to address the question of what HOX protein is required for tarsus determination in *Drosophila*. To answer this question, a method is required that inhibits HOX activity only during larval stages and not during embryogenesis as HOX activity is essential for the development of viable larvae (Brown et al., 2002). Additionally, the method needs to inhibit HOX activity in all cells of the larva such that no hypothetical HOX-dependent cell signalling molecule is produced. I propose to use a heat shock-inducible nanobody (*UAS- NSlmb-vhhGFP4* driven by *hsp-GAL4*) activated during the third larval stage in all cells to degrade thoracically expressed HOX proteins (SCR, ANTP, and UBX) tagged with XFP (GFP derivatives; GFP, YFP, CFP, or 17 amino acid (aa) epitope of GFP; Figure 14). I will use the GFP-nanobody degrader (nanobody) to determine whether the requirement for ANTP matches that of the requirement for Ptl in *Tribolium.* This would suggest that requirement is conserved in insects. If the requirement is not conserved, then tarsi may be determined by SCR alone, or in conjunction with other HOX proteins, suggesting that HOX requirement for tarsus determination has diverged during insect evolution. Alternatively, if ANTP, SCR and

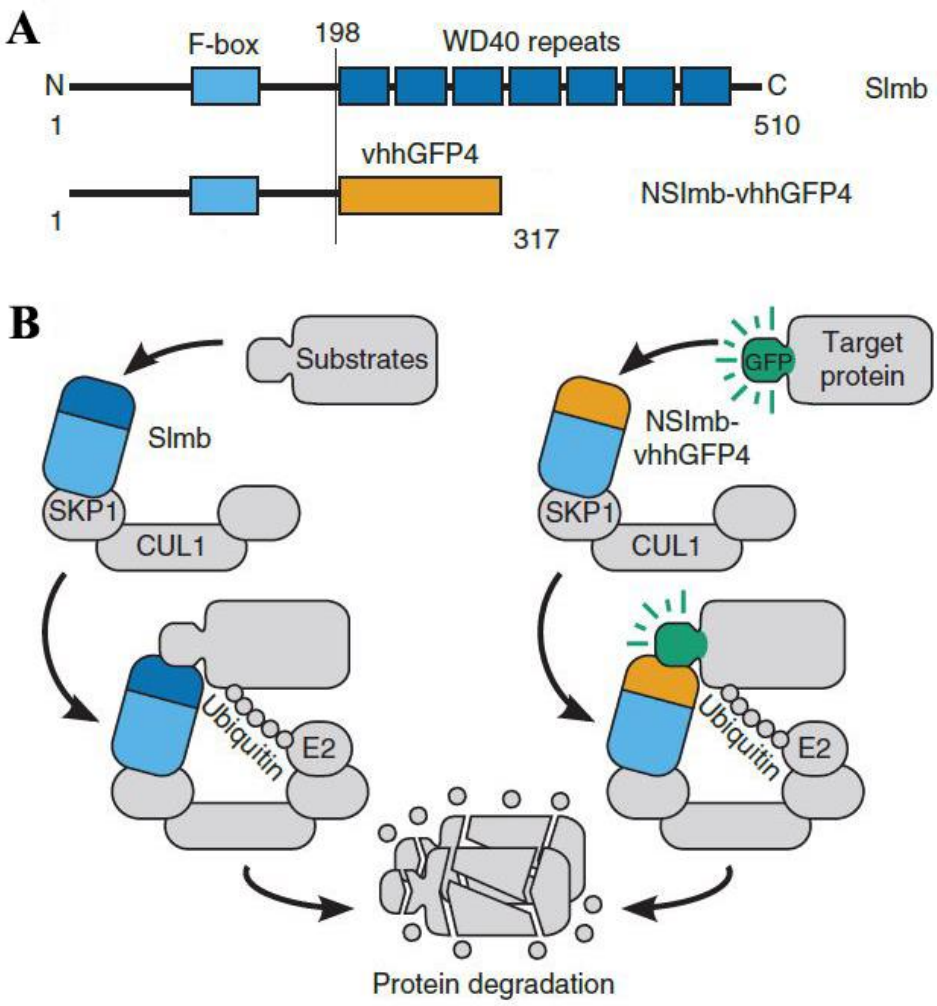

Figure 12. Schematic Illustration of GFP-nanobody Degrader.

(A) Linear representation of Slmb, and NSlmb-vhhGFP4. Numbers refer to amino acid positions from N terminus (N) to C terminus (C). (B) SKP1–CUL1–F-box protein ligase complexes (SCFs) mediate the ubiquitin transfer from E2 enzymes to target substrates. Ubiquitin-tagged substrates are then degraded by the proteasome. The specificity of the SCFs depends on the FBPs, which are either endogenous like Slmb or engineered like NSlmb-vhhGFP4 (left and right sides, respectively; Caussinus et al., 2012).

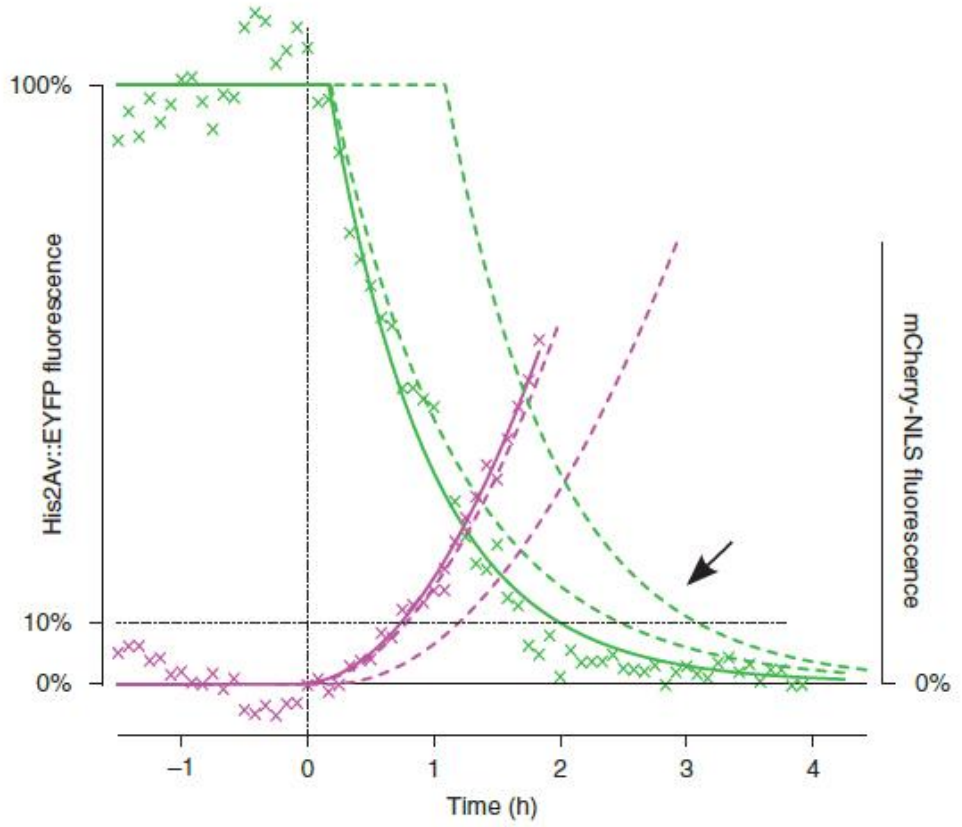

Figure 13. Kinetics of Target Protein Degradation by GFP-nanobody Degrader in *Drosophila*.

Live confocal imaging of embryos in stages 9 to 13 was used to show the degradation of a target protein (His2Av::EYFP; green) compared to expression of an NSlmb-vhhGFP4 reporter (mCherry-NLS; purple). The data points measured in the embryo are represented (crosses) and summarized by regression curves (continuous and dashed lines). The data is normalized and aligned in time with respect to mCherry-NLS expression and maturation (vertical dashed line is time *t*). On average, the His2Av::EYFP signal is reduced to 10% of its maximum (horizontal dashed line) 151 min (arrow) after time *t* (Caussinus et al., 2012).

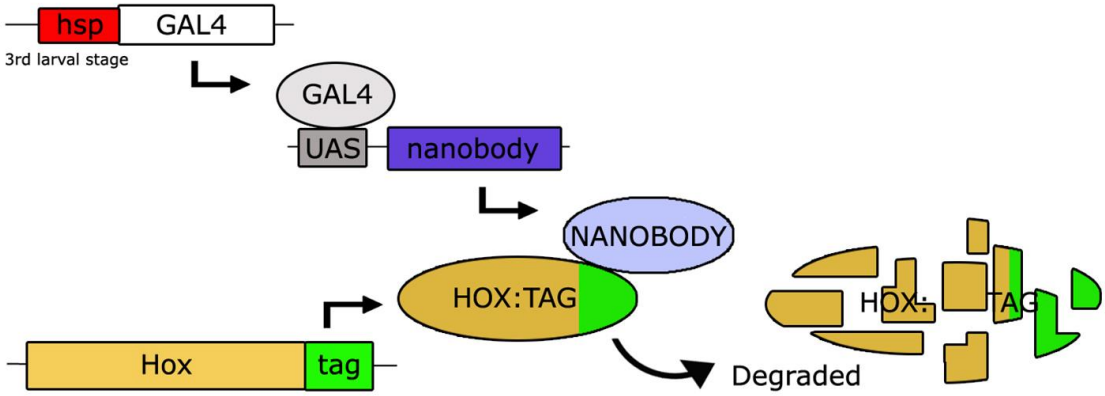

Figure 14. Model: Nanobody Expression to Target and Degrade XFP-tagged Proteins.

A *heat-shock promoter* (*hsp*) activated at the third larval stage of *Drosophila* development induces expression of the transcriptional activator, *GAL4*. This GAL4 driver line induces the *GAL4* responsive promoter, or upstream activation sequence (*UAS*). UAS activation causes expression of the GFP-nanobody degrader ("nanobody"), which then targets GFP derivatives (XFP; tag) for poly-ubiquitination. Consequently, HOX fusion proteins with a XFP tag are degraded by the intrinsic proteasome through the endogenous ubiquitin pathway.

UBX are not required for tarsus determination in *Drosophila*, then the mechanism of determination is HOX-independent.

2 Materials and Methods

2.1 Plasmids

Table 1 documents the origin of the plasmids used in this study.

2.2 *Drosophila* Strains

Drosophila were maintained at 25ºC with 60-65 % relative humidity on standard *Drosophila* media supplemented with baker's yeast ("Media Recipes and Methods", Bloomington *Drosopila* Stock Center website). Table 2 documents the genotype and origin of the fly strains used in this study.

2.3 chiRNA for CRISPR-mediated HR

chiRNA specific for *Scr*, *Antp*, and *Ubx* coding regions were designed to target a DSB as close to the stop codon as possible ("Protocols: U6-gRNA (chiRNA) cloning", flyCRISPR website). Primers phosphorylated at the 5' end were designed to encode the 20 nt corresponding to the target sequence (Appendix B.a). The forward and reverse primers were annealed together, and the double stranded product was ligated (T4 DNA Ligase – Invitrogen) into the pU6-BbsI-chiRNA vector that had been digested with BbsI. The ligation reaction was used to transform *Escherichia coli* Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) and ampicillin resistance was selected for on LB agar plates. The plasmid DNA was isolated (Geneaid miniplasmid prep kit) and digested with the restriction enzyme BbsI. Selected plasmid clones that had lost the BbsI recognition site (i.e. did not get cleaved) were sent for sequence analysis (Robarts Research Institute DNA Sequencing Facility).

2.4 Repair Vectors for CRISPR-mediated HR

The donor templates for HR were designed to consist of: *5'Hox*::(*XFP* or tag) *loxP* y^+*loxP 3'Hox* inserted in a pFUS_A plasmid (Figure 15.B). The following are the six repair vectors constructed: *5'Scr*::*GFP loxP* y^+*loxP 3'Scr, 5'Scr*::tag *loxP* y^+*loxP 3'Scr, 5'Antp*::*CFP lox2272* y^+*lox2272 3'Antp, 5'Antp*::tag *lox2272* y^+*lox2272 3'Antp,*

Plasmid Name	Supplier	Reference	Purpose	Annotated in Appendix
MiMIC (GenBank: GU370067)	Drosophila Genomics Resource Center	Venken et al. (2011)	*yellow+* sequence	N/A
pHsp70-Cas9	Addgene (45945)	Gratz et al. (2013)	Cas9 plasmid	A. a
pU6-BbsI-chiRNA	Addgene (45946)	Gratz et al. (2013)	chiRNA plasmid	A. b
pFUS_A	Addgene (31028)	Cermak et al. (2011)	repair vector backbone	A. c
pEGFP	APS lab	N/A	*GFP* sequence	N/A
pFA6a-EYFP-natMX6	APS lab	Van Driessche et al. (2005)	*YFP* sequence	N/A
pUBXTT	APS lab	N/A	3X FLAG peptide	N/A

Table 1. Origin of Plasmids.

Plasmid names, supplier, reference, and purpose for use in this work, as well as indication of whether an appendix exists are given. Legend: N/A, not applicable; APS, Dr. Anthony Percival-Smith.

Stock	Genotype	Origin	Reference	Short Form
y w	*Df(1)w[67c23.2]*	APS lab	N/A	*y w*
496	*y w ; L / CyO ; TM6B, Tb, P{walLy} / Ki ftz[11]*	APS lab	N/A	balancer
805	*y w ; P{arm-GAL4, w+} ; TM6B, Tb, P{walLy} / Scr[14] e*	APS lab	Sivanantharajah, L. & Percival-Smith, A. (2014)	armGAL4
2077	*w ; P{GAL4-Hsp70.PB}2*	Bloomington Stock Centre	Tenney, K. et al. (2006)	GAL4(hs)
7108	*w ; P{tubP-GAL80[ts]}10 ; TM2 / TM6B, Tb*	Bloomington Stock Centre	McGuire, S. E. et al. (2003)	GAL80(ts)
7019	*w ; P{tubP-GAL80[ts]}20 ; TM2 / TM6B, Tb*	Bloomington Stock Centre	McGuire, S. E. et al. (2003)	GAL80(ts)
38422	*y w ; P{UAS-Nslmb-vhhGFP4}2*	Bloomington Stock Centre	Caussinus, E. et al. (2012)	UAS-nano
54590	*y M{Act5C-Cas9.P}ZH-2A w*	Bloomington Stock Centre	Port, F. et al. (2014)	*act-Cas9*
54591	*y M{nos-Cas9.P}ZH-2A w*	Bloomington Stock Centre	Port, F. et al. (2014)	*nos-Cas9*

Table 2. Genotypes and Origins of Fly Stocks.

Abbreviations: *Df, deficiency; y, yellow*; *w, white*; P{}, P element transposon; *arm, armadillo*; *TM6b*, balancer of chromosome 3; *Tb, Tubby*; *walLy*, fragment containing *y+* gene; *Ki, Kinked*; *ftz, fushi-tarazu*; *Hsp, heat-shock promoter*; *tubP, tubulin promoter*; ts, temperature sensitive; *TM2*, balancer of chromosome 3; *UAS, upstream activation sequence*; *Nslmb-vhhGFP4*, GFP-nanobody degrader, *L, Lobe*; *CyO, Curly*; *Scr, Sex combs reduced; e, ebony; nos, nanos promoter; act5C, actin5C promoter;* N/A, not applicable; APS, Dr. Anthony Percival-Smith.

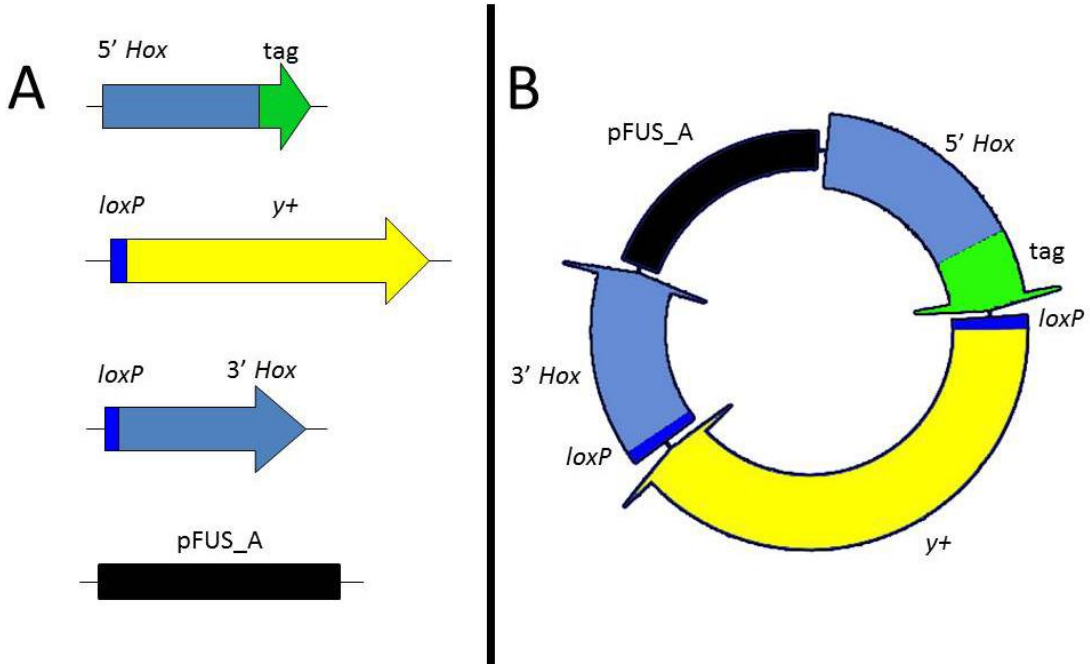

Figure 15. General Design of Repair Vectors.

(A) The *5'Hox::XFP* or *5'Hox*::tag (DNA fragment 1), *loxP y+* (DNA fragment 2) and *loxP 3'Hox* (DNA fragment 3) fragments are all created by standard PCR with unique cohesive ends when digested with the restriction endonuclease, BsaI. The pFUS_A vector is digested with BsaI and dephosphorylated. (B) All four DNA fragments ligate together in a single ligation reaction to form a circularized repair vector for use in injecting CRISPR components for HR.

5'Ubx::*YFP loxP* y^+ *loxP 3'Ubx,* and *5'Ubx*::tag *loxP* y^+ *loxP 3'Ubx* (annotated sequence in Appendix A.d). The pFUS_A vector was digested with BsaI and dephosphorylated.

For each of the six constructs, three DNA fragments were created using standard PCR mutagenesis technique (McPherson & Møller, 2000) with a Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). The following thermocycling parameters were generally used: 94ºC for 2 min; 30 cycles of 94ºC for 45 s, 58ºC for 45 s and 72ºC for 20 min; 72ºC for 10 min; and held at 4ºC. Primers used for amplification are included in Appendix B.

DNA fragment 1 contains the 5' portion of a *Hox* gene fused either to *XFP* (*GFP*, *YFP*, or *CFP*) or a tag (Table 3) and was created in two steps (Figure 15.A). First, genomic DNA was isolated from adult *y w* flies. The 5'*Scr*, 5'*Antp*, and 5'*Ubx* portions were amplified with the reverse primers containing identical sequence to *GFP* or the tag (An illustrative example is given in Figure 16)

Second, a series of products were created with identical sequence to 5'*Scr*, 5'*Antp*, and 5'*Ubx*.

A pEGFP plasmid was used to amplify *GFP* with a forward primer containing identical sequence to 5'*Scr* (Figure 16).

The pEGFP plasmid was also used to amplify a modified *CFP* with a forward primer containing identical sequence to 5'*Antp*. In addition, primers were used containing sequence changes to alter aa positions 66, 153, and 163 to create a *CFP* from the *GFP* sequence that is recognized by the nanobody. The GFP aa 66 position was changed from Tyr (DNA sequence TAC) to Trp (TGG). The GFP aa 153 position was changed from Met (ATG) to Thr (ACC). The GFP aa 163 position was changed from Val (GTG) to Ala (GCA).

A pFA6a-EYFP-natMX6 plasmid was used to amplify *YFP* with a forward primer containing identical sequence to 5'*Ubx*.

Sequence of the "tag" 17 aa epitope of GFP (17aa) fused to a **3X FLAG peptide (3 DYKXXD repeats)**	
Protein	ILGHKLEYNYNSHNVYI**DYKDHDGDYKDHDIDYKDDDDK**
DNA	ATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGT CTATATC**GACTACAAAGACCATGACGGTGATTATAAAGATCA TGACATCGACTACAAGGATGACGATGACAAG**

Table 3. Protein and DNA Sequence of the Tag Fused to 5' *Hox* Genes.

The tag consists of a fusion of the 17 aa epitope on GFP (17aa) that is recognized by the nanobody (normal) with a 3X FLAG peptide (bold).

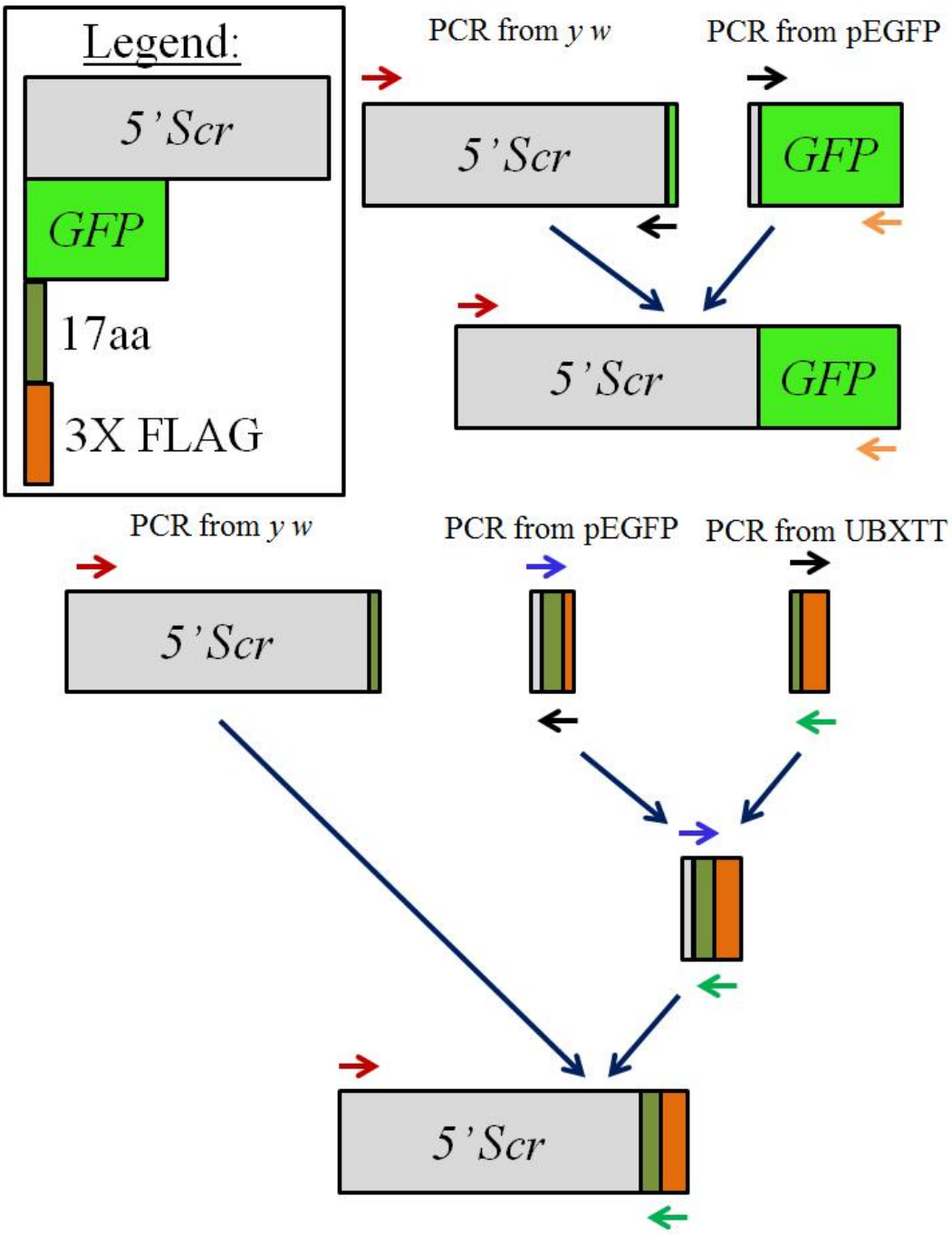

Figure 16. Schematic Example of Generating DNA Fragment 1.

DNA source is indicated above and arrows indicate position and orientation of primers used to PCR each DNA fragment resulting in *5'Scr::GFP* and *5'Scr*::tag. "tag" refers to a fusion of the 17 aa epitope of GFP (17aa) and a 3X FLAG peptide.

To fuse 5' *Scr* to *GFP*; the isolated product, *5'Scr* containing identical sequence to *GFP*, was used in a reaction with the isolated product, *GFP* with identical sequence to *5'Scr* (Figure 16).

To fuse 5' *Antp* to *CFP*; the isolated product, *5'Antp* containing identical sequence to *GFP*, was used in a reaction with the isolated product, *CFP* with identical sequence to *5'Antp.*

To fuse 5' *Ubx* to *YFP*; the isolated product, *5'Ubx* containing identical sequence to *GFP*, was used in a reaction with the isolated product, *YFP* with identical sequence to *5'Ubx*.

The pEGFP plasmid was used to amplify the 17 aa epitope of GFP that is recognized by the nanobody (ILGHKLEYNYNSHNVYI) with a forward primer containing identical sequence to 5'*Scr*, 5'*Antp*, and 5'*Ubx* and a reverse primer containing identical sequence to a 3X FLAG peptide. The product was used in a subsequent reaction with the following: A pUBXTT plasmid was used to amplify a 3X FLAG peptide with a forward primer containing identical sequence to the 17 aa epitope of GFP (ILGHKLEYNYNSHNVYI). The resulting product was an amplified fragment of 17 aa epitope of GFP (17aa) fused with the 3X FLAG peptide (collectively called "tag") with a forward primer containing identical sequence to 5'*Scr*, 5'*Antp*, and 5'*Ubx* (An illustrative example is given in Figure 16)

To fuse 5' *Scr* to tag; the isolated product, *5'Scr* containing identical sequence to tag, was used in a reaction with the isolated product, tag with identical sequence to *5'Scr* (Figure 16).

To fuse 5' *Antp* to tag; the isolated product, *5'Antp* containing identical sequence to tag, was used in a reaction with the isolated product, tag with identical sequence to *5'Antp*.

To fuse 5' *Ubx* to tag; the isolated product, *5'Ubx* containing identical sequence to tag, was used in a reaction with the isolated product, tag with identical sequence to *5'Ubx*.

DNA fragment 2 contains either the *loxP* or *lox 2272* site fused to the *y+* gene (Figure 15.A). A *minos*-mediated integration cassette (MiMIC; GenBank accession code GU370067) vector was digested, and used as the template for amplification of *yellow+* with forward primers that incorporated on the 5' end a *loxP* site or a *lox2272* site.

DNA fragment 3 contains a *loxP* fused to the 3' of *Scr* and *Ubx* or a *lox2272* site fused to the 3' of *Antp* (Figure 15.A). Genomic *y w* DNA was used to create 3'*Antp*, 3'*Scr*, and 3'*Ubx* portions with primers that incorporated on the 5' end a *loxP* site for 3'*Scr* and 3'*Ubx* and a *lox2272* site for 3'*Antp*.

The products were verified by 1% (w/v) agarose gel electrophoresis, which was stained with RedSafe (FroggaBio), and visualized on a gel doc (FlourChem8900 - Alpha Innotech). They were subsequently purified using a PCR Product purification kit (BioBasic Inc). The primers used to create all three DNA fragments contained the restriction endonuclease site, BsaI, which is a restriction enzyme that recognizes a six base sequence but generates a 4 base 5' overhang outside the recognition site (Figure 17.A). In the design, all eight 5' overhangs of pFUS_A and the three DNA fragments had unique but complementary sequences such that the fragments would assemble in order during ligation (Figure 17.B). The three DNA fragments were digested with BsaI and used in a ligation reaction (T4 DNA Ligase – Invitrogen) with the digested and dephosphorylated pFUS_A. *E. coli* Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were transformed and spectinomycin/streptomycin resistance was selected for on X-gal and IPTG containing LB agar plates. When the fragments insert into pFUS_A , the *lacZ* gene is lost and therefore white colonies, but not blue colonies of bacteria, were chosen for PCR screening. The first PCR screen amplified a product from the 5' end of pFUS_A to the 5' end of the *y+* marker gene. The second PCR screen amplified a product from the 3' end of *y+* to the 5' end of pFUS_A. Primers used for screening are included in Appendix B. If a sample showed products for both screens, the plasmid DNA was isolated (Geneaid miniplasmid prep kit). The DNA was digested with selected restriction enzymes BamHI, BglII, HincII, Tth111, XbaI, XhoI, and XmnI. The plasmid clones that gave the expected patterns of cleavage for all of the restriction

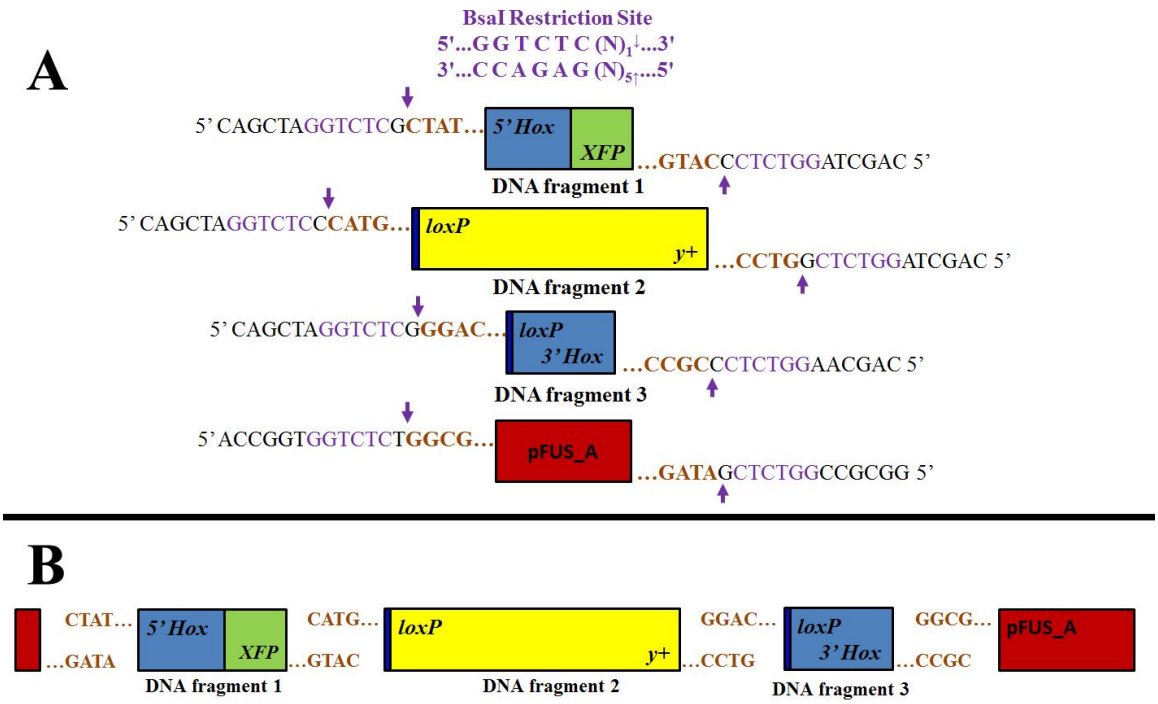

Figure 17. Schematic of Using BsaI Unique Ends for a Single Ligation Reaction of the Repair Vectors.

(A) The primers used to create all three DNA fragments contained the restriction endonuclease site, BsaI, which is a restriction enzyme that recognizes a six base sequence but generates a 4 base 5' overhang outside the recognition site. (B) After being digested with BsaI, all eight 5' overhangs of pFUS_A and the three DNA fragments had unique but complementary sequences such that the fragments would assemble in order during ligation.

enzymes were isolated (QIAGEN QIAfilter midiprep kit). The six repair vectors were given the short forms: *Scr*::*GFP*, *Scr*::tag, *Antp*::*CFP*, *Antp*::tag, *Ubx*::*YFP*, and *Ubx*::tag.

2.5 CRISPR Germline Transformant Flies

2.5.1 Injections

Drosophila embryos were injected using standard protocols (Murphy & Carter, 1993). *Drosophila* eggs were collected on apple or grape juice agar plates smeared with yeast paste. 3% bleach was used to dechorionate the embryos on the plates and the embryos were collected in mesh covered baskets. Embryos were then transferred to agar strips and lined up so they were all in the same orientation before getting transferred to double-sided tape on a microscope slide. The embryos were placed under a dryer for 4 min and 40 sec before covering the embryos with halocarbon oil. The slide was positioned on an inverted microscope equipped with a micromanipulator, micropipette holder, and a microinjector. Syncytial blastoderm embryos expressing or not expressing *Cas*9 were injected with DNA (in a 10% Glycerol and 1X PBS solution) at the posterior end with a glass capillary injection needle (Table 4). The injected embryos on the tape were transferred to apple juice agar plates and kept at 18ºC for 48 hours before being allowed to hatch into larva and then eclose as adults in vials.

To assess germline transmission of targeted genome modifications, G_0 adults that developed from injected embryos were individually crossed to *y w* flies. The G_1 offspring of crosses were screened for the phenotype of the marker used, w+ or y+. The number of vials that produced no offspring (i.e. were sterile) were recorded along with the total.

2.5.2 Verification of Injected Germline Transformant

A transformant fly candidate was tested to confirm its genotype of *Antp*::*CFP*. A G_0 adult injected fly was completely y+, indicating the presence of *y*+ locus in its genome. Genomic DNA was isolated from the y+ flies.

To test for the presence of *Antp*::*CFP* at the *Antp* locus, PCR amplification of DNA fragments unique to the *Antp::CFP* gene was performed. The first product spans within

Injection	Fly Genotype	Cas9 Plasmid	chiRNA Plasmid	Repair Vector	Other
***Frost* and P Element**	*y w*	**200 ng/µl**	*Frost* **200 ng/µl**	*Frost (w+)* **200 ng/µl**	–
	y w	–	–	–	P element (*w*+) **400 ng/µl** Δ2-3*wc* helper **200 ng/µl**
Cas9 Plasmid	*y w*	**500 ng/µl**	*Scr*, *Antp*, or *Ubx* **500 ng/µl**	*Scr:gfp*/tag, *Antp:cfp*/tag, or *Ubx:yfp*/tag **120 ng/µl**	–
***Cas9* Transgenic Flies**	*nos-Cas9*	–	*Scr*, *Antp*, or *Ubx* **600 ng/µl**	*Scr:gfp*/tag, *Antp:cfp*/tag, or *Ubx:yfp*/tag **500 ng/µl**	–
	act-Cas9	–	*Scr*, *Antp*, or *Ubx* **600 ng/µl**	*Scr:gfp*/tag, *Antp:cfp*/tag, or *Ubx:yfp*/tag **500 ng/µl**	–

Table 4. Injected Flies and DNA.

The concentrations of the DNAs injected into flies.

5'Antp only as a positive control (659 bp). The second product spans 5*'Antp* to the middle of *CFP* (848 bp). The third product spans 5'*Antp* to the 3' end of *CFP* (1352 bp). Genomic *y w* DNA and genomic *y w* DNA spiked with *Antp::CFP* repair template DNA, were compared to the y+ genomic DNA using standard PCR mutagenesis technique (McPherson & Møller, 2000) with a Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). The following thermocycling parameters were used: 94ºC for 2 min; 30 cycles of 94ºC for 45 s, 58ºC for 45 s and 72ºC for 20 min; 72ºC for 10 min; and held at 4ºC. Primers used for amplification are included in Appendix B.

The candidate *Antp::CFP* flies were crossed with fly line 496 (containing balancer chromosomes) to analyze the segregation pattern of Lobe, Curly, Tubby, and y+ to determine whether the *y+* locus is on the 2nd or the 3rd chromosome, which contains the *Antp* locus.

2.6 Crossing Schemes

The goal is to create flies with the genotype for nanobody degradation of HOX::XFP proteins. This genotype will be homozygous for the *Hox::XFP* locus/loci and have the genetic elements for GAL4-dependent expression of the nanobody. Expression of the nanobody will be regulated in one of two ways: first by using an *hsp-GAL4* fusion gene for heat shock-induced GAL4 expression, and second a ubiquitously expressed GAL4 source, *arm-GAL4*, that is silenced by expression of a temperature sensitive (ts) GAL80 from a ubiquitously expressed *tubP-GAL80^{ts}* transgene. GAL80^{ts} activity is inhibited at 30ºC, allowing expression of the nanobody. Each fly line was made homozygous with standard crosses (Greenspan, 2004).

The following short forms are used in the crossing schemes: GAL4(hs), *GAL4-Hsp70;* armGAL4, *arm-GAL4;* UAS-nano, *UAS-Nslmb-vhhGFP4;* and GAL80+UAS-nano, *tubP-GAL80[ts], UAS-Nslmb-vhhGFP4.*

Below are the four separate crossing schemes to result in two final genotypes: *y w ; P{GAL4-Hsp70.PB}2 / P{UAS-Nslmb-vhhGFP4}2 ; Hox*::*XFP y*$^{+}$, referred to as the homozygous *Hox*::*XFP* stock with GAL4(hs) and UAS-nano, and *y w ; P{arm-GAL4} /*

P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 ; Hox::*XFP* y^+, referred to as the homozygous *Hox*::*XFP* with armGAL4 and GAL80+UAS-nano.

2.6.1 Crossing Scheme 1: GAL80+UAS-nano

Crossing scheme 1 (Figure 18) is the recombination of the GAL80 source onto the same second chromosome as the UAS-nano source resulting in a stock homozygous for the GAL80 and UAS-nano and a balanced lethal third chromosome:
y w ; P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 ; TM6, Tb, P{walLy} / Ki* ftz^{11}
This stock is used in crossing scheme 3.

2.6.2 Crossing Scheme 2: GAL4(hs) and UAS-nano

Crossing scheme 2 (Figure 19) creates two balanced stocks. The GAL4(hs) stock is homozygous for GAL4(hs) on the 2^{nd} chromosome with a balanced 3^{rd} chromosome . The UAS-nano stock is homozygous for UAS-nano on the 2^{nd} chromosome with a balanced 3^{rd} chromosome. They are, respectively:
y w ; P{GAL4-Hsp70.PB}2 ; TM6B, Tb, P{walLy} / Ki ftz^{11} and
y w ; P{UAS-Nslmb-vhhGFP4}2 ; TM6B, Tb, P{walLy} / Ki ftz^{11}
Both of these stocks are used in crossing scheme 3.

2.6.3 Crossing Scheme 3: Heterozygous *Hox*::*XFP* with either GAL4(hs), UAS-nano, GAL80+UAS-nano, or armGAL4

Crossing scheme 3 (Figure 20) creates balanced flies with either *Scr*::*GFP*, *Scr*::tag, *Antp*::*CFP*, *Antp*::tag, *Ubx*::*YFP*, or *Ubx*::tag and one nanobody component, GAL4(hs), UAS-nano, or GAL80+UAS-nano. The genotypes are:
y w ; P{GAL4-Hsp70.PB}2 / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+ and
y w ; P{UAS-Nslmb-vhhGFP4}2 / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+ and
y w; P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 / L ; TM6B, Tb, P{walLy} / Hox*::*XFP* y^+ and
y w ; P{arm-GAL4 w+} / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+
These flies are used in crossing scheme 4.

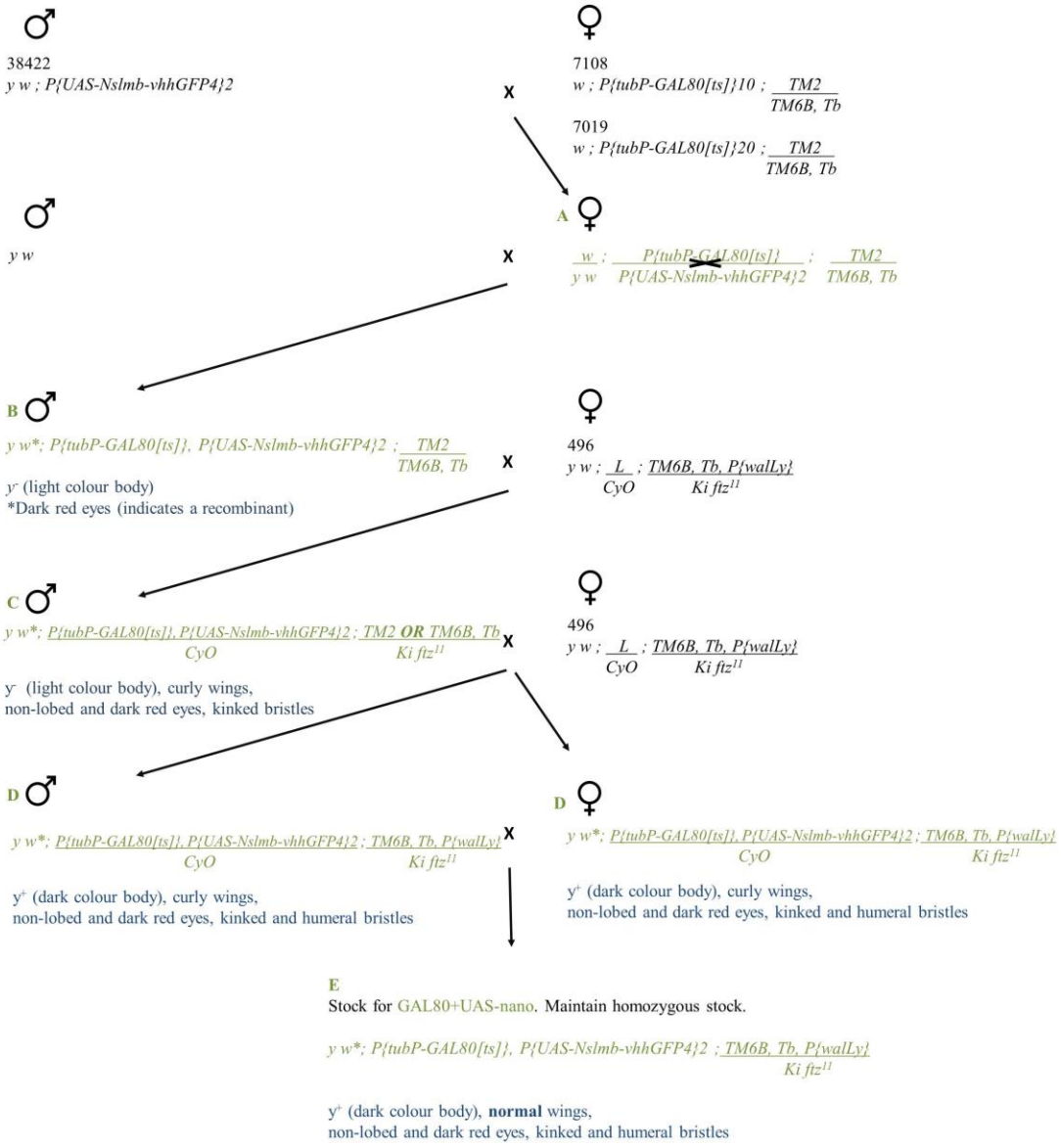

Figure 18. Scheme to Create the GAL80+UAS-nano Genotype:

y w ; P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 ; TM6, Tb, P{walLy} / Ki ftz*^{11}.

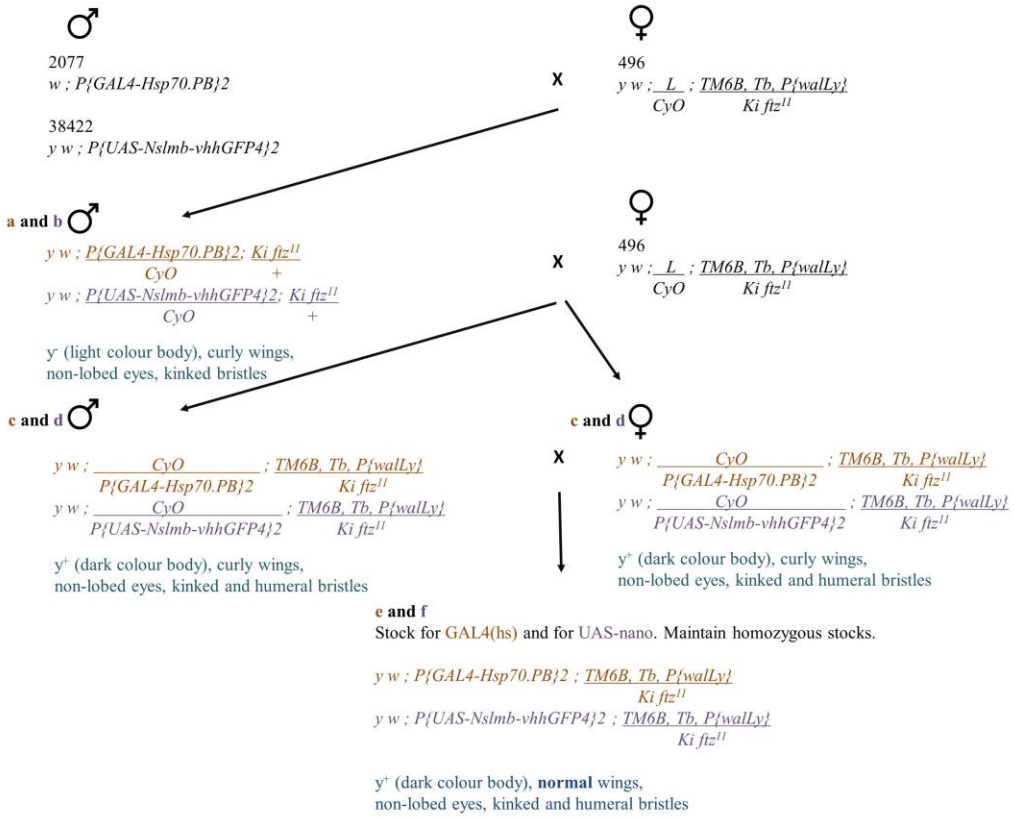

Figure 19. Scheme to Create the GAL4(hs) Genotype and UAS-nano Genotype:

GAL4(hs): *y w ; P{GAL4-Hsp70.PB}2 ; TM6B, Tb, P{walLy} / Ki ftz*^{11}

UAS-nano: *y w ; P{UAS-Nslmb-vhhGFP4}2 ; TM6B, Tb, P{walLy} / Ki ftz*^{11}

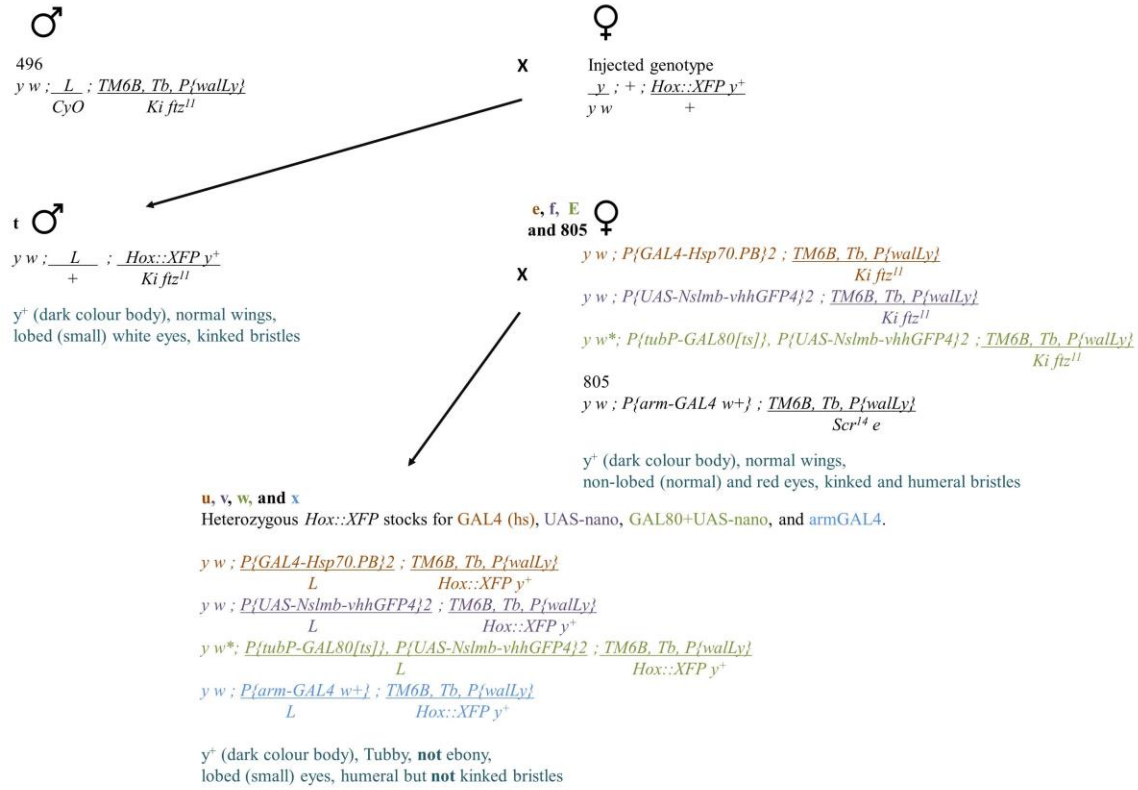

Figure 20. Scheme to Create the Heterozygous *Hox*::*XFP* with GAL4(hs), UAS-nano, GAL80+UAS-nano, and armGAL4 (Respectively) Genotypes:

y w ; P{GAL4-Hsp70.PB}2 / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+

y w ; P{UAS-Nslmb-vhhGFP4}2 / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+

y w; P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 / L ; TM6B, Tb, P{walLy} / Hox*::*XFP* y^+

y w ; P{arm-GAL4 w+} / L ; TM6B, Tb, P{walLy} / Hox::*XFP* y^+

2.6.4 Crossing Scheme 4: Homozygous *Hox*::*XFP* with GAL4(hs) and UAS-nano & Homozygous *Hox*::*XFP* with armGAL4 and GAL80+UAS-nano

Crossing scheme 4 (Figure 21) creates the final flies that are homozygous at *Hox*::*XFP* locus/loci and carrying one of the two GAL4 sources and the UAS-nanobody source. The final genotypes are:

y w ; P{GAL4-Hsp70.PB}2 / P{UAS-Nslmb-vhhGFP4}2 ; Hox::*XFP* y^+ and

y w ; P{arm-GAL4} / P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 ; Hox::*XFP* y^+

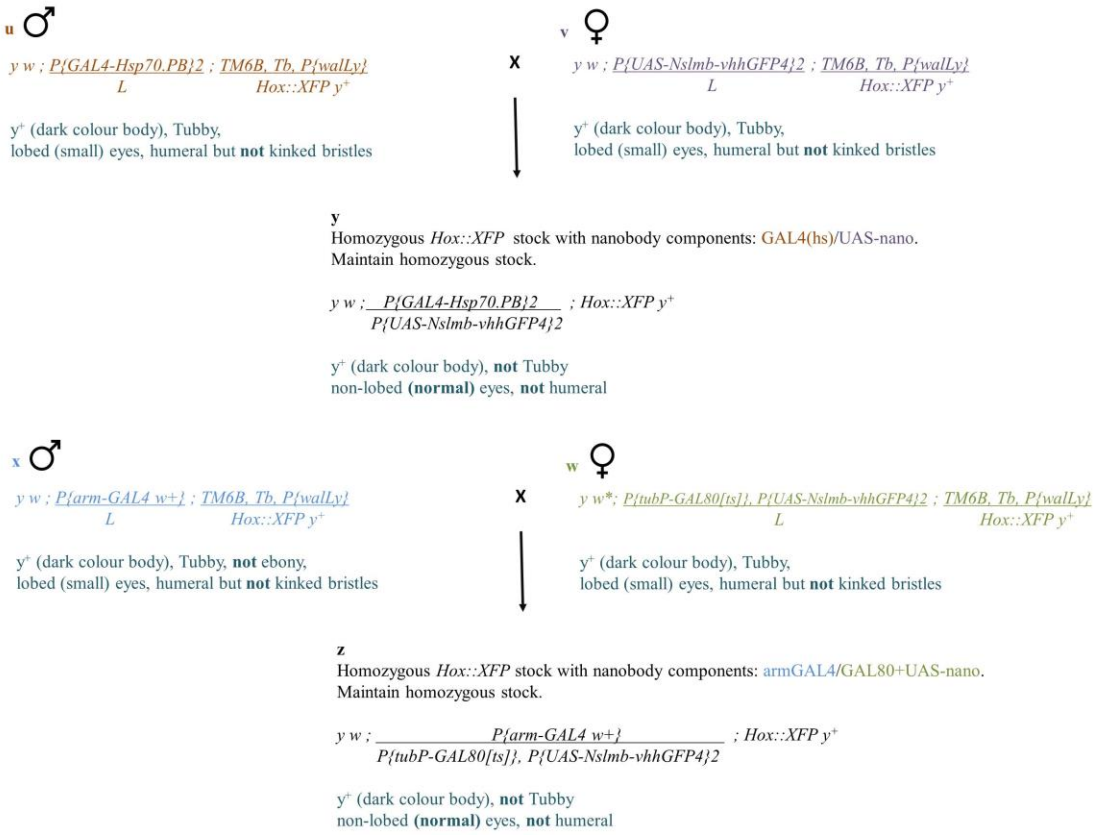

Figure 21. Scheme to Create the Homozygous *Hox*::*XFP* with GAL4(hs) and UAS-nano Genotype as well as the Homozygous *Hox*::*XFP* with armGAL4 and GAL80+UAS-nano Genotype:

Homozygous *Hox*::*XFP* with GAL4(hs) and UAS-nano:

y w ; P{GAL4-Hsp70.PB}2 / P{UAS-Nslmb-vhhGFP4}2 ; Hox::*XFP* y^+

Homozygous *Hox*::*XFP* with armGAL4 and GAL80+UAS-nano:

y w ; P{arm-GAL4} / P{tubP-GAL80[ts]}, P{UAS-Nslmb-vhhGFP4}2 ; Hox::*XFP* y^+

3 Results

Nanobody Degradation of HOX::XFP Tagged Proteins

The goal of this study was to degrade HOX proteins specifically during the larval stage using the nanobody degradation system. To do this, first the *Hox* loci have to be modified using CRISPR-mediated HR to create *Hox::XFP* loci. Second, these *Hox::XFP* transformants must be combined with genetic elements that will allow regulated expression of the nanobody and degradation of the HOX::XFP proteins.

3.1 chiRNA for CRISPR-mediated HR

The first step in implementing CRISPR-mediated HR is to design chiRNA. chiRNA specific for *Scr, Antp,* and *Ubx* were designed to be as close to the stop codon as possible. 20 nt of sequence within the 3' end of each *Hox* was chosen as the target for a DSB by a chiRNA/Cas9 riboprotein. This target sequence was the closest 20 nt of sequence to the TAG stop codon with a NGG PAM sequence. Table 5 shows: the sequences of the 3' end of each *Hox* (*Scr, Antp, and Ubx*), the primers used to create the specific chiRNA, and confirmation of insertion into the expression plasmid.

3.2 Repair Vectors for CRISPR-mediated HR

I created six repair vectors: *5'Scr*::*GFP loxP* y^+ *loxP 3'Scr, 5'Scr*::tag *loxP* y^+ *loxP 3'Scr, 5'Antp*::*CFP lox2272* y^+ *lox2272 3'Antp, 5'Antp*::tag *lox2272* y^+ *lox2272 3'Antp, 5'Ubx*::*YFP loxP* y^+ *loxP 3'Ubx,* and *5'Ubx*::tag *loxP* y^+ *loxP 3'Ubx* (annotated sequence in Appendix A.d). The tag consists of the 17 aa epitope of GFP (17aa) and a 3X FLAG peptide. When integrated, they will create the following six novel HOX fusion proteins: SCR::GFP, SCR::17aa::3XFLAG, ANTP::CFP, ANTP::17aa::3XFLAG, UBX::YFP, and UBX::17aa::3XFLAG (Figure 22).

***Hox* Gene**	**Sequence Derived From**	**Sequence**
Scr	3' *Scr*	CCTCGAT**GAACATCGTACCCTACCACA*TGG***GTC CATATGGCCACCCGTACCACCAGTTCGATATCCA TCCGTCGCAGTTCGCGCACCTAAGCGCA**TAG**
	Designed chiRNA	CTTC**GAACATCGTACCCTACCACA CTTGTAGCATGGGATGGTGT**CAAA
	Sequencing Analysis	*TTTATATAGGTATGTTTTCCTCAATACTTC***GAACATC GTACCCTACCACA**GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCGAGTCGGTGC**TTTTTT**
Antp	3' *Antp*	GAAGGGC**GAGCCGGGATCCGGAGGCGA*AGG***CG ACGAGATAACACCACCCAACAGTCCGCAG**TAG**
	Designed chiRNA	CTTC**GAGCCGGGATCCGGAGGCGA CTCGGCCCTAGGCCTCCGCT**CAAA
	Sequencing Analysis	*TTTATATAGGTATGTTTTCCTCAATACTTC***GAGCCGG GATCCGGAGGCGA**GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCGAGTCGGTGC**TTTTTT**
Ubx	3' *Ubx*	CGGCT**GCGGCGGCGGCGGTCCAAGG*TGG***ACAC TTAGATCAG**TAG**
	Designed chiRNA	CTTC**GCGGCGGCGGCGGTCCAAGG CGCCGCCGCCGCCAGGTTCC**CAAA
	Sequencing Analysis	*TTTATATAGGTATGTTTTCCTCAATACTTC***GCGGCGG CGGCGGTCCAAGG**GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCGAGTCGGTGC**TTTTTT**

Table 5. The Design Strategy of chiRNAs.

The matching 20 nt guide sequence before a PAM sequence (NGG) is highlighted in each of the three *Hox* loci for: 3' end of the gene, the double-stranded primer oligonucleotide pair for insertion into the chiRNA expression plasmid, and the sequence analysis of the ligated chiRNA. Legend: 20 nt guide sequence (bold and underlined), NGG PAM sequence (bold and italicized), TAG stop codon or terminator (bold), pU6 promoter (italicized), crRNA repeat and tracrRNA (wavy underline).

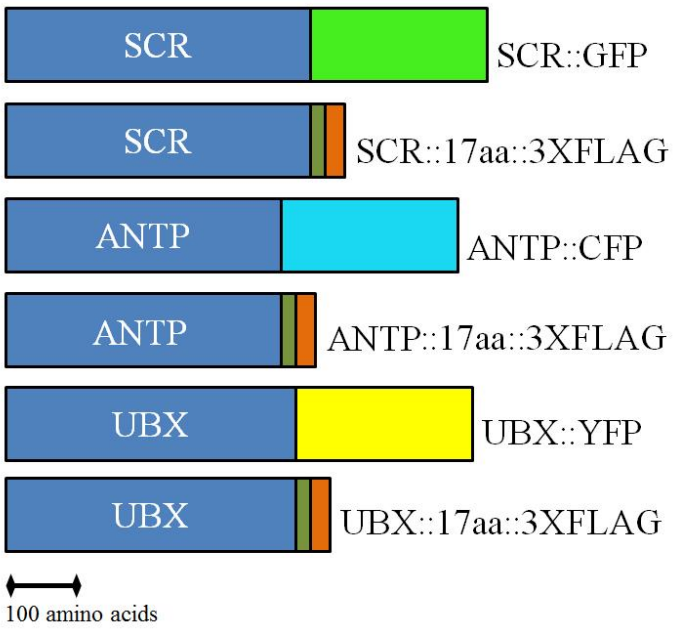

Figure 22. Novel Proteins Produced from HOX Protein Fusions.

Abbreviations: SCR, Sex combs reduced; GFP, green fluorescence protein; ANTP, Antennapedia; CFP, cyan fluorescence protein; UBX, Ultrabithorax; YFP, yellow fluorescence protein; 17aa, 17 aa epitope of GFP.

3.3 Screens of Injection Flies for CRISPR Germline Transformation

3.3.1 Injection of Flies with P Element or *Frost*

P element mediated transgenesis requires injection of both a P element plasmid and a helper plasmid. P elements insert into the genome with an efficiency of between 5 and 20 % (Dr. Anthony Percival-Smith, personal communication). P element and a Δ2-3*wc* helper plasmid were injected into embryos and these gave 13 fertile, viable G_0 progeny, of which none produced w+ progeny (Table 6).

Frost (Fst) is a *Drosophila* cold tolerance gene (Colinet et al., 2010) that has been targeted for CRISPR-mediated HR previously with an efficiency of 10% (Dr. Anthony Percival-Smith, personal communication). Three CRISPR components; Cas9 vector, Fst chiRNA vector, and *Fst* repair vector, were injected into embryos to test the recombination efficiency. 63 fertile, viable G_0 progeny were recovered, of which none produced w+ progeny (Table 6).

3.3.2 Injection of Flies with the Cas9 Source Encoded on a Plasmid

Three CRISPR components; Cas9 vector, one of three chiRNA vectors, and one of six repair vectors, were injected into embryos and these gave 679 fertile, viable G_0 progeny (Table 7). No germ line transformation was observed at any of the three loci or with any of the six repair vectors when G_1 progeny were screened for the y+ phenotype.

3.3.3 Injection of Flies Expressing *Cas9* from a Transgene.

Injecting one of three chiRNA vectors and one of six repair vectors into *Cas9* transgenic flies gave 147 fertile, viable adult flies (G_0; Table 8). Two *Cas9* transgenic flies were used: *act-Cas9* where *Cas9* is expressed from a constitutively expressed *actin5C* promoter, and *nos-Cas9* where *Cas9* is expressed from a germline-specific *nanos* promoter. The G_1 generation was screened for successful transformants. One y+ candidate transformed fly was identified from the *Antp*::*CFP* injected into *nos-Cas9* flies (*Table 8). When the G_1 male was crossed to *y w* females, the phenotypes of the G_2

	DNA		Total
	P Element	***Fst***	
TOTAL	14	82	96
STERILE	1	19	20
VIABLE	13	63	76

Table 6. Screen for Germ-line Transformation with P Element or *Frost*.

y w flies were injected with either a P element and a Δ2-3*wc* helper plasmid or with a *Fst* repair vector, Fst chiRNA, and a plasmid encoding *Cas9*. Values represent the number of vials of flies (each vial contains 50-100 flies).

	DNA						Total
	Scr*::*GFP	***Scr*::*tag***	***Antp*::*CFP***	***Antp*::*tag***	***Ubx*::*YFP***	***Ubx*::*tag***	
TOTAL	226	106	160	97	100	149	838
STERILE	42	15	35	12	17	38	159
VIABLE	184	91	125	85	83	111	679

Table 7. Screen for Germ-line Transformation with the Cas9 Source Encoded on a Plasmid.

y w flies were injected with one of six repair vectors, one of three chiRNA plasmids and a plasmid encoding *Cas*9. Values represent the number of vials of flies (each vial contains 50-100 flies). Abbreviations: *Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; CFP*, cyan fluorescence protein; *GFP*, green fluorescence protein; *YFP*, yellow fluorescence protein; tag, a 17 aa epitope of GFP with a 3X FLAG peptide.

	DNA						Total
	Scr*:: *GFP	***Scr*:: tag**	***Antp*:: *CFP***	***Antp*:: tag**	***Ubx*:: *YFP***	***Ubx*:: tag**	
nos total	12	8	22	30	16	21	109
act total	6	1	9	5	15	31	67
nos sterile	1	3	5	3	2	6	20
act sterile	0	1	0	0	2	6	9
nos viable	11	5	17*	27	14	15	72
act viable	6	0	9	5	13	25	58
TOTAL	**18**	**9**	**31**	**35**	**31**	**52**	**176**
STERILE	**1**	**4**	**5**	**3**	**4**	**12**	**29**
VIABLE	**17**	**5**	**26**	**32**	**27**	**40**	**147**
y+ observed	0	0	1	0	0	0	1

Table 8. Screen for Germ-line Transformation with the Cas9 Source Encoded on a Transgene.

nos-cas9 (*nos*) or *act-Cas9* (*act*) transgenic flies were injected with one of six repair vectors and one of three chiRNA plasmids. The set of injections that resulted in a successful HR event is marked with an Asterix (*). Values represent the number of vials of flies (each vial contains 50-100 flies). Abbreviations: *nos*, *nos-Cas9* flies; *act*, *act-cas9* flies; *Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; Fst, Frost; CFP*, cyan fluorescence protein; *GFP*, green fluorescence protein; *YFP*, yellow fluorescence protein; tag, a 17 aa epitope of GFP with a 3X FLAG peptide.

progeny showed that the w+ phenotype , which is the marker for integration of the *P{nos-cas9 w+}* transposon on the X chromosome, exhibited X linkage with all females being w+ and all males being w-. However, the inheritance of *y*+ showed no X linkage indicating that the *y*+ gene is integrated on an autosome (The endogenous *y* locus is on the X chromosome; Table 9). The single w+ male is likely a non-disjunction event of the X chromosome of a female.

3.4 Verification of Injected Germline Transformant

PCR on genomic DNA extracted from transformed flies was performed to verify the presence of *Antp*::*CFP*. A fragment within *Antp* was used as a positive control and *y w* genomic DNA spiked with repair template DNA was also used as a control. To specifically detect *Antp::CFP*, the amplified fragments spanned the *5'Antp* to *CFP* junction to confirm the *5'Antp*::*CFP lox2272 y+ lox2272 3'Antp* integration (illustrated in Figure 23.B). All products were observed as expected for genomic *y w* DNA and genomic *y w* DNA spiked with *Antp::CFP* repair template, however y+ genomic DNA failed to produce the correct products, indicating the y+ locus is not present at the expected locus within the genome (Figure 23.A).

Furthermore, analysis of the segregation pattern of Lobe, Curly, Tubby, and y+ in the progeny from crossing the candidate *Antp::CFP* flies with fly line 496 (containing balancer chromosomes) revealed that the y+ locus is on the 2nd chromosome and not on the 3rd chromosome, which contains the *Antp* locus.

3.5 Survival Assay

The efficiency of HR is low and this may be due to a low level of survival to the injection. To determine the survival index, the number of embryos injected was counted along with larval, pupal, and adult survivers (Table 10). The survival of injected embryos was 4.9% to larva and 3.0% to eclosed adults. This is low as generally the goal is 10-20 % survival.

Gender	Number	Body Phenotype	Eyes Phenotype
Male	37	y+	w-
Male	33	y-	w-
Male	1	y-	w+
Female	51	y+	w+
Female	64	y-	w+

Table 9. Progeny from Cross Between Male Transformant Candidate and Female *y w*.

Segregation of w+ and y+ phenotypes are shown for the male transformant crossed with *y w* females.

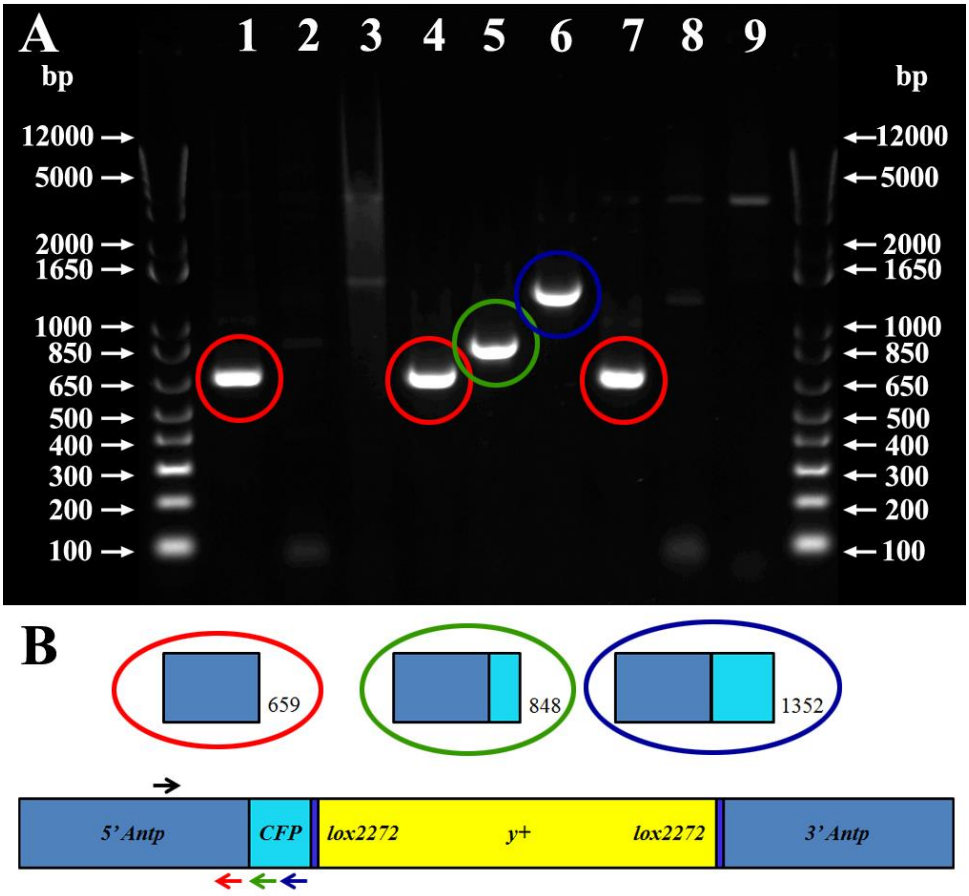

Figure 23. PCR of Genomic DNA Extracted from Transformants and Illustration of the Associated Amplified Fragments.

(A) PCR gel image displaying amplified fragments flanked by 1 kb+ DNA Ladder to indicate sizes in base pairs (bp). Lanes 1-3 used genomic DNA from *y w* flies as template, lanes 4-6 used genomic DNA from *y w* flies that was spiked with *Antp::CFP* repair vector DNA as template, and lanes 7-9 used genomic DNA of y+ transformant fly candidates as template. (B) The amplified fragments and their expected sizes (in bp) are illustrated compared to the transgene: *5'Antp::CFP lox2272 y+ lox2272 3'Antp*. The arrows indicate the position and orientation of the primers used in the PCR.

Injected			Larva (#)	Pupa (#)	Adults (#)	Survivors	
Fly Genotype	**DNA**	**Embryos (#)**				**Larval (%)**	**Adult (%)**
nos-Cas9	*Antp*::*CFP*	210	9	4	4	4.3	1.9
nos-Cas9	*Antp*::*CFP*	215	12	2	2	5.6	0.9
nos-Cas9	*Antp*::tag	210	14	9	9	6.7	4.3
nos-Cas9	*Antp*::tag	210	29	12	11	13.8	5.2
act-Cas9	*Scr*::*GFP*	228	3	0	0	1.3	0.0
act-Cas9	*Scr*::tag	326	5	1	1	1.5	0.3
act-Cas9	*Ubx*::*YFP*	264	4	1	1	1.5	0.4
act-Cas9	*Ubx*::tag	293	2	1	1	0.7	0.3
y w	P element	210	7	7	8	3.3	3.8
y w	*Fst*	191	22	25	26	11.5	13.6
y w	*Fst*	215	11	6	4	5.1	1.9
y w	*Fst*	206	7	7	7	3.4	3.4
	Average:	232	10	6	6	4.9	3.0

Table 10. Rate of Survival to Injections.

Abbreviations: *Antp, Antennapedia; Scr, Sex combs reduced; Ubx, Ultrabithorax; Fst, Frost; CFP*, cyan fluorescence protein; *GFP*, green fluorescence protein; *YFP*, yellow fluorescence protein; tag, a 17 aa epitope of GFP with a 3X FLAG peptide.

3.6 Fly Lines with Nanobody Components

Multiple balanced fly lines have been generated.

From crossing scheme 1, two fly lines homozygous on the 2nd chromosome for the GAL80 and UAS-nano with a balanced lethal third chromosome were created (referred to as GAL80+UAS-nano 7108 and GAL80+UAS-nano 7019). The two lines differ in the location and expression activity of the GAL80 insertion in the chromosome.

From crossing scheme 2, two fly lines were created with a balanced 3rd chromosome. The 2nd chromosome is either homozygous for GAL4(hs), referred to as the GAL4(hs) stock, or homozygous for UAS-nano, referred to as the UAS-nano stock.

3.7 Summary of Results

A summary of plasmids and flies that were obtained and created as well as experiments conducted are summarized in Table 11.

	Plasmids		**Flies**	
Obtained	Cas9 Plasmid		Cas9 Transgene	act-Cas9
				nos-Cas9
Created	chiRNA	*Scr*	Balanced Stocks	UAS-nano
		Antp		GAL4(hs)
		Ubx		GAL80(7108)+UAS-nano
	Repair Vectors	*Scr::GFP*		GAL80(7019)+UAS-nano
		*Scr::*tag		armGAL4
		Antp::CFP		
		*Antp::*tag		
		Ubx::YFP		
		*Ubx::*tag		
	Injection		**Other**	
Experiments	Injection Control	P element	Survival Assay	
		Frost	Transformant Progeny Assay (y+ segregation)	
	Cas9 Plasmid	*Scr*	PCR of y+ Transformant Genomic DNA	
		Antp		
		Ubx		
	Cas9 Transgene	*Scr*		
		Antp		
		Ubx		

Table 11. Summary of Results.

Plasmids and flies that were obtained and created as well as experiments conducted are summarized using short-forms.

4 Discussion

4.1 Efficiency and Optimization

CRISPR/Cas9 genome editing in *Drosophila* and many other organisms has only been established for two years. Several groups have performed CRISPR/Cas9 genome editing in *Drosophila* with differing methods of supplying the Cas9 protein and chiRNA components (Bassett et al., 2013; Gratz et al., 2013; Kondo & Ueda, 2013; Ren et al., 2013; Sebo et al., 2014; Yu et al., 2013). In genome editing there are two distinct approaches employed to repair a DSB: first is NHEJ which creates small deletions, and HR, which creates precise genome modifications.

The efficiency of NHEJ in *Drosophila* is well documented. Initial mutagenesis with CRISPR involved co-injection of two plasmids into syncytial blastoderm *Drosophila* embryos; one plasmid expressing *Cas*9 under the *Hsp70* promoter and one driving chiRNA expression through the *U6* gene promoter (Gratz et al., 2013). The efficiency of mutagenesis was low, at 5.9% of injected flies giving rise to a mutant offspring; however, they were successful in demonstrating that full deletions of sequence was possible (Gratz et al., 2013). An alternative means that has been applied in *Drosophila* involves *in vitro* transcription to synthesize *Cas9* mRNA and chiRNA that are then co-injected into syncytial blastoderm embryos. This method achieves higher mutagenesis rates, up to 80%, making it possible to target multiple loci simultaneously throughout the genome (Bassett et al., 2013; Yu et al., 2013). Differences in efficiency may be explained by lower expression levels of Cas9 protein and chiRNA from injected plasmids relative to direct injection of *Cas9* and chiRNA mRNA (Bassett & Liu, 2014). Another method uses transgenic flies to express *Cas9* in the germline and express the chiRNA ubiquitously, leading to higher efficiency of mutagenesis with up to 90% of flies producing mutant offspring and allowing the creation of longer deletions of up to 1.6 kb (Kondo & Ueda, 2013). However, producing a new transgenic fly for each chiRNA is very time consuming, as is the removal of the *Cas*9 and chiRNA transgenes after mutagenesis (Bassett & Liu, 2014). An alternative to this method is to inject chiRNA plasmids into germline-expressed *Cas*9 transgenic lines, which prevents somatic

mutagenesis since *Cas9* expression is limited to the germline and can yield efficiencies of 12-75 % (Ren et al., 2013; Sebo et al., 2014). Most of these methods created mutants via deletions or disruptions to genes; however, HR when a donor template is supplied creates precise genomic alterations.

HR of a DSB requires either endogenous homologous sequences or exogenously introduced sequences. When exogenously introduced sequences are used to repair a DSB, the repaired DSB has the sequence of the exogenous DNA allowing precise genome editing. The donor template supplying exogenous sequence may come in two forms. One is in the form of short (around 200 nt in length) single-stranded DNA (ssDNA) oligonucleotides, which can be used to integrate short transgene sequences. The second form of donor template, and the one used in this study, is longer double-stranded DNA (dsDNA) constructs containing hundreds to thousands of nt of homologous sequence on either side of the DSB site, which is capable of integrating longer sequences (Beumer & Carroll, 2014). For example, Bassett et al. (2013) used longer homology arms to insert a 1.8 kb cassette with a 4% efficiency rate. This technique can be used in conjunction with site-specific recombinase sites (such as *loxP*) to delete, replace, or otherwise modify intervening sequence (Bassett & Liu, 2014). The process of using longer dsDNA constructs as repair vectors has a relatively low efficiency, but it allows the use of marker genes, which is particularly advantageous simplifying identification of HR events (Bassett et al., 2013). In addition to positive markers, negative selectable markers, such as UAS-rpr outside of the homology arm, can also be used to optimize homologous targeting by enabling selection against non-homologous integrations (Baena-Lopez et al., 2013). Evidence has suggested that 1 kb of homology at either side of the integration site is sufficient to allow efficient HR, simplifying the construction of these vectors (Bassett et al., 2013). Larger homology arms enable the integration of longer features, for example tagging with fluorescent proteins such as GFP (Bassett & Liu, 2014). When a protein (such as GFP) is fused to an endogenous gene, expression of the protein is subject to the same transcriptional and post-transcriptional controls as the endogenous gene, and therefore the tag is expressed at similar levels of functionality and expression (Bassett & Liu, 2014). This was demonstrated when a circular double-

stranded plasmid containing the *GFP*-coding sequence flanked by 1.4 and 1.7 kb homology arms from the *wingless* (*wg)* locus was injected into double transgenic *nos-cas9, wg*-chiRNA fly embryos to produce an in-frame insertion of *GFP* within the *wg* coding region, leading to a secreted Wg::GFP protein (Port et al., 2014). 38% of the offspring of injected embryos expressed a secreted GFP protein in the Wg expression pattern (Port et al., 2014). Comparison of the integration efficiency across different CRISPR methods is difficult because of the use of different chiRNAs, donors, and target genes in each study. Roughly though, when all three DNA components (Cas9, chiRNA, and donor) are injected together, 0.3% of offspring integrated the exogenous sequence at the target site (Gratz et al., 2013). Larger constructs were integrated at a rate of 0-11 % by injecting the donor and chiRNA plasmids into *Cas9* transgenic embryos (Gratz et al., 2014). Injecting donor plasmids into embryos expressing *Cas9* and chiRNA transgenes produced the highest integration rates of 11-38 % of all offspring (Port et al., 2014). In one comparison where the same chiRNA and donor repair vector construct were used across all three major CRISPR methods; 3% of all offspring from G_0 flies contained the integration when a *Cas9*/*chiRNA* dual plasmid was co-injected with the donor plasmid, which was significantly lower than both the 5-15 % when the chiRNA expressed on a plasmid was injected with the donor into single transgenic (*Cas*9) embryos, and the 19-25 % when donor plasmid was injected into double transgenic (*Cas9* and chiRNA) embryos (Port et al., 2015).

Additionally, the promoter used for chiRNA expression in the chiRNA vector is an important element that affects the activity of chiRNA. Port et al. (2014) demonstrated that the same chiRNA expressed from different *U6 snRNA* promoters (*U6:1, U6:2*, and *U6:3*) have different activities. The previously untested *U6:3* promoter gives the most potent affect and leads to the highest efficiency of genome editing with the CRISPR system (Port et al., 2014).

When a target gene is cleaved (DSB), either inaccurate NHEJ, or HR when a repair template is supplied, are stimulated (Lieber et al., 2003; West, 2003). NHEJ uses the NHEJ pathway and HR mainly uses the invasion-mediated synthesis-dependent strand-annealing pathway (Bozas et al., 2009). Deleterious mutations in genes of the NHEJ

pathway, such as *lig4* (DNA ligase IV), can significantly improve the proportion of HR events (Bozas et al., 2009).

4.2 *Hox*::*XFP* Transformation

I attempted to induce homologous integration of a *GFP*, or a derivative of *GFP* tag, into the endogenous *Scr*, *Antp*, and *Ubx* loci, either by co-injection of Cas9 and chiRNA plasmids or by injection of a chiRNA plasmid into *Cas9* transgenic flies. Both approaches involved supplying a repair vector containing a *yellow* marker gene flanked by *loxP* sites with 1-2 kb homology arms. Unfortunately, the first method yielded no edited flies, even after screening through 679 vials of G_1 progeny (Table 7). This suggests that the efficiency of genome editing is below 0.2%. A number of different transgenic *Cas9* strains have been described and are publicly available, but differ in their capacity to make efficient DSBs (Port et al., 2015). Assessments of comparative performance revealed substantial differences in their pattern and activity level (Port et al., 2015). Therefore, two different fly strains, *nos-Cas9* and *act-Cas9* were used for the second CRISPR approach. Screens of 147 vials of progeny (Table 8) resulted in one injected fly showing evidence of being edited by the integration of the new sequence; however this was off-target. This suggests that the efficiency of genome editing is less than 1%.

There are many reasons to explain this low efficiency of editing. First, the survival rate of injected flies may explain the low efficiency rate for homologous integration. Only 3% of injected embryos eclose (Table 10), which may simply be explained by: poor injection technique and lack of experience by the experimenter. Laura Garofalo and I (working 50/50 together) injected *Fst* DNA repair vector along with chiRNA and the Cas9 plasmid as a control for the injection method. Dr. Anthony Percival-Smith found that the efficiency of editing was 10%, but screening 63 vials of fertile adult flies (Table 6), we found no editing when we should have seen in the range of 3-8 events. One potential explanation is the low 3% survivorship of injected flies. The normal survivorship in good injections is in the range of 10-20 % (Dr. Anthony Percival-Smith, personal communication).

Low efficiency of editing at *Scr*, *Antp*, and *Ubx*, may be due to different editing frequencies at each locus. To date, it is not known how many genomic target sites can be effectively edited in CRISPR/Cas experiments because most previous studies do not report results of non-editing (Bassett et al., 2013; Lee et al., 2014; Ren et al., 2014; Zhang et al., 2014). To date there has been no systematic study on the range of editing rates at different loci. Furthermore, most experiments have not attempted to insert large sequences, such as the ~6 kb in this study.

Fly lines containing components for the GFP-nanobody degrader (NSlmb-vhhGFP4; "nanobody") were successfully generated with relevant balanced background genotypes for use in this study. The nanobody components (the *UAS* driven nanobody, UAS-nano; GAL4 under control of a *hsp*, GAL4(hs); GAL4 under control of the *armadillo* promoter, armGAL4; and the UAS-nano expressed alongside a ts GAL80 negative regulator of GAL4, GAL80+UAS-nano) were crossed to create flies that are balanced on the chromosome 3 and homozygous for the nanobody component on chromosome 2. In a few crosses, flies that are homozygous for the desired genotype change (*Hox::XFP*) and containing one UAS-nanobody source and one GAL4 source can be created.

4.3 Future / Next Steps

4.3.1 Germline Transformants

Co-injections of relevant chiRNA and repair templates in *Cas9* transgenic flies will result in the generation of the six *Hox*::*XFP* (*Scr*::*GFP, Scr*::tag, *Antp::CFP, Antp*::tag, *Ubx*::*YFP, and Ubx*::tag) transformants. Once y+ transformants have been identified, these heterozygous transformants can be crossed with the generated single nanobody component balanced stock lines.

To test whether combinations of HOX proteins work together to determine the tarsus, the use of single, double and triple *Hox*::*XFP* transformants is required so that multiple HOX (SCR, ANTP, or UBX) can be degraded at the same time. To create double and triple mutants, single mutant *Hox*::*XFP* lines must first have the *yellow (y+)* marker gene removed. Removal of the y^+ gene can be accomplished by crossing with flies that carry a

Cre recombinase source and site-specific recombination between the *loxP* sites. For the *Scr* and *Ubx* integration events, the y^+ marker has been flanked with *loxP* sites. For *Antp*, the y^+ is flanked with *lox2272* sites that do not recombine with *loxP*, which is important to allow multiple, sequential recombination events, especially of *Scr*::*GFP Antp*::*CFP* genotypes, which are close together on the chromosome. Co-injection of relevant chiRNA and *Hox*::*XFP* repair vectors would establish transformants with the secondary and/or tertiary transgene integrations.

4.3.2 Homozygous *Hox*::*XFP* with Nanobody Components

The *Hox::XFP* transformants may then be crossed to generate heterozygous *Hox::XFP* with all four single nanobody components (GAL4(hs), UAS-nano, GAL80+UAS-nano, and armGAL4). A few simple crosses will generate the homozygous *Hox::XFP* with GAL4(hs) and UAS-nano as well as the homozygous *Hox::XFP* with armGAL4 and GAL80+UAS-nano. These two fly lines can be exposed either to a 37ºC heat shock at the third larval stage to activate expression of the nanobody using hsp-GAL4, or to 29ºC to inactivate GAL80. This would cause degradation of the tagged HOX proteins within the developing larva during the stage that determination of segment identity occurs. The tarsi of the resulting mature adult can be analyzed to determine whether the tarsus has been transformed to an arista.

4.3.3 Expected Results

If the requirement of ANTP is conserved in insects, the degradation of ANTP will result in a tarsus to arista transformation on all legs, as is observed in *Tribolium*. However, if this is not the case, the use of the other *Hox*::*XFP* transformants is required to test the alternative hypotheses.

One alternate is that SCR is responsible for tarsus determination or that multiple *Hox* genes may be working together to determine tarsus development. Tarsus to arista transformations in one of these cases would suggest that HOX requirement for tarsus determination has diverged during insect evolution and is not conserved in insects.

Another alternate is that none of the three HOX proteins, ANTP, SCR, or UBX, are required for tarsus determination in *Drosophila* and the mechanism of determination is HOX-independent.

4.4 Conclusion

Establishing CRISPR mediated HR was difficult and had a very low efficiency. However, the initial steps have been completed and the system is ready for further experimentation. *Hox::XFP* transformants can be used in conjunction with expression of the nanobody and subsequent recombination events to test the hypotheses and further define the role of HOX in tarsus determination. The system is established to determine which HOX protein is required for tarsus determination in *Drosophila melanogaster* and whether HOX requirement for tarsus determination is conserved in insects. This information will provide insight into the scope of conservation of requirement of genetic mechanisms of development that exist in all bilaterally symmetric organisms.

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Appendices

Appendix A. Sequences for CRISPR

Annotated sequences of (a) codon-optimized *Cas9* under the control of the *Drosophila hsp70* promoter and 3' UTR (Gratz et al., 2013), (b) chiRNA expression vector for generating site-specific, *U6*-driven chiRNAs (Gratz et al., 2013), (c) the pFUS_A vector used as the backbone for the repair vectors, and (d) the six repair vectors used to add a *GFP* derivative tag to *Hox* with *y+* marker gene flanked by *loxP* sites.

A.a. pHsp70-Cas9

DmHsp70 promoter and 3'UTR
3X FLAG
NLS
Cas9

ATCCCCCTAGAATCCCAAAACAAACTGGTTATTGTGGTAGGTCATTTGTTTGGCAGAAAGAAA
ACTCGAGAAATTTCTCTGGCCGTTATTCGTTATTCTCTCTTTTCTTTTTGGGTCTCTCCCTCTCT
GCACTAATGCTCTCTCACTCTGTCACACAGTAAACGGCATACTGCTCTCGTTGGTTCGAGAGA
GCGCGCCTCGAATGTTCGCGAAAAGAGCGCCGGAGTATAAATAGAGGCGCTTCGTCTACGGA
GCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAA
CAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAATTAA
AAGTAACCAGCAACCAAGTAAATCAACTGCAACTACTGAAATCTGCCAAGAAGTAATTATTG
AATACAAGAAGAGAACTCTGGGGGATCTGATCGATATGGACTATAAGGACCACGACGGAGAC
TACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCG
GAAGGTCGGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCG
GCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTC
AAGGTGCTGGGCAACACCGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTT
CGACAGCGGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACC
AGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGA
CGACAGCTTCTTCCACAGACTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGC
GGCACCCCATCTTCGGCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCT
ACCACCTGAGAAAGAAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTG
GCCCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGAC
AACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGA
AAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGA
GCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGA
AACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAG
GATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCCCA
GATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCT
GAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCA
AGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGCAGCTG
CCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCCGGCTACATTGA
CGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACG
GCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACCTTC
GACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGCA
GGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCC
GCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGATGACCAGA
AAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAAGTGGTGGACAAGGGCGCTTCCGC
CCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGC

CCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATAC
GTGACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTGGA
CCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGA
AAATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGG
GCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAAC
GAGGACATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGA
GGAACGGCTGAAAACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGC
GGAGATACACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCA
GTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCA
GCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCC
AGGGCGATAGCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGC
ATCCTGCAGACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGA
GAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGC
CGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAG
AACACCCCGTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAAT
GGGCGGGATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGA
CCATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACAACAAGGTGCTGACCAGAAG
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AACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGAC
CAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAGCTGG
TGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAACACTAAG
TACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGTCCAAGCTGGT
GTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACTACCACCACGC
CCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAGTACCCTAAGCTGG
AAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGC
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CGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTGA
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TCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAA
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AAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATCATGGAA
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AAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGA
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GCAGAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCA
GCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACA
ACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTG
ACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTAC
ACCAGCACCAAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGA
GACACGGATCGACCTGTCTCAGCTGGGAGGCGACAAAAGGCCGGCGGCCACGAAAAAGGCC
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GATTTTCTGATTTTTTCCGAACGGATTTTCGTAGACCCTTTCGATCTCATAATGGCTCATTTTAT
TGCGATGGACGGTCAGGAGAGCTCCACTTTTGAATTTCTGTTCGCAGACACCGCATTTGTAGC
ACATAGCCGGGACATCCGGTTTGGGGAGATTTTCCAGTCTCTGTTGCAATTGGTTTTCGGGAA
TGCGTTGCAGGCGCATACGCTCTATATCCTCCGAACGGCGCTGGTTGACCCTAGCATTTACAT
AAGGATCAGCAGCAAAATTTGCCTCTACTTCATTGCCCGGAATCACAGCAATCAGATGTCCCT
TTCGGTTACGATGGATATTCAGGTGCGAACCGCACACAAAGCTCTCGCCGCACACTCCACACT
GATATGGTCGCTCGCCCGTGTGGCGCCGCATATGGATCTTAAGGTCGTTGGACTGCACAAAGC

TCTTGCTGCACATTTTGCAGGAGTACGGCCTTTGACCCGTGTGCAATCGCATGTGTCGCGCCA
GCTTGTTCTGCGAAATAAACTAACGGGAATTCCTGCAGCCCGGGGGATCCGCGGCCGC

A.b. chiRNA Expression Vector pU6-BbsI-chiRNA

Dm snRNA:U6:96Ab promoter
BbsI sites for inserting guide sequence
crRNA repeat-derived sequence
tracrRNA
U6 terminator

GTTCGACTTGCAGCCTGAAATACGGCACGAGTAGGAAAAGCCGAGTCAAATGCCGAATGCAG
AGTCTCATTACAGCACAATCAACTCAAGAAAAACTCGACACTTTTTTACCATTTGCACTTAAA
TCCTTTTTTATTCGTTATGTATACTTTTTTTGGTCCCTAACCAAAACAAAACCAAACTCTCTTAG
TCGTGCCTCTATATTTAAAACTATCAATTTATTATAGTCAATAAATCGAACTGTGTTTTCAACA
AACGAACAATAGGACACTTTGATTCTAAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTA
AGACCATTTGCCAATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGT
ATGTTTTCCTCAATACTTCGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT

A.c. pFUS_A Vector to Insert Repair Sequence

pFUS_A backbone for repair vector sequences
Bsa1 recognition site
digested out section

TTGATGCCTGGCAGTTCCCTACTCTCGCGTTAACGCTAGCATGGATGTTTTCCCAGTCACGACG
TTGTAAAACGACGGCCAGTCTTAAGCGTCTCCCCCTGAACCTGACCCCGGACCAAGTGGTGGC
TATCGAGACCGGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGG
AAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCA
AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGT
GAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCCTC
CAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTT
CCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGT
AAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCACCGGTGGTCTCTGGCGGCAAGCAAGCGCTCGAAACGGTGCAGCGGCTGTTGCCGGTGC
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TGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTA
AACATTATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGT
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CGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAAC
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TCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCT
AAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCA
GCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTG
GTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTA
AATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGT
GCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGC

TGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACA
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CCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAACCCTCGAGCCACCCATGAC
CAAAATCCCTTAACGTGAGTTACGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCA
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GCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGG
CTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTT
CAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC
AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCA
GCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCG
AACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCG
GACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGG
GAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTT
GTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGT
TCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGAT
AACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGC
GAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTG
GCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCA
ACGCAATTAATACGCGTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCG
TCAGGATGGCCTTCTGCTTAGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCC
TCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGC
GTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTAT

A.d. Repair Vectors

A.d.1 *Scr*::*GFP*

5’ and 3’ homology arms
GFP
loxP site
y+ marker
Target sequence for chiRNA + PAM

CTATTTGTGGCCACGGCCACAGCAGTTGTCCAAGTAGAAAGAGTCCCTCTCTGTTGCCGGCTG
ATCCGGATGACTGATGGAGACGATGCCGGTTGTCTGGTTACTGTCTGCTCGCTGTTTACTTGGT
CATTAGTTTAAGCCCGTTCAGAATGGCAGCCAGCCGGGGAAGTGCAGGAGCTTTGATGTCGGC
GCTATCCCTGCGCTTTGGCCACACTCAACACACTGAAACTCGCTTTAATTAAATGAGCGGTAA
TTAATTTCAACTTTAATCAGAAATTCCATTTAGATTTTGGCCGCTGATTGATGTGGCCCGACAT
CTTCCGCCAACGTCACATCGAAGACCACTAAACTGTGACAGCGGCAACAACGGGGGAAAACT
TTTGGAAGTGTGTGGGCGGTTTTCTTTTTTGCGTGTGTGTGTTTTTGTTTTGTTTTTGTTAAAGA
AGCAACAGTTGCAATATGTATCTTTAAGTAGGCAACATGAATAATAAACTAACGTACAACATC
AGTTTTATAGGAGACAAAGCTCTACGAAATAGAATTGCAGAATAGGTCTTTTGAATATCAGTT
GCACAAATATACATAACTATTACATAGAGTCAATTGAAGGGAAAACCATTGACTTAAACTCTT
ATTAAGAATAAACAACCTGTTCTAAGCAGTAAGCCTAACTGCCTTTCATTTAGCATGTATTCC
ATGACCCATATAAACTATGAACCAAGCGAGACAACAATACCCAAAAATCAAAGAGAGACGCA
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AAATACTAGAAATACAGATACAGTGTCTATAGCCGAACTGCCTCTGTCTCAGCTCAACTCTTT
ATTGGAAATCCAAAACAAAGCGGGCGGGCCAATTGAGTTGGCCGTAATAACACGCCACGGTA
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CGCCCTCCTGCGGTTCGCCTCGAATAAAAGAGGAAAACCCGATACAGATGACAGAATAGTTTT
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CCTAGATACGCCTTAAAATAGTTTGCTGCAGAAACAAATGGCTTACATAAAAGAGTAAGGAA
CGAGTGTTTAAACAAAAATTGTTTTACTTAATATATTCAATTTAATTGATTTTCGTTTATTATGT
ATTGATATTGTACACGTTTGAACTTCCCGTCTAGTCTATAGGTATTCCAAGCACTGTTAAAAAG
AAAACTCCTTTAGAGCGCCAAATACTTGATCGACGCGTTAACTACGTTATTCAGAAACTTCAT
TTCTAATTGTTTCCTTTAAATTATTAATATTAAATAAGCACCTCTATTCCTGTATTGCAACAGC
AACTGACAAAACTATTCCGGTTTTTCCTCCAGGTACTGTGAATGCCAATGGCGAGACGAAACG
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GCTACCTGACCCGCCGCCGCAGAATCGAGATCGCGCATGCCCTGTGTCTCACGGAGCGGCAG
ATCAAGATCTGGTTCCAGAACCGGCGCATGAAGTGGAAGAAGGAGCACAAGATGGCCTCGAT
GAACATCGTACCCTACCACATGGGTCCATATGGCCACCCGTACCACCAGTTCGATATCCATCC
GTCGCAGTTCGCGCACCTAAGCGCAGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGC
CCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC
GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCC
CGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCC
CGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGC
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A.d.2 *Scr*::tag

5’ and 3’ homology arms
17 aa tag
3X FLAG
loxP site
y+ marker
Target sequence for chiRNA + PAM

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A.d.3 *Antp*::*CFP*

5’ and 3’ homology arms
CFP
modified aa sites
lox2272 site
y+ marker
Target sequence for chiRNA + PAM

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A.d.4 *Antp*::tag

5’ and 3’ homology arms
17 aa tag
3X FLAG
lox2272 site
y+ marker
Target sequence for chiRNA + PAM

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A.d.5 *Ubx*::*YFP*

5’ and 3’ homology arms

YFP
loxP site
y+ marker
Target sequence for chiRNA + PAM

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ACAAACCGTTTG

A.d.6 *Ubx*::tag

5’ and 3’ homology arms
17 aa tag
3X FLAG
loxP site
y+ marker
Target sequence for chiRNA + PAM

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AC

Appendix B. List of Primers.

(a) List of primers and their sequences used for: integrating 20 nt of target sequences into chiRNA, generating the six repair vectors, colony PCR screening for candidate repair vectors, and genomic extraction PCR of *Antp*::*CFP* transformant flies. (b) Forward and reverse primer pairs with corresponding DNA template for: generating the six repair vectors (fragment 1, 2, and 3), colony PCR screening for candidate repair vectors, and genomic extraction PCR of *Antp*::*CFP* transformant flies.

B.a. List of Primers

Primer Name	Sequence
chiRNA	
F.UbxChi	5'CTTCGCGGCGGCGGCGGTCCAAGG 3' (5'phosphate)
R.UbxChi	5'AAACCCTTGGACCGCCGCCGCCGC 3' (5'phosphate)
F.AntpChi	5'CTTCGAGCCGGGATCCGGAGGCGA 3' (5'phosphate)
R.AntpChi	5'AAACTCGCCTCCGGATCCCGGCTC 3' (5'phosphate)
F.ScrChi	5'CTTCGAACATCGTACCCTACCACA 3' (5'phosphate)
R.ScrChi	5'AAACTGTGGTAGGGTACGATGTTC 3' (5'phosphate)
Repair Vectors	
F.17FLAG	5'AACGTCTATATCGACTACAAAGACCATGACGGTG 3'
R.17FLAG	5'GTCTTTGTAGTCGATATAGACGTTGTGGCTGTTG 3'
F.UBXXFP	5'CACTTAGATCAGGTGAGCAAGGGCGAGGAGCTG 3'
R.UBXXFP	5'GCCCTTGCTCACCTGATCTAAGTGTCCACCTTG 3'
F.ANTPXFP	5'AACAGTCCGCAGGTGAGCAAGGGCGAGGAGCTG 3'
R.ANTPXFP	5'GCCCTTGCTCACCTGCGGACTGTTGGGTGGTG 3'
F.SCRXFP	5'CACCTAAGCGCAGTGAGCAAGGGCGAGGAGCTG 3'
R.SCRXFP	5'GCCCTTGCTCACTGCGCTTAGGTGCGCGAACTG 3'
F.UBX17	5'CACTTAGATCAGATCCTGGGGCACAAGCTGGAG 3'
R.UBX17	5'GTGCCCCAGGATCTGATCTAAGTGTCCACCTTG 3'
F.ANTP17	5'AACAGTCCGCAGATCCTGGGGCACAAGCTGGAG 3'
R.ANTP17	5'GTGCCCCAGGATCTGCGGACTGTTGGGTGGTG 3'
F.SCR17	5'CACCTAAGCGCAATCCTGGGGCACAAGCTGGAG 3'
R.SCR17	5'GTGCCCCAGGATTGCGCTTAGGTGCGCGAACTG 3'
F.5Ubx	5'CAGCTAGGTCTCGCTATCTCCAAAATGCCAAGTTAACATG 3'
F.x5Antpx	5'CAGCTAGGTCTCGCTATCTCTTCGGCCTCCTACCCCTC 3'
F.5Scr	5'CAGCTAGGTCTCGCTATTTGTGGCCACGGCCACAGCAG 3'
F.x5Scrx	5'CAGCTAGGTCTCGCTATGAATAGTTTTCGGGGGCGAC 3'
R.5XFP	5'CAGCTAGGTCTCCCATGTTACTTGTACAGCTCGTCCATG 3'
R.5FLAG	5'CAGCTAGGTCTCCCATGTTACTTGTCATCGTCATCCTTGTAG 3'
F.loxPy	5'CAGCTAGGTCTCCCATGATAACTTCGTATAGCATACATTATACG AAGTTATGTCGACTATTAAATGATTATC 3'
F.lox2272y	5'CAGCTAGGTCTCCCATGATAACTTCGTATAGGATACTTTATACG AAGTTATGTCGACTATTAAATGATTATC 3'
R.loxy	5'CAGCTAGGTCTCGGTCCTCGACCTGCAGGTCAACGGATC 3'
F.loxPUbx	5'CAGCTAGGTCTCGGGACATAACTTCGTATAGCATACATTATACG AAGTTATATCCTTAGATCCTTAGATCCTTAG 3'

R.loxPUbx	5’CAGCTAGGTCTCCCGCCGTATGAACGGAGCCTGCGGGTC 3’
R.xloxPUbxx	5’CAGCTAGGTCTCCCGCCCAAACGGTTTGTCGCACTCCTC 3’
F.lox2272Antp	5’CAGCTAGGTCTCGGGACATAACTTCGTATAGGATACTTTATACG AAGTTATGATCGACGGAGTCTACCCAC 3’
R.lox2272Antp	5’CAGCTAGGTCTCCCGCCGCTTTCCGCTTTGCAGCCCTTTC 3’
F.loxPScr	5'CAGCTAGGTCTCGGGACATAACTTCGTATAGCATACATTATACG AAGTTATGACGCGTGGCACTTTTCGGGTAC 3’
R.loxPScr	5’CAGCTAGGTCTCCCGCCGCGAAAATCAGTATGGTTCCGAC 3’
R.xloxPScrx	5’CAGCTAGGTCTCCCGCCTCGACAGCTTTGTCTGCTCATC 3’
F.CFP1	5’ACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGC 3’
R.CFP1	5’GCACTGCACGCCGGTGGTCAGGGTGGTCACGAGGGTG 3’
F.CFP23	5’GCCGACAAGCAGAAGAACGGCATCAAGGCAAACTTCAAGATCC GCCACAAC 3’
R.CFP23	5’CTTGATGCCGTTCTTCTGCTTGTCGGCCCAGATATAGACGTTGT GGCTGTTGTAG 3’

Colony PCR Screening

F.S2.a	5’ CTGGCAGTTCCCTACTCTCG 3’
R.S2.a	5’ GGTAAATCAGCGGGCTGCGTTCG 3’
F.S1.S2.b	5’ CAGGGAAAGTTCAACTTAATCGC 3’
R.S2.b	5’ CTGTCCTGGCTGGTCTAGACGTC 3’

Genomic extraction PCR

F.x5ANTPx	5’CAGCTAGGTCTCGCTATCTCTTCGGCCTCCTACCCCTC 3’
R.CFP1	5’GCACTGCACGCCGGTGGTCAGGGTGGTCACGAGGGTG 3’
R.5XFP	5’CAGCTAGGTCTCCCATGTTACTTGTACAGCTCGTCCATG 3’
F.S1.S2.b	5’CAGGGAAAGTTCAACTTAATCGC 3’
R.S1.Antp	5’GTTTAGGTTTGTTCACGCGAAG 3’
R.lox2272Antp	5’CAGCTAGGTCTCCCGCCGCTTTCCGCTTTGCAGCCCTTTC 3’

B.b. PCR Primers and Templates

DNA template 1	DNA template 2	Forward Primer	Reverse Primer
Repair Vector Fragment 1			
UBXTT		F.17FLAG	R.5FLAG
eYFP		F.UBXXFP	R.5XFP
pEGFP		F.SCRXFP	R.5XFP
pEGFP		F.ANTPXFP	R.CFP1
pEGFP		F.CFP1	R.CFP23
pEGFP		F.CFP23	R.5XFP
pEGFP		F.UBX17	R.17FLAG
pEGFP		F.SCR17	R.17FLAG
pEGFP		F.ANTP17	R.17FLAG
y w		F.5Ubx	R.UBXXFP
y w		F.5Scr	R.SCRXFP
y w		F.x5Antpx	R.ANTPXFP
y w		F.5Ubx	R.UBX17
y w		F.x5Scrx	R.SCR17
y w		F.x5Antpx	R.ANTP17
ANTPXFP.CFP1	CFP1.CFP23	F.ANTPXFP	R.CFP23
CFP1.CFP23	CFP23.5XFP	F.CFP1	R.5XFP
ANTPXFP.CFP23	CFP1.5XFP	F.ANTPXFP	R.5XFP

x5Antpx.ANTPXFP	ANTPXFP.5XFP	F.x5Antpx	R.5XFP
5Scr.SCRXFP	SCRXFP.5XFP	F.5Scr	R.5XFP
5Ubx.UBXXFP	UBXXFP.5XFP	F.5Ubx	R.5XFP
UBX17.17FLAG	17FLAG.5FLAG	F.UBX17	R.5FLAG
SCR17.17FLAG	17FLAG.5FLAG	F.SCR17	R.5FLAG
ANTP17.17FLAG	17FLAG.5FLAG	F.ANTP17	R.5FLAG
5Ubx.UBX17	UBX17.5FLAG	F.5Ubx	R.5FLAG
x5Scrx.SCR17	SCR17.5FLAG	F.x5Scrx	R.5FLAG
x5Antpx.ANTP17	ANTP17.5FLAG	F.x5Antpx	R.5FLAG
Repair Vector Fragment 2			
y+ (digested miMIC)		F.loxPy	R.loxy
y+ (digested miMIC)		F.lox2272y	R.loxy
Repair Vector Fragment 3			
y w		F.loxPScr	R.loxPScr
y w		F.loxPScr	R.xloxPScrx
y w		F.loxPUbx	R.loxPUbx
y w		F.loxPUbx	R.xloxPUbxx
y w		F.lox2272Antp	R.lox2272Antp
Colony PCR Screening			
white bacteria colony		F.S2.a	R.S2.a
white bacteria colony		F.S1.S2.b	R.S2.b

Genomic Extraction PCR (of y+ transformants)			
y w		F.x5ANTPx	R.CFP1
y w		F.x5ANTPx	R.5XFP
y w		F.S1.S2.b	R.S1.Antp
y w		F.S1.S2.b	R.lox2272Antp
y+ transformants		F.x5ANTPx	R.CFP1
y+ transformants		F.x5ANTPx	R.5XFP
y+ transformants		F.S1.S2.b	R.S1.Antp
y+ transformants		F.S1.S2.b	R.lox2272Antp

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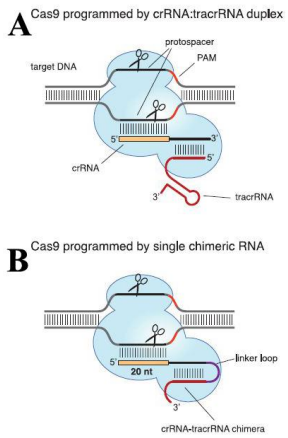

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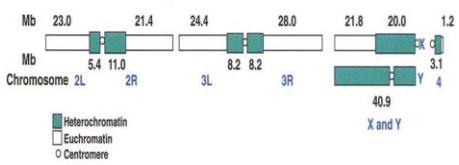

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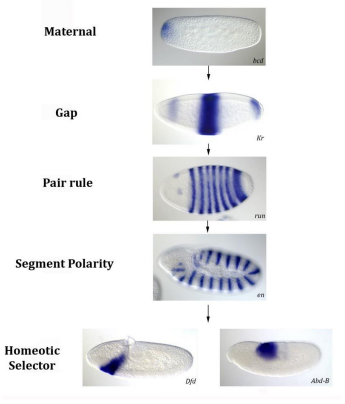

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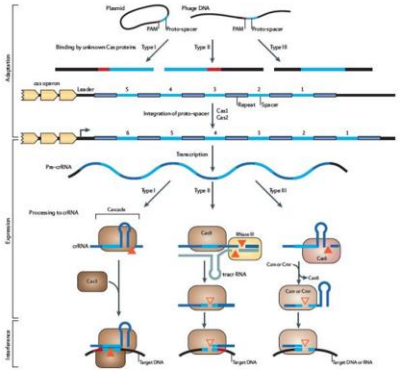

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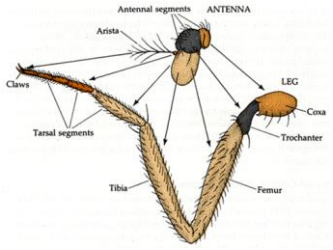

Figure 7. Gilbert, 2013 modified from Postlethwait & Schneiderman, 1971.

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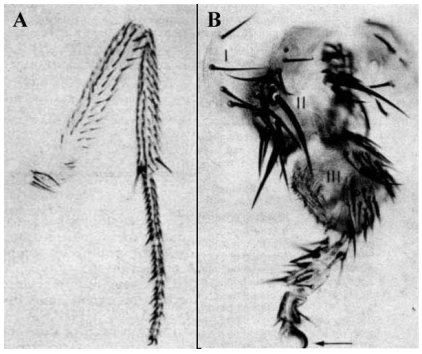

Figure 8. Struhl, 1981b.

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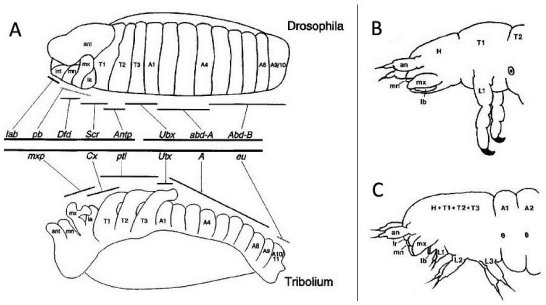

Figure 9. Beeman et al., 1993.

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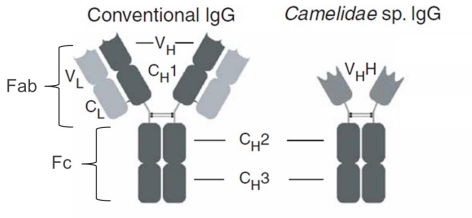

Figure 10. Rothbauer et al., 2006.

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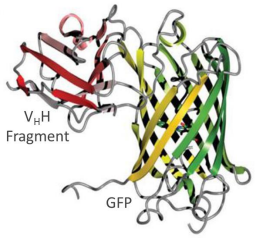

Figure 11. Kubala et al., 2010.

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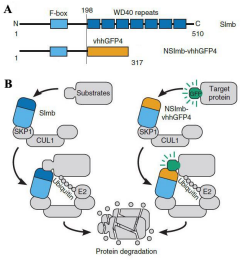

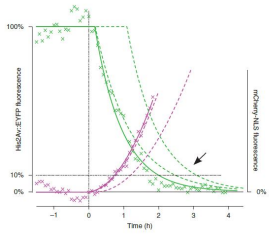

Figure 12 and 13. Caussinus et al., 2012.

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