

8-2010

GENETIC ANALYSIS OF THE FUNCTION OF THE DROSOPHILA DOUBLESEX-RELATED FACTOR dmrt93B

Diana O'Day

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations



Part of the [Behavioral Neurobiology Commons](#), [Developmental Biology Commons](#), and the [Genetics Commons](#)

Recommended Citation

O'Day, Diana, "GENETIC ANALYSIS OF THE FUNCTION OF THE DROSOPHILA DOUBLESEX-RELATED FACTOR dmrt93B" (2010). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 54.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/54

This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

GENETIC ANALYSIS OF THE FUNCTION OF THE DROSOPHILA

DOUBLESEX-RELATED FACTOR *dmrt93B*

by

Diana O'Day, B.S.

APPROVED:

William Mattox, Ph.D., Supervisory Professor

Richard Behringer, Ph.D.

Andreas Bergmann, Ph.D.

Gilbert Cote, Ph.D.

Pierre McCrea, Ph.D.

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

GENETIC ANALYSIS OF THE FUNCTION OF THE DROSOPHILA
DOUBLESEX-RELATED FACTOR *dmrt93B*

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Diana O'Day, B.S.
Houston, Texas

August, 2010

For my mother

Acknowledgements

I would like to thank my advisor Dr. William Mattox for his guidance throughout my graduate career. His patience during the gene targeting strategy allowed me to develop valuable skills in *Drosophila* genetics. I would also like to thank the members of my various committees: Drs. Richard Behringer, Andreas Bergmann, Gilbert Cote, David Hewett-Emmett, Randy Johnson and Pierre McCrea for all of their helpful comments.

I thank Dr. Audrey Christansen for all her support and advice these past few years. I am indebted to Elaine McGuffin for techniques she passed on to me for *Drosophila* research. Sam Su has been very helpful with staining protocols and RNA *in situ* analysis. I also thank Shanzhi Wang for his help in the lab and comments in lab meetings. I'm grateful to Rong Dong not only for making fly food, but also for her concern about my everyday life.

Special thanks goes to Hank Adams for work in the microscopy core. I would also like to thank Clare Richards and Kathleen Gajewski for teaching me how to do embryo microinjections. Their help was key to the generation of the donor transgenic line used to generate the targeted strains.

I give special thanks to the following University of Houston members that provided great assistance in the analysis of the mutant phenotype: Brigitte Dauwalder, Valbona Hoxha, Gregg Roman, and Shiyu Xu. Bona was vital to the development of my technique for adult brain dissections. Shiyu was essential in the analysis of the feeding behavior with the CAFÉ assay.

Abstract

Genetic analysis of the function of the *Drosophila*
Doublesex-related factor *dmrt93B*

Publication No. _____

Diana O'Day, B.S.

Supervisory Professor: William Mattox, Ph.D.

DMRT (Doublesex and Mab-3 related transcription factor) proteins generally associated with sexual differentiation in many organisms share a common DNA binding domain and are often expressed in reproductive tissues. Aside from *doublesex*, which is a central factor in the regulation of sex determination, *Drosophila* possesses three different *dmrt* genes that are of unknown function. Because the association with sexual differentiation and reproduction is not universal and some DMRT proteins have been found to play other developmental roles we chose to further characterize one of these *Drosophila* genes. We carried out genetic analysis of *dmrt93B*, which was previously found to be expressed sex-

specifically in the developing somatic gonad and to affect testis morphogenesis in RNAi knockdowns. In order to disrupt this gene, the GAL4 yeast transcriptional activator followed by a polyadenylation signal was inserted after the *dmrt93B* start codon and introduced into the genome by homologous recombination. Analysis of the knock-in mutation as well as a small deletion removing all *dmrt93B* sequence demonstrate that loss of function causes partial lethality at the late pupal stage. Surprisingly, these mutations have no significant effect on gonad formation or male fertility. Analysis of GAL4-driven GFP reporter expression indicates that the *dmrt93B* promoter activity is highly specific to neurons in the suboesophageal and proventricular ganglion in larva and adult of both sexes suggesting a possible role in digestive tract function. Using the Capillary Feeder (CAFÉ) assay to measure daily food intake we find that reduction in this gene's function leads to an increase in food consumption. These results suggest *dmrt93* plays an important role in the formation or maintenance of neurons that affect feeding and support the idea that *dmrt* genes may not be restricted to roles in sexual differentiation.

Table of Contents

Approval	i
Title Page	ii
Dedication	iii
Acknowledgements	iv
Abstract	vi
Table of Contents	viii
List of Illustrations	xi
List of Tables	xiii
Chapter 1: Introduction	1
1. Genetic control of sex determination in <i>Drosophila</i>	1
2. DM domain proteins	4
3. <i>Drosophila</i> DM proteins	6
4. Morphogenesis of the male reproductive tract	9
5. The role of the CNS and digestive tract in feeding	12
6. Endogenous expression of <i>dmrt93B</i>	13
7. RNAi knockdown of <i>dmrt93B</i> function	16
8. Targeted knockout by homologous recombination in <i>Drosophila</i>	21
Chapter 2: Generation of the <i>dmrt93B</i>^{GAL4KI} allele	25
Introduction	25
Materials and Methods	26
1. Design of the <i>dmrt93B</i> -GAL4 construct	27

2. Embryo microinjection	30
3. Genomic DNA extraction	31
4. Creation of a positive plasmid control	31
5. Southern blot analysis	32
6. RT-PCR analysis	32
Results	33
1. Construct design for ends-out gene targeting	33
2. GFP-based screen for gene targeting	37
3. Separation of two donor alleles by meiotic recombination	39
4. Identification of targeted events using a DNA-based screen	43
5. Confirmation of the <i>dmrt93B</i> ^{GAL4KI} allele by RT-PCR	48
Discussion	53
Chapter 3: Analysis of the <i>dmrt93B</i>^{GAL4KI} allele	57
Introduction	57
Materials and Methods	58
1. Isogenized lethal-free strain BDGP3	59
2. Meiotic recombination to remove exogenous mutations	60
3. Immunohistochemistry	60
4. Establishing GFP/RFP double reporter	61
5. CAFÉ assay	62
Results	62

1. Analysis of the <i>dmrt93B</i> ^{GAL4KI} allele reveals late pupal lethality.	63
2. Analysis of fertility in <i>dmrt93B</i> ^{GAL4KI} mutants.	68
3. The <i>dmrt93B</i> GAL4 driver forces UAS-GFP expression in the suboesophageal ganglion of the central brain.	73
4. Expression of the reporter GFP is altered in hemizygous mutant brains in comparison to heterozygous wild-type.	78
5. CAFÉ assay indicates the <i>dmrt93B</i> ^{GAL4KI} allele results in increased consumption of food.	82
Discussion	86
Chapter 4: Conclusion and Future Directions	92
Bibliography	94
Vita	111

List of Illustrations

1-1	Abbreviated signaling cascade for sex determination	3
1-2	<i>Drosophila</i> DMRT proteins share a conserved DNA binding domain	8
1-3	Gonad morphogenesis in <i>Drosophila melanogaster</i>	11
1-4	Diagram of the adult brain and proventriculus	14
1-5	Tissue specific RT-PCR for <i>dmrt93B</i> transcripts	15
1-6	RNA <i>in situ</i> hybridization of <i>dmrt93B</i> transcripts in the developing testis	17
1-7	RNA knockdown of <i>dmrt93B</i> results in defects in testis morphogenesis	18
1-8	Comparison of testicular morphogenesis in wild-type and <i>dmrt93B</i> RNAi knockdown males	20
2-1	Gene targeting of <i>dmrt93B</i>	34
2-2	Southern blot to confirm donor transgenes separated by meiotic recombination	40
2-3	PCR to confirm the two separated donor transgenes are able to excise the targeting construct	42
2-4	PCR amplification of positive control plasmid DNA	45
2-5	PCR amplification of genomic DNA diluted in a pool of flies	46
2-6	Mating scheme for gene targeting	47
2-7	Southern blot confirming targeting	50

2-8	Radio-probed blot of RT-PCR	52
3-1	The <i>dmrt93B^{GAL4KI}</i> mutants have reduced adult viability	64
3-2	The <i>dmrt93B^{GAL4KI}</i> homozygotes have late pupal lethality	66
3-3	The <i>dmrt93B^{GAL4KI}</i> hemizygous mutants have late pupal lethality	67
3-4	Testicular defect of a <i>dmrt93B^{GAL4KI}</i> hemizygous mutant	71
3-5	Expression of <i>dmrt93B</i> in larvae	74
3-6	Expression of <i>dmrt93B</i> in adults	77
3-7	Reporter GFP not detected in the proventricular ganglion of ablated animals	79
3-8	Comparison of wild-type and mutant reporter GFP	81
3-9	CAFÉ assay indicates <i>dmrt93B^{GAL4KI}</i> mutants consume more food than control	84
3-10	CAFÉ assay indicates <i>dmrt93B^{GAL4KI}</i> has a dominant effect on food intake	85

List of Tables

2-1	Recovery of targeted lines from PCR screen	49
3-1	Summary of fertility assay	69

Chapter 1: Introduction

The molecular mechanism of sex determination varies significantly among animal species. For example, in mammals the male Y-linked gene SRY is sufficient to elicit male identity. Although the mechanism for sex determination is different for worms and flies, the primary signal for both is the ratio of X chromosome to autosomes. In worms this primary signal works through a signal transduction cascade, and in flies it works through a splicing cascade. The recent identification of a family of proteins with a conserved DNA binding domain and important roles in sex determination provides a potential link between the mechanisms used in a wide variety of organisms. In this chapter, more detail will be given to the sex determination pathway in *Drosophila* with an emphasis on male development. Also, background information about the digestive system and the gene targeting strategy is provided as these topics appear in the next chapters.

1. Genetic control of sex determination in *Drosophila*

Sexual differentiation in *Drosophila* occurs through alternative splicing of several genes (Nagoshi et al.,

1988; MacDougall et al., 1995). A major downstream target of this cascade is Doublesex (DSX), a gene vital for somatic sexual differentiation (Baker and Ridge, 1980; Burtis and Baker, 1989). Males develop from the default pathway in the absence of female influencing factors such as the proteins Sex-lethal (SXL) and Transformer (TRA) (Baker and Ridge, 1980). These factors act to control the expression of the sex specific isoforms of Doublesex, DSX-F (found in females) and DSX-M (found in males).

These two Doublesex proteins differ in the carboxy-terminus of the translated protein. In females, TRA acts in combination with a second protein, Transformer-2 (TRA2), to promote alternative splicing of *doublesex* mRNA and the expression of the DSX-F protein, which promotes female development and represses male development. In males the TRA protein is absent and default splicing occurs using an alternate acceptor site that leads to expression of the DSX-M protein, which has an opposite function relative to that of DSX-F (figure 1-1).

Though several genes have been identified downstream of the sex differentiation cascade, only a few direct targets of *doublesex* have been identified. The *yolk-protein* (*Yp*) gene, which is turned on by DSX-F and off by DSX-M, was the first direct target of *doublesex* identified

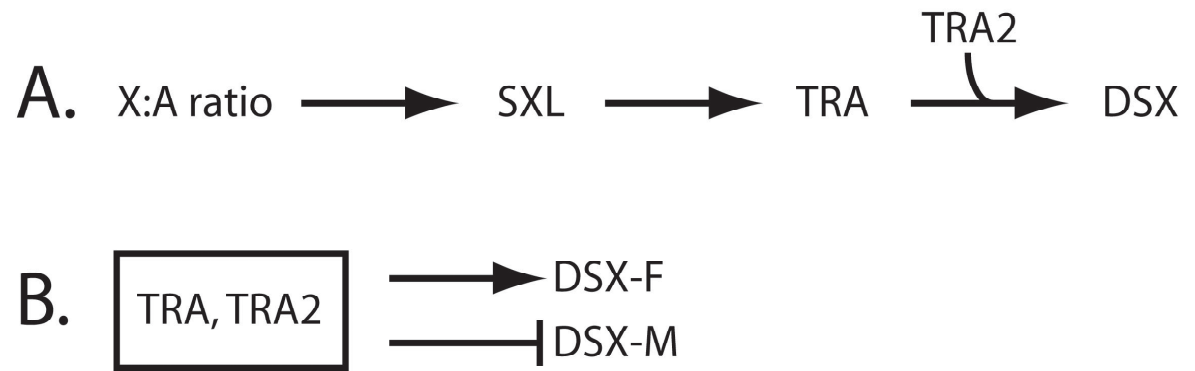


Figure 1-1: Abbreviated signaling cascade for sex determination. (A) The order of genes and signals influencing DSX expression. (B) Effects of TRA and TRA2 on DSX. Arrowhead indicates activation. Barred line indicates inhibition.

(Burtis et al., 1991; Coshigano and Wensink, 1993). It has also been shown that DSX acts with Abdominal-B to directly regulate a cis-regulatory element of the gene *bric-a-brac* to control sex specific pigmentation (Williams et al., 2008). Recently, female specific *desatF*, a gene encoding sex pheromones that enhance female attractiveness to males, was also identified as a direct target of the female specific form of *doublesex* (Shirangi et al., 2009).

As previously mentioned, *doublesex* is an important target of the sex determination cascade in *Drosophila*. In the next section I will discuss a family of proteins that share a conserved DNA binding domain with *doublesex* and are found in a range of higher organisms.

2. DM domain proteins

The DSX-F and DSX-M proteins both contain a characteristic DNA binding domain that contains variant intertwined zinc fingers. This DM domain defines a family of protein factors known as DMRTs (Doublesex and MAB-3-related transcription factors), that have been found to play an important role in sexual differentiation in vertebrate and invertebrate organisms and are known to be expressed specifically in the gonad of many species (Volff

et al., 2003). For example, the mouse *Dmrt1* gene affects maintenance of sexual differentiation in the postnatal testis (Raymond et al., 2000; Lei et al., 2007). In chickens, *Dmrt1* is the primary sex determination factor (Smith et al., 2009). In the fish medaka, the male specific DMY gene appears to be the primary sex-determining factor (Matsuda et al., 2002).

The DM domain is a DNA binding variant of the zinc-finger motif and is typically located near the N-terminus of the protein (Erdman and Burtis, 1993). At the C-terminus of the DM domain is an alpha helical region believed to function as a recognition helix involved in DNA binding (Narendra et al., 2002). In general, DMRTs share little homology outside of the DM domain, although this is not universal. Interestingly, it has been shown that the male isoform of the *Drosophila doublesex* gene is sufficient to rescue the mutant phenotypes in *C. elegans* associated with the lack of the *mab-3* gene despite the fact that these proteins share similarity only in their DM domains (Raymond et al., 1998).

Another interesting feature of the *dmrt* genes is their ability to bind DNA as both homodimers and heterodimers through C-terminal alpha helical regions (Murphy et al., 2007; Yang et al., 2008). This suggests the possibility

that this family of factors can form various combinations to alter downstream gene transcription. However, current evidence suggests that not all DMRT proteins are able to heterodimerize and *in vitro* assays have only so far shown associations between the vertebrate proteins *Dmrt3* and *Dmrt5* to *Dmrt1* (Murphy et al., 2007).

While DMRT proteins are identified primarily by the conserved DNA binding domain, some "DM-less" proteins with homology outside of this conserved region have also been classified into the family. One example of this is the mouse *Dmrt8*, which has sequence homology to *Dmrt7*. *Dmrt8* encodes several protein isoforms and at least one that is specific to the Sertoli cells of the testis (Veith et al., 2006). Another example of the "DM-less" *dmrt* genes is an alternative isoform of *dmrt99B* from the water flea *Daphnia magna* (Kato et al., 2008). According to Kato et al., the truncated *dmrt99B* is believed to have dimerization activity, however this is not demonstrated.

3. Drosophila DM proteins

After the realization that the DNA binding domain of *doublesex* was conserved in other organisms, it was found that the *Drosophila* genome encodes three additional *dmrt*

genes (figure 1-2). These uncharacterized genes were named based on their cytological location: *dmrt11E* is located on the X chromosome, *dmrt93B* is located on chromosome 3R, and *dmrt99B* is also on chromosome 3R.

RNAi knockdown and expression studies carried out in our lab subsequently suggested roles for two *dmrt* genes in gonad morphogenesis and formation of the testicular duct. In adults, this duct forms part of the reproductive tract and serves as a conduit for mature sperm. Development of the male reproductive system in *Drosophila melanogaster* depends on the formation of this duct, which connects the gonad to the genital disc early in pupation.

During gonad morphogenesis, the testicular duct serves as a substrate for migratory cells that move from the genital disc to the gonad. These cell movements are essential for the adult testis to take on its normal elongation and coiled shape. RNA *in situ* hybridization showed that two *Drosophila dmrt* genes, *dmrt11E* and *dmrt93B*, are expressed in the male gonad. *dmrt93B* expression is localized to the terminal epithelium in the posterior region of the testis where the duct arises. *dmrt11E* is expressed in a complementary pattern to *dmrt93B* in the larval gonad and is seen broadly in the anterior testis. RNAi experiments targeted at each gene individually

indicated that both of them are required for the formation of the testicular duct and for the subsequent development of an intact male reproductive tract. The next section will provide detail on testicular morphogenesis in the developing fly.

4. Morphogenesis of the male reproductive tract

The formation of the *Drosophila* male reproductive system is a continuous process that initiates in the embryo and develops until the fly ecloses as a sexually mature adult. During the embryonic stage, primordial germ cells migrate toward the posterior end of the animal and combine with somatic cells to form the gonad (Sonnenblick, 1941; Jaglarz et al., 1994). The expression of Sox100B and the male specific ABD-B in these somatic cells is essential to the development of the male gonad (DeFalco et al., 2003). In males, fertility is dependent on the Jak/Stat pathway expression in the germ cells (Sheng et al., 2009)

In the larval stage, sexual differences in the gonad are visible as these cells divide earlier and grow larger in males relative to females. The genital disc is a somatic tissue that forms posterior to the gonad during the larval stage. This tissue responds to the sex pathway to

form the vas deferens and, subsequently, the seminal vesicle (Keisman and Baker, 2001). The formation of the male genitalia is activated by *decapentaplegic* (*dpp*) and repressed by *wingless* (*wg*) in the genital disc (Keisman and Baker, 2001).

During the pupal stage, the genital disc and gonad join as the terminal epithelium of the gonad connects to the seminal vesicle derived from the genital disc (figure 1-3). In the process of this connection, the testicular duct is formed. Male specific pigment cells that initially coat the testis migrate to the seminal vesicle during morphogenesis and muscle precursor cells migrate from the genital disc to the testis to form the coiled shape found in adults.

Based on tissue-specific RT-PCR analysis, the head was identified as a non-sex specific tissue expressing *dmrt93B*. During the development of this dissertation I identified discrete expression of this gene in regions of the brain associated with feeding behavior. The next section introduces these additional organ systems that are also affected by *dmrt93B*.

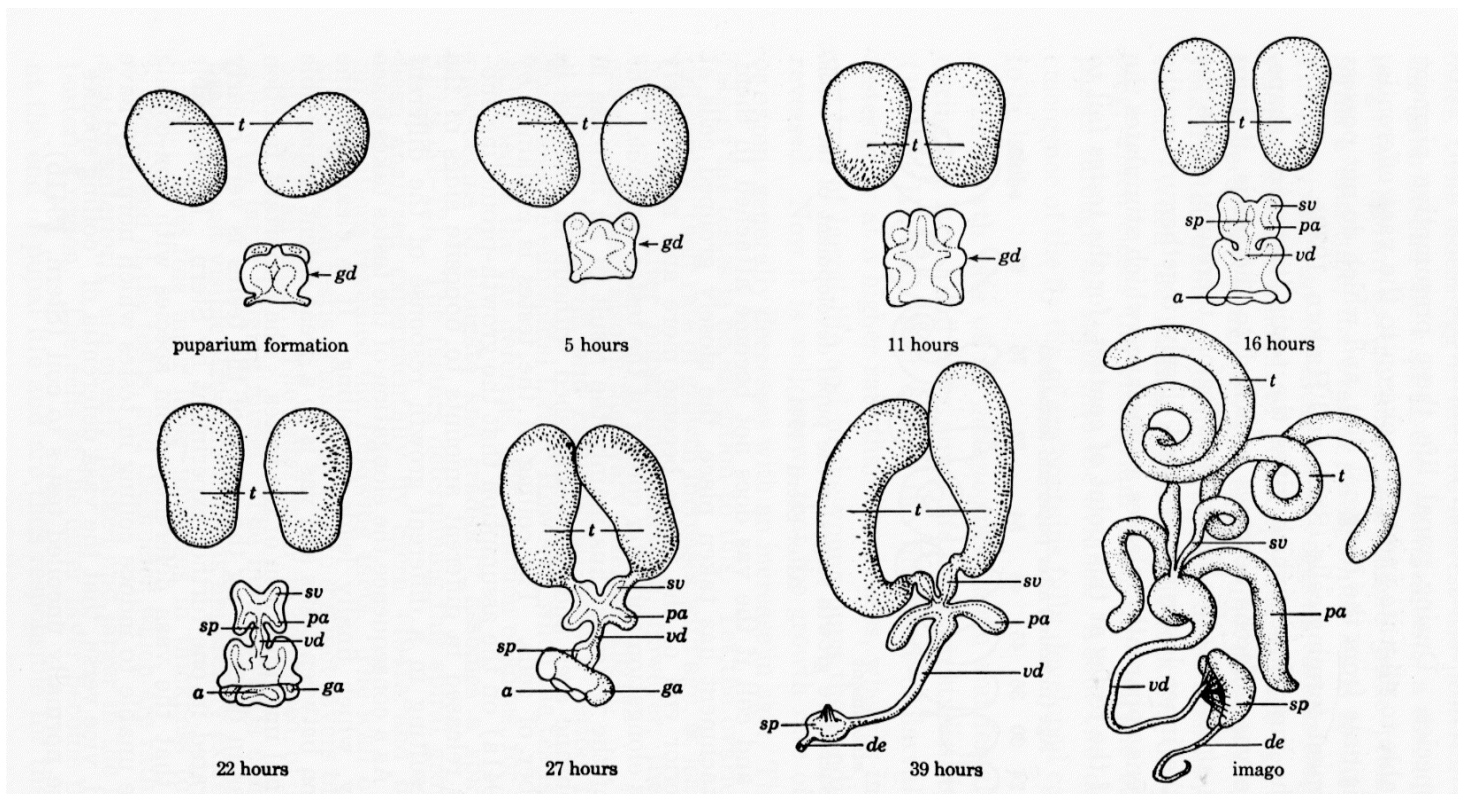


Figure 1-3: Gonad morphogenesis in *Drosophila melanogaster*. Gonad and genital disc initially develop as distinct tissues. By 27 hours after pupation, these two structures connect at the terminal end of the testis (t) and the seminal vesicle (sv) from the genital disc (gd). Additional structures labeled are anal plates (a), ejaculatory duct (de), genital arch (ga), paragonia (pa), sperm pump (sp), and the vas deferens (vd). (image reproduced from Bodenstein, 1994).

5. The role of the CNS and digestive tract in feeding

Studies on *Drosophila* have identified roles for the central nervous system (CNS) in processing information to control feeding behavior. Examples of feeding behavior regulated by the CNS include taste for food preference and insulin signaling for sated appetite. Serotonergic neurons identified in the *Drosophila* brain indicate that specific neurons can negatively regulate feeding behavior by releasing serotonin (Valles and White, 1988; Bao et al., 2010).

In *Drosophila*, the esophagus passes through the brain, below the antennal lobes and above the suboesophageal ganglion, to the rest of the digestive tract. The esophagus joins the mouthparts from the pharynx to the proventriculus, the organ that regulates the passage of food into the gut (Budnik et al., 1989). During larval metamorphosis the majority of the gut cells are histolyzed and replaced by adult gut. These larval gut cells slough off in the meconium of newly eclosed adults.

The frontal nerve that innervates the proventriculus runs along the esophagus from the more anterior structures of the digestive tract. After the nerve passes through the esophagus it is referred to as the recurrent nerve and branches out at the proventriculus to form the

proventricular ganglion (Spiess et al., 2008) (figure 1-4). The proventricular ganglion is associated with the contractions of the proventriculus, although it is not essential for this function as shown by nerve severing experiments in the blowfly (Schoofs et al., 2007).

6. Endogenous expression of *dmrt93B*

As previously mentioned in section 3 of this chapter, three additional *Drosophila* genes encoding DM domain proteins exist. For the purpose of this dissertation, I discuss one of these three genes, *dmrt93B*, in greater detail. This unpublished data from our lab obtained prior to my dissertation formed the basis of my original hypothesis that *dmrt93B* is required for the morphogenesis of the male reproductive tract.

RT-PCR analysis of *dmrt93B* showed that this gene has at least three different alternatively spliced transcripts encoding different isoforms (figure 1-5). Only one of these isoforms contains the conserved recognition helix in the C-terminus of the DM domain (S. Wen, Master's thesis 2002). This recognition helix has been implicated in DNA binding specificity in other DM proteins; therefore,

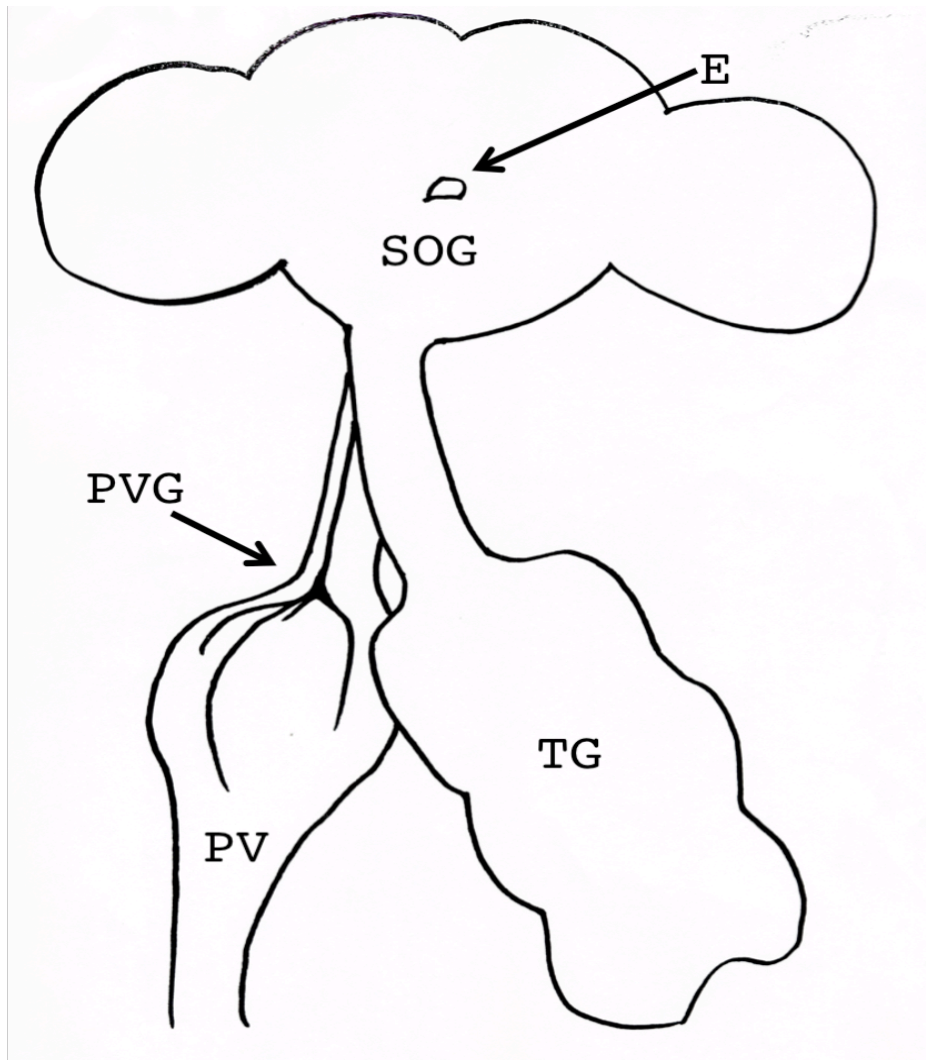


Figure 1-4: Diagram of the adult brain and proventriculus. The esophagus (E) passes through the central brain above the suboesophageal ganglion (SOG) and connects to the proventriculus (PV). The proventricular ganglion (PVG) branches at the anterior portion of the PV. The thoracic ganglion (TG) is the adult equivalent of the ventral ganglion seen during the larval stages of development.

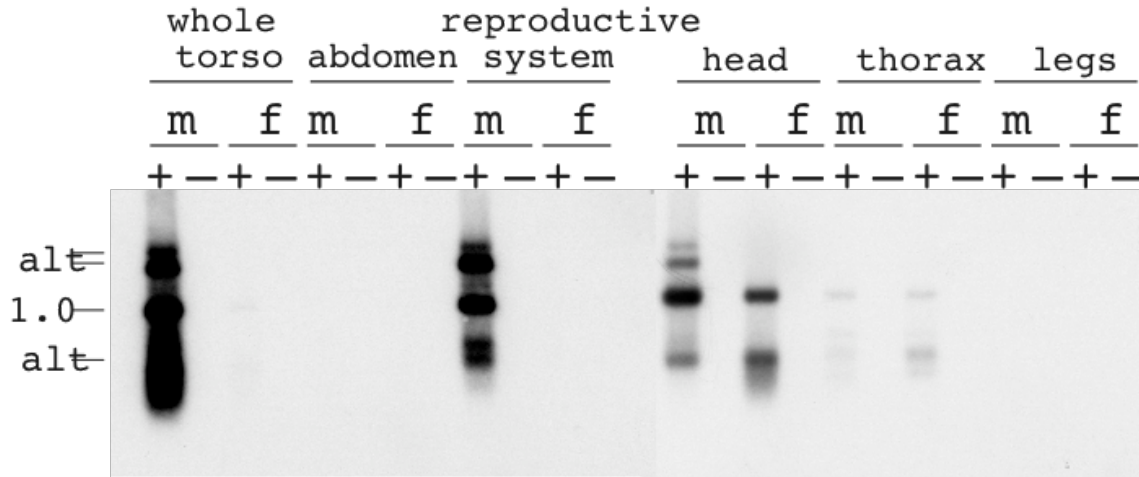


Figure 1-5: Tissue specific RT-PCR for *dmrt93B* transcripts. Total RNA was extracted from the head, thorax, legs, abdomen, and reproductive tissue of both male and female adult flies. After first strand cDNA synthesis and PCR to amplify *dmrt93B*, the products were run on an agarose gel and blotted to a nylon membrane. A *dmrt93B* specific probe was used to amplify the signal from the transcripts. (Elaine McGuffin, unpublished).

variation in this region may indicate different functions for the isoforms.

RNA *in situ* hybridization of *dmrt93B* reveals expression in the most posterior region of the larval gonad of wild-type *Drosophila* (figure 1-6). By the later pupal developmental stage, after the gonad forms a connection with the rest of the reproductive system originating from the genital disc, *dmrt93B* expression can be seen in the seminal vesicle and testicular duct, as well as the most posterior region of the gonad.

7. RNAi knockdown of *dmrt93B* function

Previous work in the Mattox lab shows that RNAi knockdown of *dmrt93B* function by ubiquitous drivers resulted in approximately 70% lethality. Of the few escapers, most were sterile. Analysis of RNAi knockdown individuals showed significant defects in the pupal male reproductive system (figure 1-7). As described earlier, a normal adult testis has an elongated coiled shape. The most obvious defects with RNAi knockdown flies are seen in this testis morphology. In the most extreme case, the epithelium of the posterior gonad fails to connect with the genital disc to form the duct and to allow further testis

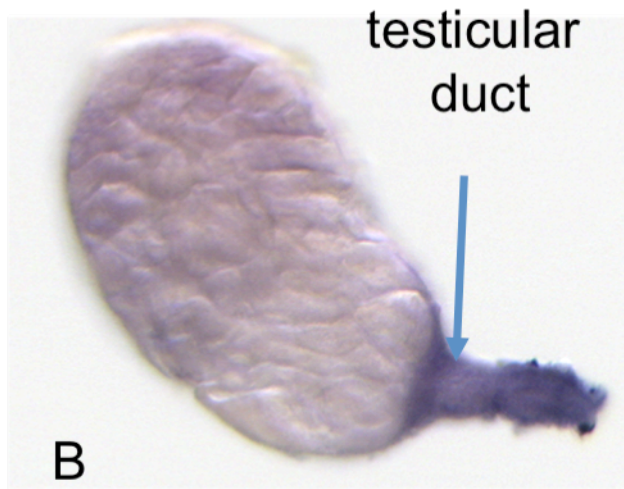
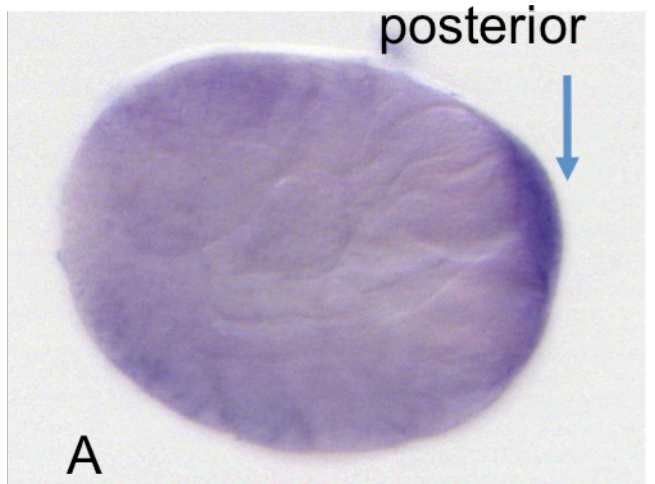


Figure 1-6: RNA *in situ* hybridization of *dmrt93B* transcripts in the developing testis. (A) 12 hours after pupation, *dmrt93B* expression is localized at the posterior end of the testis. (B) 24 hours after pupation, *dmrt93B* expression is localized to the posterior region of the testis and the testicular duct. (Sam Su, unpublished)

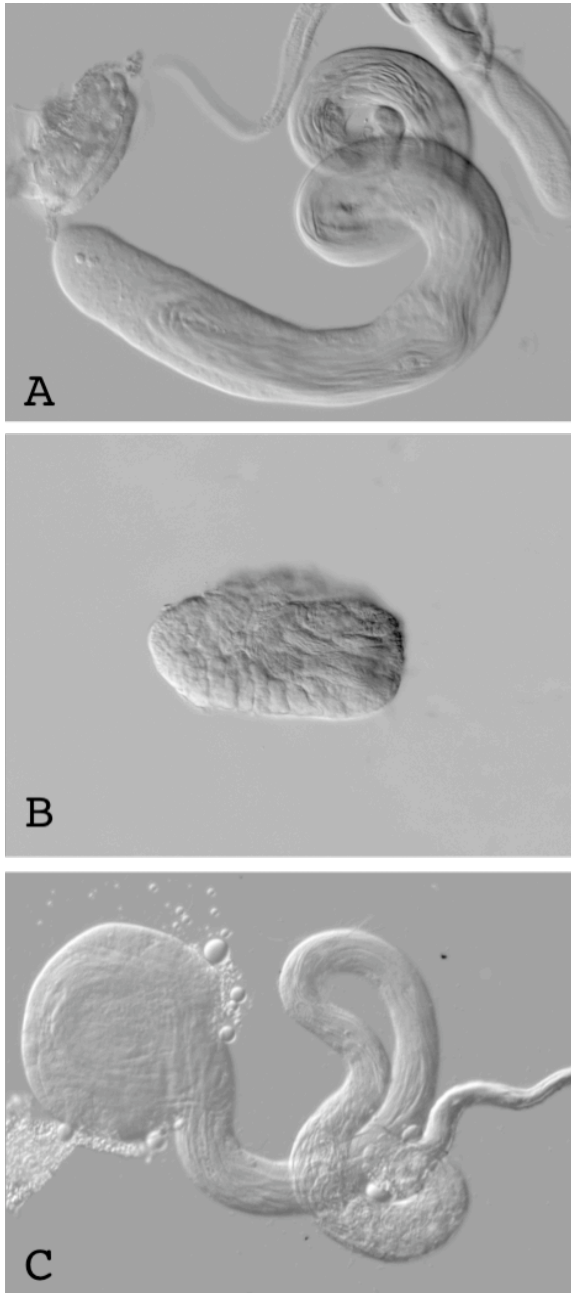


Figure 1-7: RNAi knockdown of *dmrt93B* results in defects in testis morphogenesis. (A) wild-type testis. (B) *dmrt93B* RNAi testis fails to connect with seminal vesicle. (C) *dmrt93B* RNAi testis has abnormal structure when connection is made between gonad and seminal vesicle. (Elaine McGuffin, unpublished)

morphogenesis. In cases where the gonad does connect with the genital disc, the testis partially elongates and coils but has a bulbous shape rather than the typical coiled shape due to incomplete morphogenesis.

Examination of wild-type and RNAi testes at earlier stages revealed that *dmrt93B* may play a role in morphogenic events affecting the terminal epithelium. During larval development, gonad morphogenesis is comparable to wild-type lines. As previously described, during the pupal stage a bud from the terminal epithelium forms in wild-type individuals to give rise to the testicular duct. However, the terminal epithelium of RNAi knockdown individuals seems unable to reorganize into a bud (figure 1-8). This indicates *dmrt93B* may have a role in morphogenesis events that reshape the epithelium and the process that allows the testis to connect with the seminal vesicle.

Over-expression of the UAS-*dmrt93B*-cDNA rescue construct driven by the ubiquitous alpha-tubulin-GAL4 resulted in male lethality with a few escaper females. This is consistent with ectopic expression of the male specific isoform of *doublesex* with the heat-shock promoter *hsp70*, which also results in lethality (Jursnich and Burtis, 1993). The female specific isoform of *doublesex* over-expressed with the ubiquitous Actin-5C promoter during

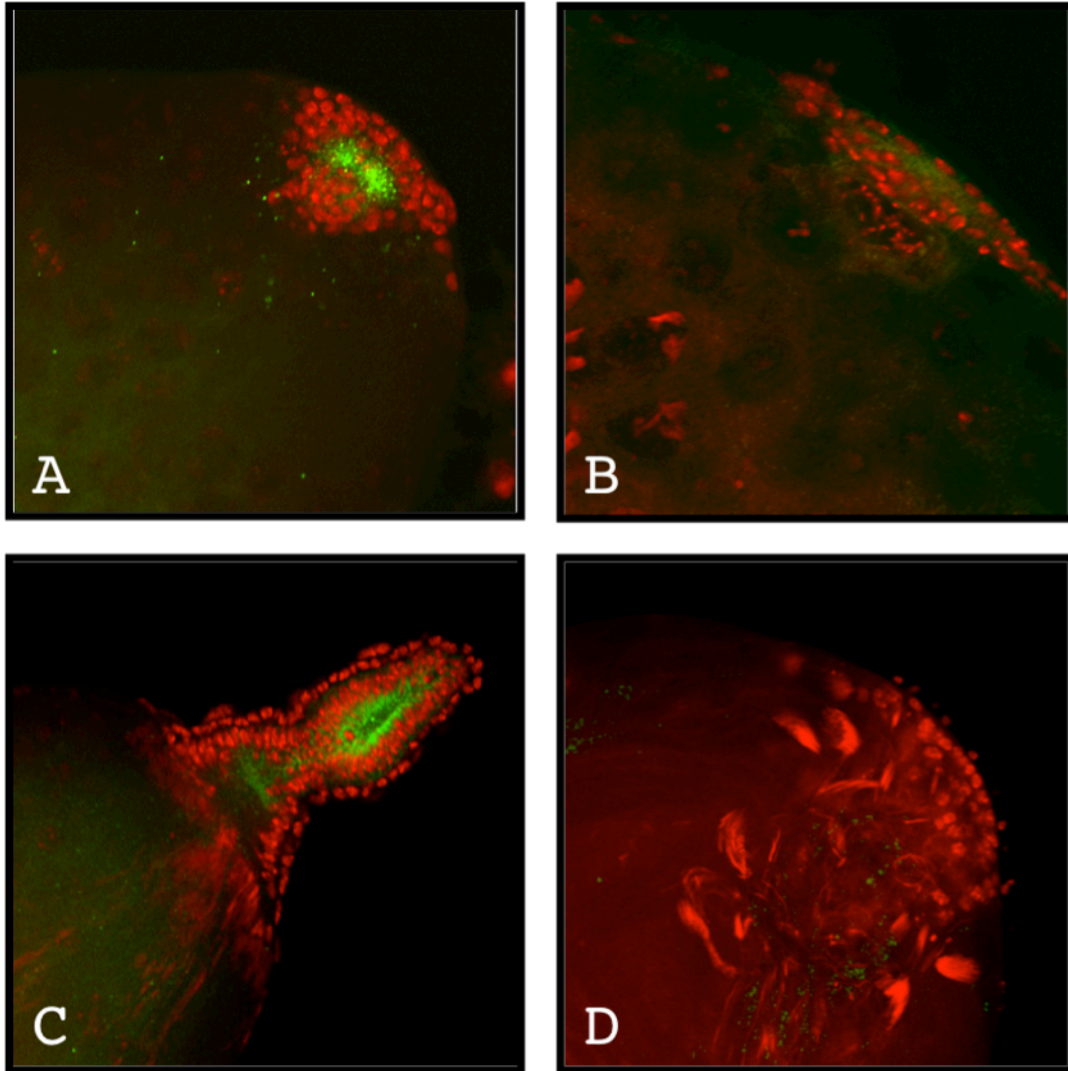


Figure 1-8: Comparison of testicular morphogenesis in wild-type and *dmrt93B* RNAi knockdown males. Testes are stained with propidium iodide in red and fasciclin III in green. (A) larval wild-type testis. (B) larval *dmrt93B* RNAi testis. (C) 30 hour pupal wild-type testis. (D) 30 hour *dmrt93B* RNAi testis. (Sam Su, unpublished)

development results in male lethality and less severe effects on female (Shen et al., 2009). This data suggests ectopic expression of the sex determination gene *doublesex* is toxic during development of the animal. Since the phenotypes seen with RNAi knockdown are variable, I further analyze *dmrt93B* gene function using a stable mutation generated by homologous recombination *in vivo*, a strategy introduced in the following section.

8. Targeted knockout by homologous recombination in *Drosophila*

In general, double stranded RNAi constructs should be gene specific. However, the phenotype of the RNAi knock-down line was not seen in all individuals and could possibly be explained by the occurrence of off-target events. To investigate gene function, I established a stable mutation in *dmrt93B* generated by gene targeting to address this issue.

As described in chapter 2, I chose to establish a GAL4 “knock-in” allele for *dmrt93B* using homologous recombination *in vivo* as the gene targeting strategy. Currently it is not possible to culture *Drosophila* totipotent stem cells for germ-line manipulations. Thus,

the conventional method of gene targeting of electroporating targeting constructs into embryonic stem cells and then microinjecting them into early stage blastocysts is not a viable option with *Drosophila*. Rong and Golic, however, developed a technique for *Drosophila* that can create targeted mutations in endogenous genes by homologous recombination in the organism itself through simple genetic manipulations.

Drosophila homologous recombination *in vivo* involves creating a transgenic line carrying a targeting construct that can be excised and linearized when crossed to a second strain containing inducible FLP recombinase (Flippase) and I-SceI endonuclease. The strategy that I chose for this project is ends-out gene replacement since it involves fewer steps to the recovery of a final targeted allele than does the ends-in gene insertion method (Gong et al., 2003).

Several plasmid vectors are available for gene targeting *in vivo* for *Drosophila* (Radford et al., 2005). In general these vectors contain P-element transposon termini and mini-white as a transformation eye phenotype marker for initial random genome integration of the targeting sequences. A key feature of these vectors is that they include FRT sites for excision of the targeting DNA by site-specific recombination and two I-SceI

restriction sites that create double stranded breaks flanking the sequence of interest in the excised circle to generate a linear recombinogenic DNA fragment. The FLP/FRT recombinase system works by recombining FRT sites from the transgenic line after another transgene expressing an inducible FLP recombinase is introduced from a second parental line to excise a circularized loop of DNA. Using flanking arms of homology, this fragment of DNA can integrate at a targeted location in the genome after the DSB is created by the I-SceI endonuclease at unique restriction sites.

The knock-in approach I selected allows the opportunity to insert the yeast transcriptional activator GAL4 into the *dmrt93B* coding region (Brand and Perrimon, 1993). The UAS-GAL4 binary transcriptional activation system uses gene specific promoter elements to direct GAL4 expression in specific tissues which in turn "drives" expression of a second transgene that includes the yeast Upstream Activating Sequences (UAS) (Duffy, 2002). A wide array of UAS driven transgenes are available to express reporter proteins (i.e. GFP or LacZ), RNAi constructs, or for over-expression of gene products that alter the survival, development, or physiology of affected cells.

The generation of a GAL4 knock-in for *dmrt93B* allows me to study the spatial expression pattern of the gene. In the characterization of this gene, our lab has found *dmrt93B* transcripts are exceedingly rare and difficult to detect by Northern blot analysis and *in situ* hybridization. A previous attempt at producing a *dmrt93B*-GAL4 driver transgene was unsuccessful when 3 kb of sequence upstream from the *dmrt93B* start codon was used (S. Wen Master's thesis, 2002). In designing my targeted gene disruption of *dmrt93B*, GAL4 was inserted in-frame after the start codon. This knock-in (KI) of GAL4 makes use of all endogenous regulatory elements for *dmrt93B*. With this design, *dmrt93B*^{GAL4KI} can drive the reporter UAS-GFP to reveal the expression pattern of the gene. Additionally, because the GAL4 insertion also disrupts *dmrt93B* function, the mutant phenotype can be analyzed with homozygotes of the knock-in allele.

Chapter 2: Generation of the *dmrt93B*^{GAL4KI} allele

Introduction

As described in chapter 1, our lab found the DMRT family member *dmrt93B* to be sex-specifically expressed in the terminal epithelium of the posterior testis, the region of the gonad that connects to structures formed by the genital disc. Additionally, RNAi knockdown of this gene suggests *dmrt93B* is essential for the proper connection of these tissues. My original hypothesis that *dmrt93B* function is required for the development of the male reproductive tract is based on these findings. To test this hypothesis, I use a reverse genetics approach to generate a mutant allele of *dmrt93B*.

Gene targeting is now an established tool in *Drosophila* genetics that can create deletions, insertions or changes in sequence at specific locations in the genome (Rong and Golic, 2000). Because mutations of choice can be specifically introduced into the target gene, this technique is advantageous over forward genetics screens with chemicals such as ethylmethane sulfonate (EMS). Random point mutations in the genome created by EMS must be screened for expected phenotypic defects, which may not be known for uncharacterized genes. Additionally, mutagenesis

may give rise to an expected phenotype that results from mutations in other genes that affect the same tissue.

Using an "ends-out" gene targeting strategy I have inserted the coding sequences for GAL4 after the start codon of *dmrt93B* to make use of endogenous enhancers for the gene. The mutant allele generated allows not only analysis of mutant phenotypes but also provides a way to observe the expression pattern of the gene using a UAS reporter gene such as GFP or lacZ. This binary system is more powerful than a simple insertion of markers like GFP and lacZ into the gene of interest because multiple reporters can be used in combination with the GAL4 to visualize expression. Additionally, the knock-in GAL4 driver can be used in combination with other UAS transgenes, such as apoptotic pathway genes *hid* and *reaper*, to ablate cells expressing *dmrt93B* and test their function.

Materials and Methods

In this section I will describe in detail the design of the *dmrt93B*-GAL4 targeting construct used to generate the *dmrt93B*^{GAL4KI} allele. Essentially, the construct was built by combining arms of homology flanking my gene specific alterations. After embryo microinjection, two

transgenic lines were established and used for the gene-targeting step. Targeted events were confirmed by Southern blot analysis before use in mutant analysis as described below.

1. Design of the *dmrt93B*-GAL4 construct

To ensure sequence fidelity to the targeted strain, the template DNA used for building the targeting construct comes from BAC DNA used in the Berkeley Drosophila Genome Project (BDGP). The DNA fragment utilized for the following cloning steps spans a portion of the *rudimentary-like* gene, the entire *dmrt93B* gene, and the neighboring CG7056 to provide a template for the arms of homology. BAC DNA library RP98 20N14 was digested with KpnI, a 9kb fragment was gel isolated, and subcloned into the KpnI site of pBluescript KS+. To easily identify clones carrying the insertion, colonies were blotted to a nylon membrane and radioprobed with a *dmrt93B* specific fragment that identified the correct insertion. This clone was named pBSKS+9kbKpnI-6A.

The low copy, high fidelity *Pfu* polymerase was used to PCR amplify the arms of homology in segments. Additionally, the amplified fragments were cloned into the pCR 2.1-TOPO TA cloning kit from Invitrogen following an A-

tailing step. The steps used to create the four DNA segments used in generating the construct are described below.

The first segment was designed to add the BamHI and SpeI restriction sites after the ATG start codon of *dmrt93B* and an XbaI site in the conserved DM domain along with a point mutation that would change the conserved Histidine (H) to a Tyrosine (Y). These unique restriction enzymes were added for further cloning steps. The sense primer was 93B-E1-BamHI-SpeI for 5'-GCG ATG gga tcc act AGT GGT GAA CC-3' (with point mutations creating the BamHI and SpeI restriction enzyme in lower case letters) and the antisense primer was 93B-E1-XbaI for 5'-GTA GGt cta gaG CTT CTT **GTA** AC-3' (with the XbaI restriction enzyme in lower case letters and an additional point mutation in bold). The amplification cycle was: 94C, 5 min; (94C, 45 sec; 55C, 45 sec; 72C, 2 min) for 30 cycles; 72C, 10 min; 4C hold. The successful cloned 184 bp fragment was named *pdmrt93B-E1-6*.

For the second segment, the 5' arm of homology, pBSKS+9kbKpnI-6A was again used as the template in the PCR using high fidelity *Pfu* polymerase. The sense primer was M13 Forward (-20) 5'-GTA AAA CGA CGG CCA GTG-3' and the antisense primer was "AS 5 prime arm" 5'-CCA CTT GAg gat ccC ATC GCA ACG CA-3' (with the BamHI restriction site in

lower case). The amplification cycle for this set was: 94C, 5 min; (94C, 45 sec; 55C, 45 sec; 72C, 6 min) for 30 cycles; 72C, 15 min; 4C hold. The clone was named p5-prime arm.

For the third segment, the 3' arm of homology, pBSKS+9kbKpnI-6A was also used as the template in the PCR using high fidelity *Pfu* polymerase for the insertion of additional mutations in the coding sequences. The sense primer was S-3-prime-arm 5'-GCT cta gaC CTA CAA GAA CTA CAA G-3' (with XbaI in lower case letters and a point mutation that would mutate the conserved Cysteine (C) to a Tyrosine (Y) in bold). The antisense primer for this 3' arm of homology was AE003733-99566AS 5'-ATA AGA GCG GGT GGG CGG AGA TAG-3'. Here, the amplification cycle was: 94C, 5 min; (94C, 45 sec; 55C, 45 sec; 72C, 8 min) for 30 cycles; 72C, 15 min; 4C hold.

The fourth and last amplified segment was the yeast transcriptional activator GAL4 using pGaTN as a template in the *Pfu* PCR amplification. The sense primer was GAL4-S1 5'-GAT Ggg atc cCT GTC TTC TAT CGA ACA A-3' (with point mutations creating the BamHI restriction site after the start codon of pGaTN in lower case). The antisense primer was GAL4-AS 5'-GAa cta gtC CGA TCT AAA CGA GTT-3' (with the SpeI restriction site in lower case).

Finally, the four segments were combined to form the targeting construct. First, the BamHI/ XbaI digested *pdmrt93B-E1-6* plasmid was inserted into the p5-prime-arm clone with the same digestion. This new plasmid was named p5-prime-E1. Next, the 3' arm of homology was cloned into this vector with the XbaI/ NotI restriction digest. The construct was named p5-prime-E1-3-prime. In the third cloning step, the GAL4 fragment was inserted using the BamHI/ SpeI restriction sites. This construct was named p5-prime-GA-E1-3-prime.

In the last cloning step, the above plasmid was KpnI/ NotI digested and inserted into the p{Whiteout2} vector (Radford et al., 2005) for targeting. The final construct was sequence verified before microinjection into embryos to establish transgenic lines by P-element mediated germ-line transformation.

2. Embryo microinjection

A lethal free third chromosome from the strain used in the Berkeley Drosophila Genome Project (BDGP) was introduced into a w^{1118} background through a series of genetics crosses. This isogenized strain was generated to ensure that the targeting construct has arms of homology matching sequences in the target chromosome; using this

sequenced chromosome avoids differences from polymorphisms that are possible in different strain backgrounds. I will refer to this strain as "BDGP3" at later parts of this dissertation.

BDGP3 wild-type flies with the genotype $w^{1118}; +; +$ were allowed an overnight egg lay and given fresh grape juice agar plates the following day. After one hour the plates were changed once again to avoid any eggs retained by the females from the previous night. The eggs were dechorionated with 30% diluted bleach and used for microinjection following standard P-element transformation protocols (Spradling and Rubin, 1982). This strain carrying the donor transgene was initially balanced over TM3, Sb.

3. Genomic DNA extraction

Genomic DNA was extracted from pools of 30-50 adult males following standard protocols (Ballinger and Benzer, 1989) unless noted otherwise.

4. Creation of a positive plasmid control

For the positive control in the PCR based DNA screen, a plasmid was made by subcloning the vector used to make

the transgenic line into the 9 kb genomic fragment isolated from the BAC library. This control is capable of producing a positive PCR band with a forward primer outside the 5' arm of homology and a GAL4 specific antisense primer. The positive control plasmid is used at 1 ng per reaction.

5. Southern blot analysis

Southern blots were performed according to standard protocols using adult male flies. Briefly, 10 micrograms of genomic DNA was digested with restriction enzymes listed in the results section, separated by gel electrophoresis, and transferred to a nylon membrane.

6. RT-PCR analysis

Transcripts analyzed by RT-PCR were extracted from males and females of indicated genotypes. Total RNA was extracted with TRIzol (Invitrogen), phenol/chloroform extracted, and ethanol precipitated. The RNA was then measured for concentration and finally used for first strand cDNA synthesis. This cDNA was used in a low cycle PCR, blotted to a nylon membrane and detected with a product specific radioprobe.

Results

1. Construct design for ends-out gene targeting

To obtain a stable mutation in the *dmrt93B* gene and determine the spatial expression pattern of the gene, I used ends-out gene targeting to introduce the yeast GAL4 transcriptional activator after the *dmrt93B* start codon. To minimize disruption of regulatory elements that might affect expression of neighboring genes *rudimentary-like* and CG7056, I chose a gene insertion mutagenesis (knock-in) strategy over gene deletion. The targeting construct was designed to insert the yeast transcriptional activator GAL4 in-frame after the translational start codon of *dmrt93B* (figure 2-1). This inserted sequence includes the GAL4 stop codon as well as a SV40 polyadenylation signal in the 3'UTR.

Even though the design of this construct should reduce the expression of the gene through transcription termination and a disruption of the translation reading frame, read-through transcripts might be produced. To ensure that these do not have the ability to produce functional protein, I also introduced point mutations into the DM DNA binding domain sequences in exon 2 of *dmrt93B* to

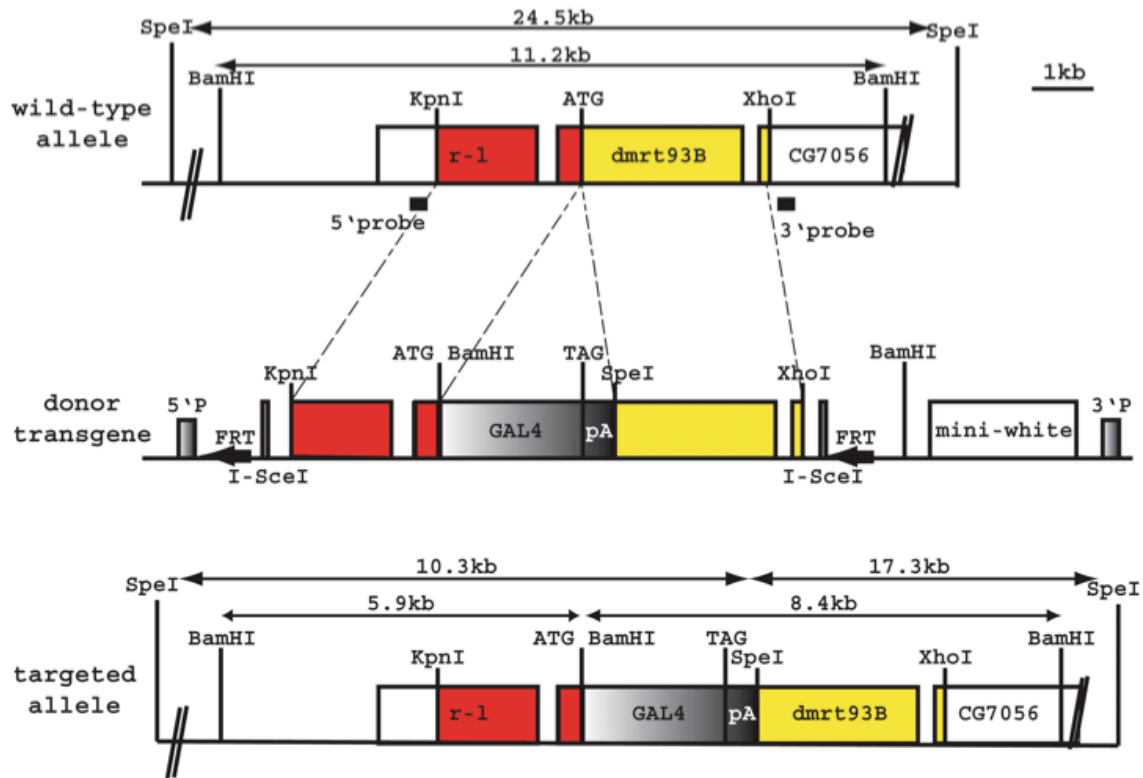


Figure 2-1: Gene targeting of *dmrt93B*. The *GAL4* knock-in is expected to eliminate *dmrt93B* function as it introduces a cleavage/polyadenylation (pA) signal upstream of the DM domain as well as a nonsense and several missense point mutations in the critical residues of the DM domain. The latter mutations are intended to inactivate any protein products that derive from spurious read-through transcription. The red area indicates the 5' arm of homology and the yellow area indicates the 3' arm of homology used for targeting.

convert conserved cysteines and histidines to tyrosine, resembling the null doublesex alleles *dsx*¹²⁸⁻¹ and *dsx*^{SDM 68} (Erdman and Burtis, 1993).

Next, the targeting construct I designed was inserted into pP{WhiteOut2}, a P-element vector suitable for insertion into the genome (Radford et al., 2005). This "donor" transgene contains targeting elements that will later be used for excision and provides sequences for recombination with the endogenous locus. To generate transgenic flies, the *pdmrt93B*-GAL4-donor construct was microinjected into pre-cellular blastoderm embryos and randomly inserted into the genome via P-element transposition to establish a stable transgenic fly strain.

I microinjected a total of 327 embryos with the targeting construct and recovered 68 viable larvae. Of these 68 individuals with potential P-element insertions, only one line was able to express the visible eye color marker indicating germ-line transformation. The stable, randomly inserted *dmrt93B*-GAL4 donor transgene was genetically linked to the third chromosome based on inheritance of the eye color marker *mini-white*. Later sections of this chapter will further discuss this transgenic line, which was found to carry a combination of

two insertions on the third chromosome based on Southern blot analysis.

Males carrying the randomly inserted *dmrt93B*-GAL4 donor transgene (DT) were mated to females that carry heat-shock inducible site-specific recombinase, flippase (FLP) (Golic and Lindquist, 1989). The recombinase is activated early in development and excises a highly recombinogenic linear fragment of DNA from the original donor transgene insertion site to target the homologous third chromosome gene, *dmrt93B*.

When mobilization of the targeting elements occurs with this donor transgene, the visible eye color marker mini-*white* (w^{+mc}) is left behind at the original insertion site while the sequence flanked by FRT sites is excised and mobilized. Mobilization of the targeting elements creates genetic mosaicism in the animal that occurs throughout the individual and can be visualized with the change in eye-color due to alterations in expression of the mini-*white* gene. The clones of cells with the excised recombinogenic DNA fragment have red mottled eyes over a background of the original color of the transgenic line.

Note that the endogenous gene is located on chromosome 3R; therefore, it would have been preferable to have an insertion on the first or second chromosome. An insertion

on a different chromosome would remove any confusion of the randomly integrated transgene with the final targeted event. However, having the transgene on a different chromosome is not essential and since the transformation efficiency of this construct was unusually low, we continued using these strains. Indeed, I was able to successfully use this transgene to produce targeted knock-in events as described in section 4 of this chapter.

2. GFP-based screen for gene targeting

The first strategy I used to attempt the identification of targeted events was a GFP-based screen utilizing the UAS-GAL4 system (Manoli et al., 2005). Males carrying the UAS-GFP transgene were crossed to “mottled-eyed” mosaic females that have excised the donor construct. The change in eye color, as seen as patches on red on an orange background, led to the assumption that the highly recombinogenic DNA fragment has excised from the original insertion site and left behind the mini-*white* eye-color marker. These mosaic females potentially carry germ-line targeted knock-in alleles of the *dmrt93B*-GAL4 construct. The progeny from this cross were screened during the third larval instar of development for GFP expression driven by

the GAL4 insertion. In previous experiments we had established that the GAL4 sequences in the donor transgene itself did not drive any significant expression of UAS-GFP, presumably because the transgene lacks sufficient promoter sequences. Based on previous ends-out targeting studies by others, one in two hundred mosaic females are expected to have a germ-line targeted event (Manoli et al., 2005).

After screening the progeny from over one thousand mosaic females for GFP expression, six independent strains were isolated. Strong GFP expression was seen in the central nervous system (CNS) in each of these lines. None of these lines, or any other lines observed in this screen, displayed GFP expression in the gonad. Southern blot analysis of DNA from these flies with external probes diagnostic of the expected knock-in mutation failed to indicate the presence of a correctly targeted allele. Thus, despite the expression of GFP, these strains did not target GAL4 to the endogenous *dmrt93B* gene.

Additional Southern blot analysis revealed that the original transgenic line carried two separate insertions of the donor transgene on the third chromosome and suggested that rearrangements of these transgenes were present in the GFP expressing lines. Based on these results I concluded that GFP expression most likely results from aberrant

recombination events between the two transgenic insertions (possibly between the FRT sites) that allowed GFP expression without a correctly targeted event.

3. Separation of two donor alleles by meiotic recombination

To alleviate the misleading rearrangement events described above, the two randomly inserted transgenes on the third chromosome were separated from each other by meiotic recombination. Females heterozygous for the chromosome bearing the two *dmrt93B*-GAL4-donor transgenes and a wild-type third chromosome were crossed to *white* mutant balancer males to recover recombinant chromosomes in which only one transgene was present. Lines were established from all balancer male progeny from this cross by individual matings regardless of their eye color. Progeny from these lines were analyzed for the presence of each transgene by Southern blotting (figure 2-2). One donor transgene (*dmrt93B*-DT 1-B) produced pale orange eyes indicating it carries a mini-*white* gene as expected. Interestingly, the strains isolated bearing DNA from the other insertion had bleach white eyes (*dmrt93B*-DT 1-1). Although the phenotype suggests a defect in the mini-*white* gene, blot and PCR analysis failed to identify any defect

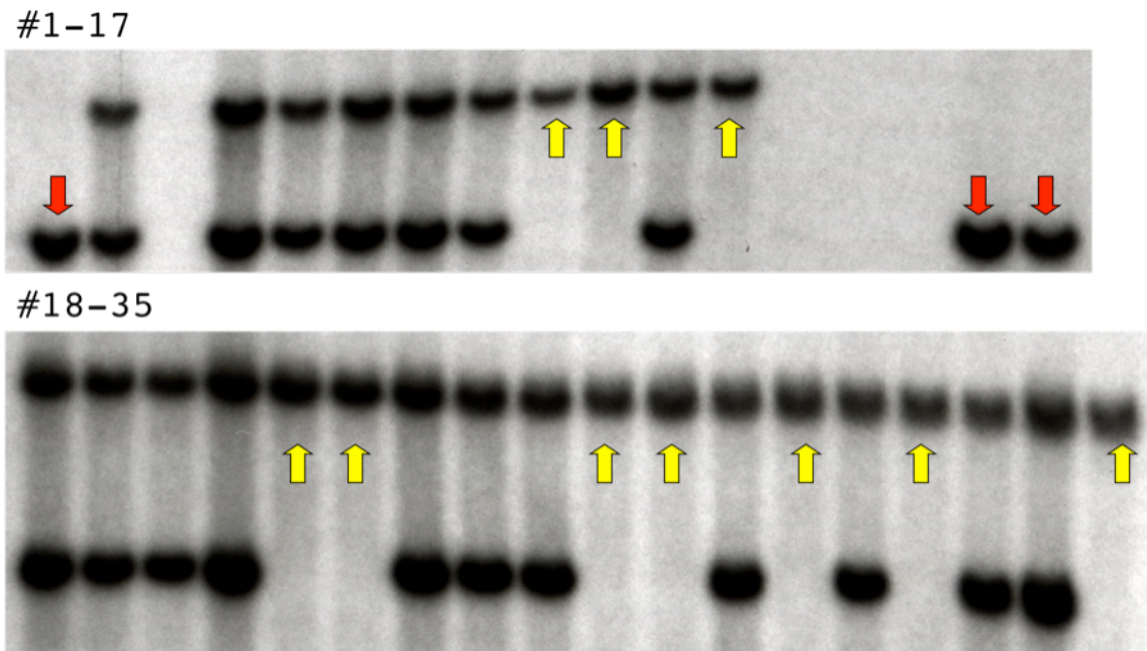


Figure 2-2: Southern blot to confirm donor transgenes separated by meiotic recombination. Using a GAL4 specific probe, genomic DNA was tested from 35 isolated strains. Three lines with a single insertion of the donor construct were found to have white colored-eyes (red arrows) and ten lines with a single insertion were found to have peach colored-eyes (yellow arrows).

in this marker gene. This suggests that the *dmrt93B*-DT 1-1 transgene insertion may have occurred in a genomic location that silences expression mini-*white*.

To verify that each transgene construct contained FRT and I-SceI sites required for mobilization, I amplified these regions by PCR from genomic DNA and sequenced them directly using an internal primer. Both of the isolated alleles contained the expected sequences required for mobilization. However, the *dmrt93B*-DT 1-B strain, which had colored eyes, had approximately 40 bp of transposed P-element sequence inserted between the 3' FRT site and the mini-*white* eye color marker. Because the altered region of *dmrt93B*-DT 1-B I outside of the FRT sites for recombination, the mutation is not expected to affect mobilization. When both donor strains were crossed separately to a constitutively active FLP recombinase strain, which can recombine the FRT sites, they were able to excise the loop of targeting DNA based on PCR analysis (figure 2-3). This indicated both strains had the ability to produce recombinogenic DNA for targeting the endogenous *dmrt93B* gene.

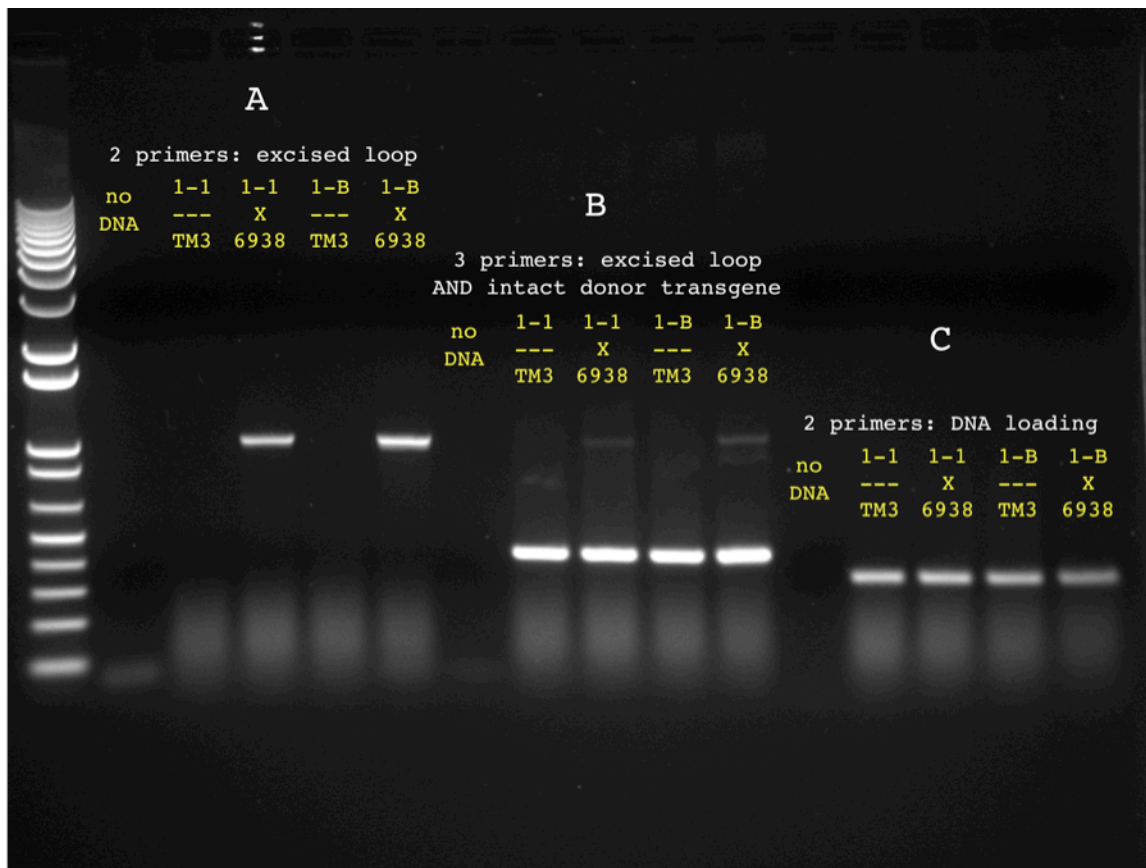


Figure 2-3: PCR to confirm the two separated donor transgenes are able to excise the targeting construct. The progeny used in this PCR result from a cross of the two separate donor transgenes 1-1 and 1-B to the constitutively active FLP recombinase strain w^{1118} ; P{70FLP}10 (Bloomington stock #6938) (A) Primers used test for an excised loop of DNA from the donor construct. (B) Three primers are included that test for two products, the excised band and the original insertion site (400bp). (C) DNA loading control.

4. Identification of targeted events using a DNA-based screen

Several factors led me to revise my screening strategy to one in which targeting events were detected directly at the DNA level. First, we found that the specific expression of GFP in the terminal epithelium of the testis (the anticipated tissue) would be difficult to detect in a screen based on trial experiments with other terminal epithelium-specific GAL4 drivers (T80 and c311). Although previous RT-PCR analysis (McGuffin and Mattox, unpublished) suggested the possibility of *dmrt93B* expression in the adult head, neither the pattern of expression nor level was known. Our previous attempt to use GFP as a marker suggested that even if a strong signal were produced in the CNS, this expression would not be a certain indication of correct targeting.

To facilitate detection of targeted events, I used a PCR based screen of pools of flies from targeting crosses. I used a sense primer outside the 5' arm of homology and an antisense primer inside GAL4, which would be unique to events with correct 5' targeting. A positive plasmid control was generated to confirm PCR conditions for this large-scale screen. By titration I determined that a control DNA was detectable at 10 fg, which is the level

needed to detect a single fly carrying a targeted event in a pool of DNA from 50 flies (figure 2-4).

In further experiments with genomic DNA from pooled flies, I found that the sensitivity of the PCR-based strategy is strong enough to find one transgenic fly in 99 wild-type flies by PCR, which agrees with previously published data (Ballinger and Benzer, 1989). Given this level of sensitivity, I elected to use pools of 20-30 flies to ensure sufficient signal strength so that detection of targeted lines on agarose gels was not missed (figure 2-5). An additional logistical advantage of using the smaller pools is that once a positive event is identified by PCR in a pool, the probability of recovering individual flies carrying the targeted event is improved.

Briefly, the pooling process involved the maintenance of flies with potentially germ-line targeted events in assigned vials. After several days of egg lay, genomic DNA is extracted from these adults and screened by PCR. When a positive event was identified from these assigned pools, all progeny from that vial were individually mated to a balancer strain to recover lines bearing the germ-line targeted event (figure 2-6).

As previously mentioned, this PCR screen is expected to identify correct 5' targeting from a pool of flies.

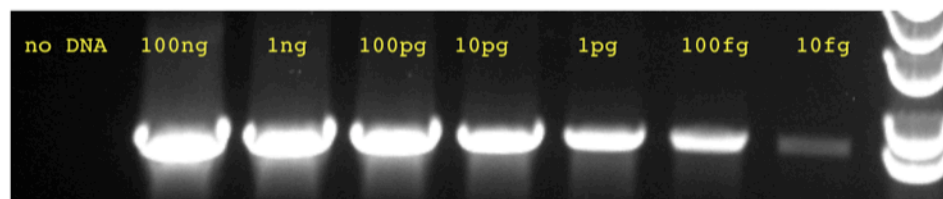


Figure 2-4: PCR amplification of positive control plasmid DNA. After the generation of the positive plasmid control for the DNA based screen, serial dilutions were used to determine that positive fragments are still detectable when 10fg of template DNA is used.

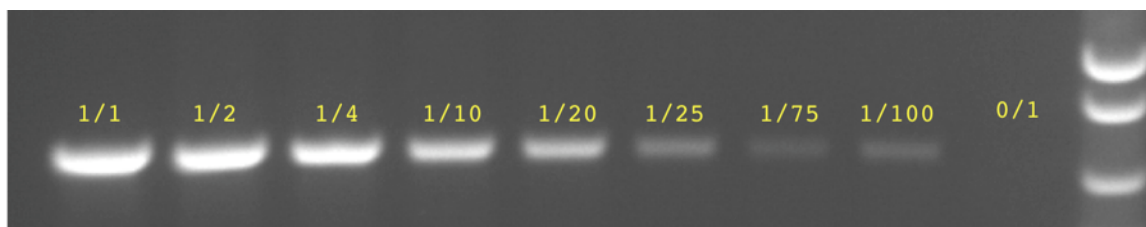


Figure 2-5: PCR amplification of genomic DNA diluted in a pool of flies. To test the optimal size for the DNA pools for the targeted event, several dilutions of the donor transgenic line were tested.

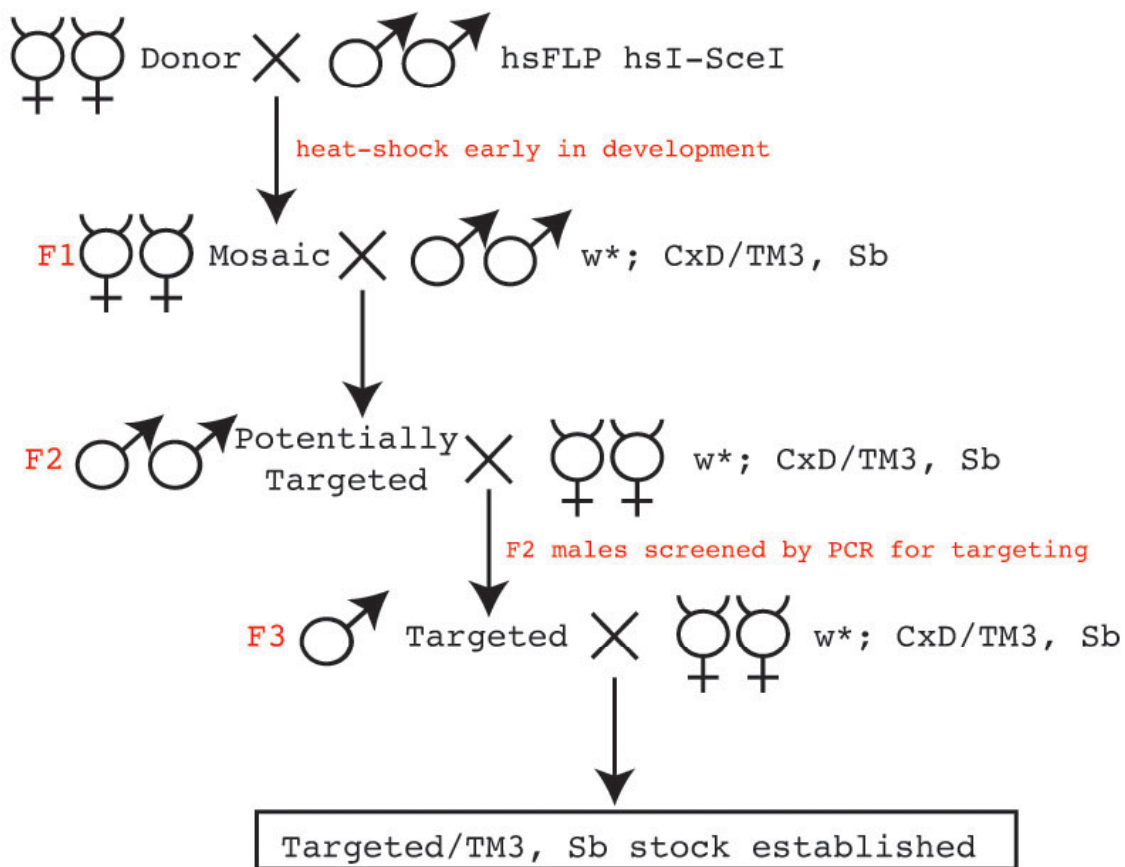


Figure 2-6: Mating scheme for gene targeting. To generate stable null mutations in *dmrt93B*, a donor GAL4 knock-in targeting vector was used to generate transgenic fly strains. These strains were then used to target mutations into *dmrt93B* by homologous recombination *in vivo*.

Eight independent targeting events were identified by PCR, but only five of these lines were reestablished out of the pooled flies (table 2-1). All five of these lines had correct 5' targeting as shown by Southern blot. However, only three of these five events were also correctly targeted in the 3' end as shown by Southern blot analysis (figure 2-7).

5. Confirmation of the *dmrt93B*^{GAL4KI} allele by RT-PCR

Using Reverse Transcriptase PCR (RT-PCR) to analyze *dmrt93B* transcripts in mutant flies, total RNA was DNaseI-treated before use in first-strand cDNA synthesis. This cDNA was amplified using a sense primer in exon two and an antisense primer in exon three. The RT-PCR product was run on an agarose gel and blotted to a nylon membrane to enhance transcript signal. A significant reduction in *dmrt93B* transcript in both hemizygous and homozygous mutants is seen on the radioprobed blot (figure 2-8). Even though very low amounts of transcript are made, it is unlikely they are able to make functional proteins. As expected, double deficiency animals have no detectable *dmrt93B* transcript by RT-PCR indicating a null allele for the gene.

Donor Transgene	two DT inserts	DT 1-B	DT 1-1
# progeny screened	2,683	3,147	6,416
# expected event	3	4	8
# positive by PCR	0	0	8
expected events (1/832 progeny) based on Gong et al., 2003			

Table 2-1: Recovery of targeted lines from PCR screen. Before both transgenes were isolated from each other, no positive events were recovered from the PCR based DNA screen. After the random transgenic insertions were separated from each other, only DT 1-1 (white-eyed) was able to generate correct targeting events at expected rates.

Figure 2-7: Southern blot confirming targeting.

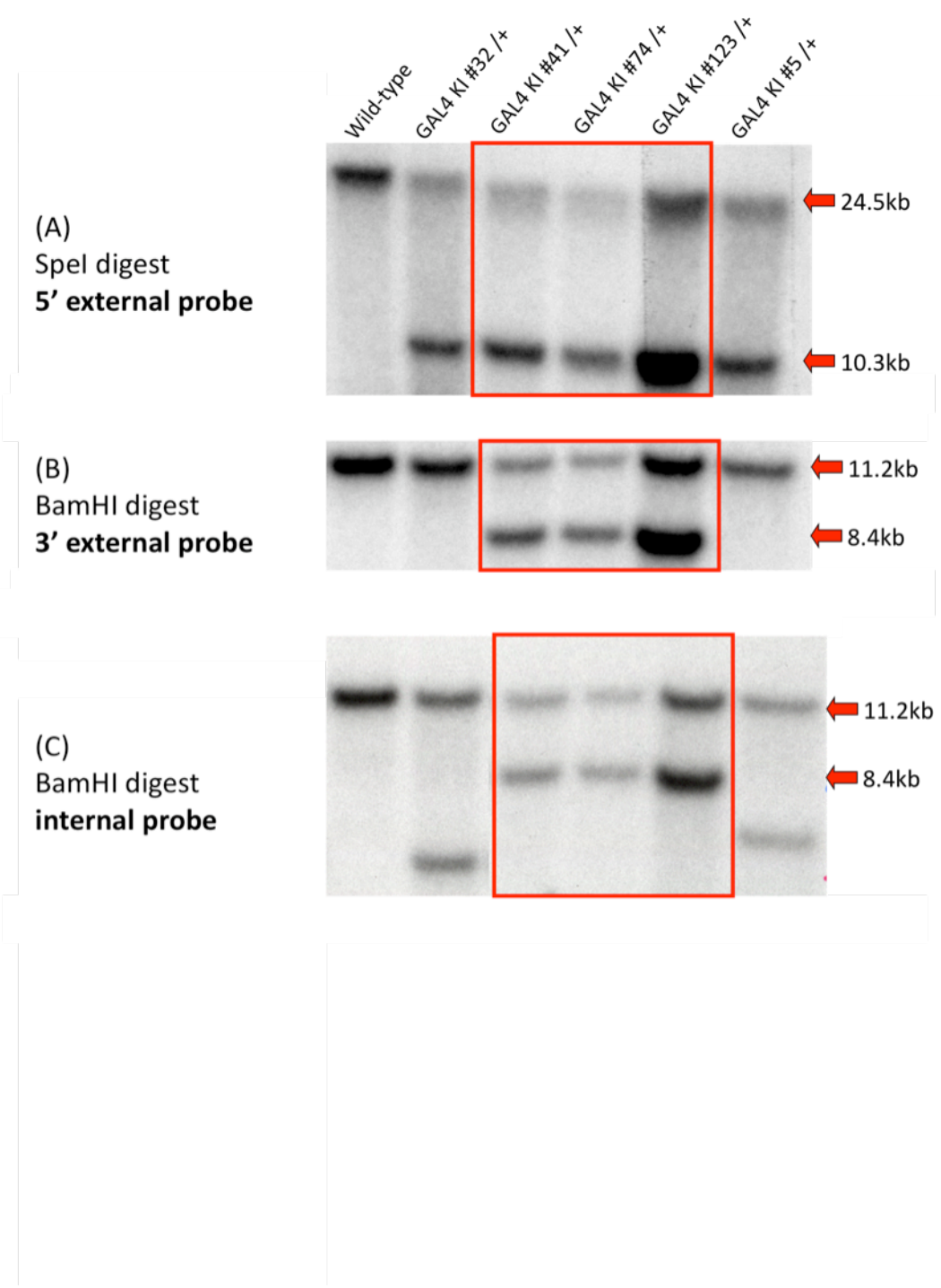


Figure 2-7: Southern blot confirming targeting. (A) The targeted event was identified for correct 5' targeting by polymerase chain reaction (PCR) and confirmed by Southern blot analysis using an external 5' probe on SpeI digested genomic DNA. The wild-type (WT) band is 24.5 kb and the targeted (KI) band is 10.3 kb. (B) Using an external 3' probe on BamHI digested genomic DNA, only three out of the five events have correctly targeted bands (highlighted by the red box). The wild-type band is 11.2 kb and the targeted band is 8.4 kb. (C) With an internal *dmrt93B* probe, the same three independent events have correct targeting.

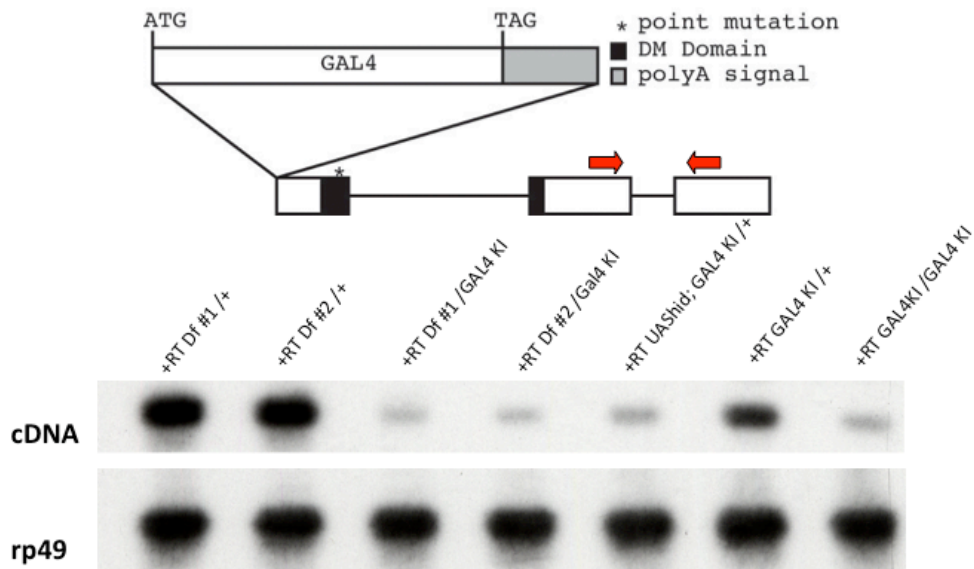


Figure 2-8: Radio-probed blot of RT-PCR. Total RNA was extracted from whole adult males with TRIzol and used for first strand cDNA synthesis in this RT-PCR using primers in exon 2 and 3 (shown as red arrows). The *dmrt93B*^{GAL4KI} allele specifically reduces transcripts of *dmrt93B* in homozygous and hemizygous mutants and also has a similar reduction of transcript in *hid* ablated heterozygotes. *rp49* is included as a loading control.

Discussion

The generation of transgenic lines by random P-element integration relies on visible eye color markers for recovery. In the case of this particular gene insertion, it was difficult to isolate a transgenic line with the P-element transformation. Typical transformation efficiency ranges from 20-50% (Rubin and Spradling, 1982). For this construct the successful transformation rate was 1.47%. Additionally, two rounds of injections performed by a professional service resulted in no transformants identifiable by the eye-color marker.

Previous experiments by others have shown that the expression of the mini-*white* transgene for eye color can depend on the P-element insertion site (Hazelrigg et al., 1984; Horn et al., 2000). The failure to express the mini-*white* gene by the *dmrt93B*-DT 1-1 suggests the random insertion occurred in a region of heterochromatic DNA that suppressed the mini-*white* expression. Data from the FRT recombination experiment, in which expression from the mini-*white* gene is seen as bright red eyes in the individuals that excised the inserted *dmrt93B* sequence, supports the idea that insertion site may have prevented mini-*white* expression.

An improvement to identifying donor transgenic lines could include the use of a fluorescent eye marker for transformation rather than using the mini-white eye color marker. This would address the issue of recovering donor transgenic lines by eye color. Alternatively, targeted insertion of a donor construct into a known site in the genome can be accomplished using the phiC31 site-specific integrase to avoid multiple copies of the transgene (Groth et al., 2004). Knowing the insertion site would also improve the recovery of the donor transgenic line.

Recently improved approaches to ends-out gene targeting have been developed. These include variations in the targeting strategy to promote recombination at the endogenous site. One example of these techniques is targeting one chromosome arm by using a Balancer chromosome that will not undergo recombination (Chen et al., 2009). Additionally, a technique referred to as "genomic engineering", which combines the ends-out gene targeting with the phiC31 integrase system, has been developed to repeatedly mutate a particular gene of interest. This is accomplished by first introducing an attP site to the endogenous gene and then using this founder line to introduce new genetic alterations with a construct containing an attB site (Huang et al., 2009).

In this chapter I used two different techniques to identify a targeted event for the GAL4 knock-in. The first approach was to rely on the UAS-GAL4 system and screen for events by GFP expression (Manoli et al., 2005). When the GFP-based screen revealed no testis expression in potentially targeted lines, I relied on the expression that was seen in the CNS. However, the exact neuronal expression pattern of *dmrt93B* was not known at that time. Not knowing the cellular expression of a gene is a common issue for a reverse genetics approach where the role of the gene is being characterized. However, genomic analysis of the GFP expressing strains revealed that a true targeting event had not occurred and may have been due to two different insertion sites of the transgene recombining with each other. Unexpected targeting events have been previously reported in attempts at gene targeting (Seum et al., 2002). In some cases, mobilization and rearrangements occur but correctly targeted events are never recovered (Funk et al., 2004).

The successful identification of the *dmrt93B*^{GAL4KI} allele was through the DNA-based PCR screen. The PCR screen was for correct 5' targeting, which was confirmed by Southern blot in all recovered events. However, Southern blot analysis of the 3' end revealed all potential events

did not target this end correctly. This result stresses the importance of checking both the 5' and the 3' ends of gene targeting strategies. The successful isolation of the targeted event by the DNA-based screen also promotes this method of identification over the use of UAS linked fluorescent markers for the confirmation of the GAL4 knock-in alleles (Manoli et al., 2005; Robinett et al., 2010).

GAL4 knock-in alleles have been successfully generated by homologous recombination *in vivo* for several genes including *fruitless*, *doublesex*, and the microRNA mir-278 (Manoli et al., 2005; Teleman and Cohen, 2006; Rideout et al., 2010; Robinett et al., 2010). The overall goal of our targeted GAL4 knock-in allele was to create a mutant allele of *dmrt93B* while minimizing disruption to nearby enhancer elements so that it can also be used as a GAL4 driver. A unique feature of our donor construct is that several point mutations were introduced into the conserved DNA binding domain of the gene. The point mutations were designed after known null alleles at conserved positions in the gene *doublesex*. Since the PCR analysis revealed the presence of read-through transcripts at low levels, the point mutations that introduced a stop codon are useful for eliminating possible translation products.

Chapter 3: Analysis of the *dmrt93B*^{GAL4KI} allele

Introduction

In this chapter I will present data on the spatial expression pattern of the *dmrt93B* transcripts and its function in both the gonads and other tissues. Previous tissue specific RT-PCR analysis in our lab has shown *dmrt93B* expression in the head of both male and female flies and male-specific expression in the reproductive system. The UAS-GAL4 binary transcriptional activation system is a powerful tool in *Drosophila* genetics (Duffy, 2002). Using this system, the *dmrt93B*^{GAL4KI} knock-in allele is able to drive various UAS reporter genes in the endogenous expression pattern of *dmrt93B*.

As mentioned in chapter 1, *dmrt* genes have been found to perform non-sex specific functions. However, RNA *in situ* analysis for the specific expression pattern of *dmrt93B* in the organism was not examined beyond the reproductive tissue. Here, I used a fluorescent UAS reporter gene in combination with the *dmrt93B*^{GAL4KI} allele to determine the gene expression at cellular resolution in the central nervous system (CNS). Specific expression is seen in the suboesophageal ganglion, a region of the brain

associated with feeding behavior. After determining the tissues expressing *dmrt93B*, I analyzed the phenotypes of mutant individuals with reduced function using the knock-in allele.

There are several UAS transgenes currently available for the *Drosophila* model organism. The two types used in this chapter are fluorescent reporters, such as UAS-Green Fluorescent Protein (GFP) and UAS-Red Fluorescent Protein (RFP); and activators of the apoptotic pathway, such as UAS-hid. With the fluorescent reporters I characterized the expression pattern of *dmrt93B* for both heterozygous wild-type and hemizygous mutant brains. Additionally, flies with the *dmrt93B*^{GAL4KI} allele were used in the Capillary Feeder (CAFÉ) assay to measure feeding behavior, revealing a dominant increase in food intake.

Materials and Methods

This chapter involves procedures used to determine spatial expression of *dmrt93B* and behavior assays to measure viability, fertility and feeding. Important methods used to examine the *dmrt93B*^{GAL4KI} knock-in allele are detailed below.

1. Isogenized lethal-free strain BDGP3

A lethal-free third chromosome from the same strain used to sequence the *Drosophila* genome (BDGP) was introduced into a w^{1118} background. I did this because *cinnabar* (*cn*) and *brown* (*bw*) on the second chromosome of the BDGP strain affect the biosynthetic pathways for eye-color. The combination of these second chromosome mutations results in white-eyed adults, a phenotype that is identical to *white* mutants (i.e. w^{1118}). Thus, the presence of these mutations would prevent us from following the presence of transgenes bearing the eye color marker *mini-w+*. The genetic crosses used to isogenize the strain are described below.

Briefly, w^{1118} ; CxD/ CyO; Sb/ TM3, Ser females were crossed to BDGP males. Male progeny with the following genotype were selected: w^{1118} ; *cn bw sp*/ CyO; +/ TM3, Ser. These males were then individually crossed to w^{1118} ; Roi/ CyO; Sb/ TM3, Ser females and siblings with the following genotype: w^{1118} ; Roi/ CyO; +/ TM3, Ser. Later, a stable stock referred to as BDGP3 was established that eliminated the balancer chromosomes.

2. Meiotic recombination to remove exogenous mutations

To remove any exogenous mutations created in the targeting step (O'Keefe et al., 2007), I used meiotic recombination to randomly replace the genome of the targeted allele with that of the BDGP3 allele while selecting for the targeted knock-in by PCR. Since meiotic recombination occurs only in the female germ-line, single female progeny are serially backcrossed at each generation to males with the desired background. The *dmrt93B*^{GAL4KI} allele was backcrossed six generations to the isogenized BDGP3 strain to remove exogenous mutations that might have been created in the generation of the targeting event. Flies bearing the targeted allele were identified by PCR of single fly genomic DNA at each generation. The final line is referred to as G6KI and has the genotype: w¹¹¹⁸; +; *dmrt93B*^{GAL4KI}.

3. Immunohistochemistry

Tissues were dissected in cold PBS and fixed in 4% Paraformaldehyde (PFA)/ PBS before immunostaining and mounting in 80% glycerol/ PBS. Generally primary antibodies were initially tested with a 1:200 dilution and secondary antibodies with a 1:2,000 dilution. Antibodies used in this chapter are listed with dilutions used,

source, and product number where applicable. All primary antibodies from Developmental Studies Hybridoma Bank (DSHB) were from concentrate. Primary antibodies include: rabbit anti-GFP (1:800; Abcam; ab6556); mouse mAb 22c10 (1:100; DSHB); mouse mAb nc82 (1:400; DSHB); mouse anti-fasciclin III (1:200; DSHB; 7G10-c). Additionally, Alexa Fluor 568 phalloidin (1:50; Invitrogen; A12380) was used to stain F-actin. Secondary antibodies include: Alexa Fluor 488 goat anti-mouse IgG (1:200; Invitrogen; A11029); Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen; A11034); Alexa Fluor 546 goat anti-mouse IgG (1:200; Invitrogen; A11030). Normal Goat Serum (NGS) was used to block non-specific binding (1:50; Sigma; G9023).

4. Establishing GFP/RFP double reporter

A double fluorescent reporter was established by meiotic recombination of w^* ; P{UAS-2xEGFP}AH2 (Bloomington stock number 6874) and w^{1118} ; P{UAS-RedStinger}4/CyO (Bloomington stock number 8546). These respectively have the transgenic insertions for GFP and a nuclear RFP on chromosome 2. A recombinant chromosome bearing both of the reporters was identified by crossing to a GAL4 driver on the third chromosome. A stable line bearing the recombined

second chromosome was established over the fused balancer chromosomes T(2;3)SM6a-TM6B, Tb[1].

5. CAFÉ assay

Animals for this experiment are raised at 25C on a 12-hour light/ dark cycle. Individuals are picked for each genotype and set up in the assay apparatus on day 2 after eclosion and given one day to adjust to the new feeding environment. Measurements are collected on day 4 through day 7. As eye color can affect vision and behavior, the genotypes of the animals in this experiment all include the UAS-2XeGFP transgene, which carries mini-w⁺, to provide a uniform eye color for all genotypes scored.

Results

Preliminary data from tissue specific RT-PCR analysis and RNA *in situ* hybridization indicate expression of the gene in the testes. Surprisingly, I found that this particular GAL4 knock-in allele was not able to drive the expression of reporter genes in the male reproductive system and did not have a significant effect on fertility. However, I found this gene to be expressed in regions of the brain and digestive system indicating a possible role

in feeding behavior. In collaboration with the lab of Gregg Roman at the University of Houston, I was able to determine that mutating this gene results in an over-eating phenotype.

1. Analysis of the *dmrt93B*^{GAL4KI} allele reveals late pupal lethality.

One of the initial objectives in characterizing the *dmrt93B*^{GAL4KI} allele was to determine if the homozygous mutants were viable. Preliminary data indicated that both male and female homozygous adult mutants survive, but at a reduced frequency compared to wild-type siblings (figure 3-1). These wild-type individuals were heterozygous *dmrt93B*^{GAL4KI} allele over the TM3 balancer. Flies carrying balancer chromosomes are usually not completely viable as they include multiple rearrangements, so mutant individuals having reduced viability compared to these heterozygous balancer lines emphasizes this phenotype. Since a reduction in viability was seen in adults, the next goal was to determine the stage in development that this lethality initially occurs.

To determine the developmental stage of lethality, I measured survival rates at several stages of development

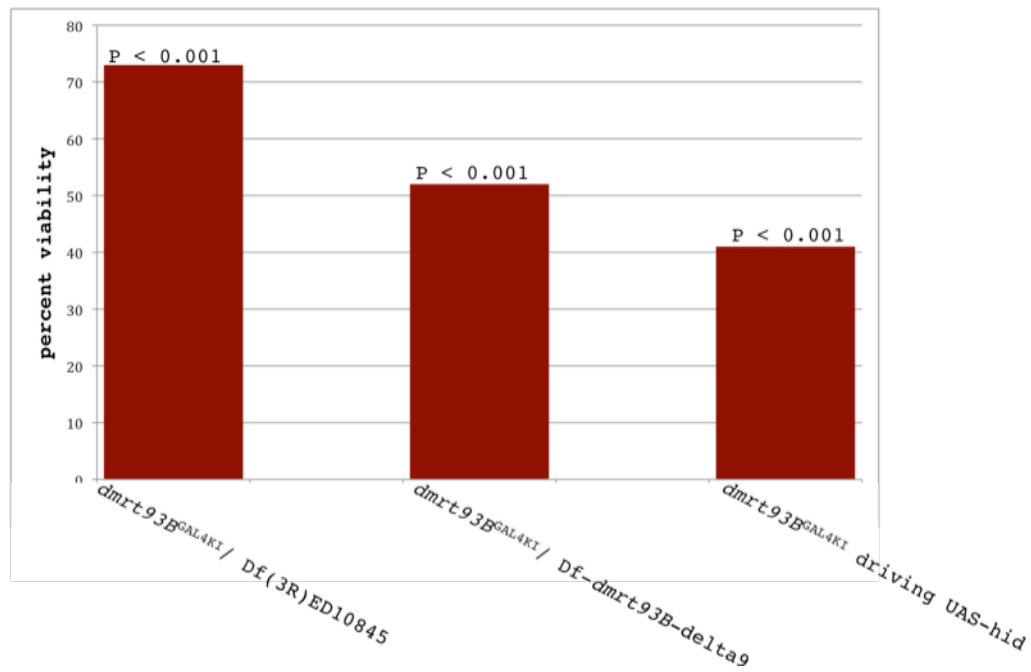


Figure 3-1: The *dmrt93B^{GAL4KI}* mutants have reduced adult viability. Percent viability calculated by dividing the number of observed individuals by the expected number based on the number of control sibling individuals. P-values shown are based on chi-squared analysis.

spaced 1-2 days apart. The life cycle of the *Drosophila* proceeds essentially as 1 day an egg/ embryo, 1 day as a first instar larvae, 1 day as a second instar larvae, 1 day as a third instar larvae, 1 day as a wandering third instar larvae, 5 days as a pupae, and then ecloses as an adult. The time points checked were divided by days and scored for number of GFP-positive individuals. Here, balancer siblings are GFP-positive and non-GFP individuals are homozygous mutant (*dmrt93B^{GAL4KI}* / *dmrt93B^{GAL4KI}*) or hemizygous mutant (*dmrt93B^{GAL4KI}* / Df-*dmrt93B*-delta9).

Based on these parameters, I determined that late pupal lethality occurs in both homozygotes (figure 3-2) and hemizygotes (figure 3-3) for the *dmrt93B^{GAL4KI}* allele. These individuals survive at approximately 30-40 percent compared to wild-type. This indicates the allele negatively affects viability, but is not essential for survival.

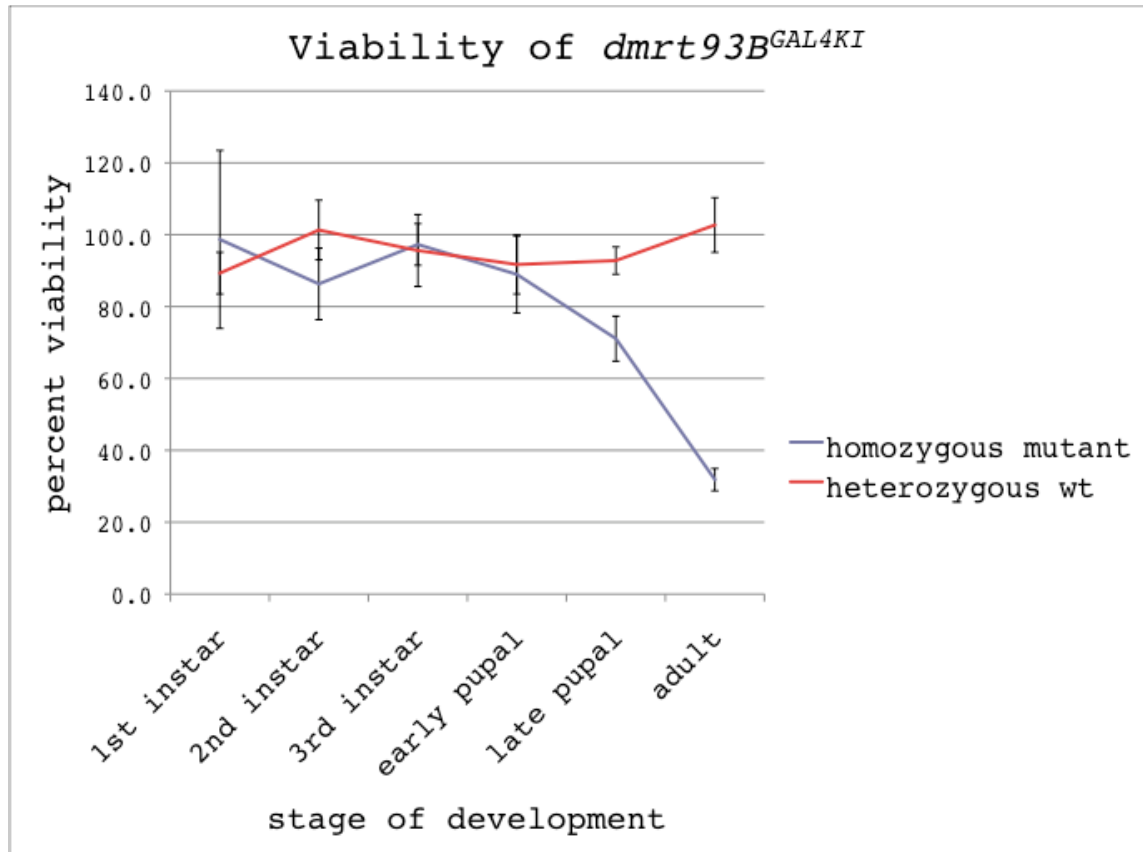


Figure 3-2: The *dmrt93B^{GAL4KI}* homozygotes have late pupal lethality. Percent viability calculated by dividing the number of observed individuals by the expected number based on the number of sibling individuals. Blue line represents the mutant homozygous class *dmrt93B^{GAL4KI} / dmrt93B^{GAL4KI}*. Red line shows the viability of heterozygous wild-type flies. Error bar represents one standard error of the mean (sem).

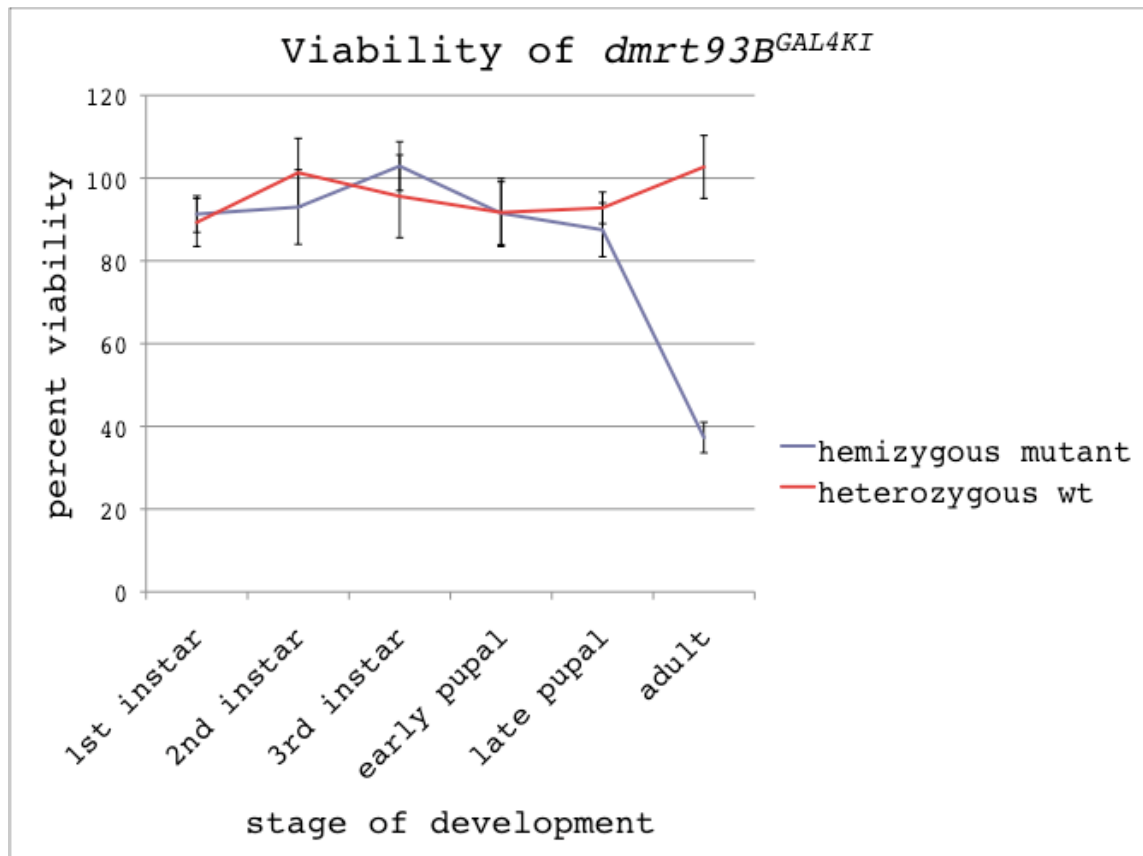


Figure 3-3: The *dmrt93B^{GAL4KI}* hemizygous mutants have late pupal lethality. Percent viability calculated by dividing the number of observed individuals by the expected number based on the number of sibling individuals. Blue line represents the mutant hemizyotes *dmrt93B^{GAL4KI} /Df-dmrt93B-delta9*. Red line shows the viability of heterozygous wild-type flies. Error bar represents one standard error of the mean (sem).

2. Analysis of fertility in *dmrt93B*^{GAL4KI} mutants.

Previous RT-PCR analysis and RNAi knockdown experiments indicate that *dmrt93B* is highly specific to the testis and is essential for its development. To determine if this stable loss-of-function mutation disrupts either gonad morphogenesis or function, I chose to test the fertility of the *dmrt93B*^{GAL4KI} individuals. Individually mated males were crossed to 3 wild-type females for a sample size of 20 males for each genotype. The same was done for individually mated females crossed to 3 wild-type males. This fertility assay compared the wild-type BDPG3 isogenized strain (control) to the hemizygous mutant strain that had one copy of the *dmrt93B*^{GAL4KI} allele over a *dmrt93B* deficiency (Df-*dmrt93B*-delta9). Surprisingly, I found no significant reduction in male fertility (table 3-1). The classifications for fertility was broken down into three classes: sterile if no progeny were seen, subfertile if 1 to 10 progeny were seen, and fertile if more than 10 progeny were seen.

The wild-type control males were 0 sterile, 2 subfertile, and 18 fertile. The hemizygous mutant over deficiency males were found to have 3 sterile, 2 subfertile, and 15 fertile. Interestingly, the double deficiency null male that deletes the entire *dmrt93B* gene

# progeny	0	1-10	>10
Classification	Sterile	Subfertile	Fertile
wild-type	0	2	18
<i>dmrt93B^{GAL4KI}/</i> <i>Df-dmrt93B-delta9</i>	3	2	15
<i>Df(3R)ED10845/</i> <i>Df-dmrt93B-delta9</i>	5	0	15

Table 3-1: Summary of fertility assay. Classification of fertility for twenty males from each genotype individually mated to three wild-type females.

as well as parts of the neighboring genes *rudimentary-like* and CG7056 was found to have 5 sterile, 0 subfertile, and 15 fertile. This result shows that the *dmrt93B* gene is not essential for male fertility as the RNAi knockdown results had earlier indicated. Instead, these results are more compatible with a minor effect of *dmrt93B*^{GAL4KI} loss-of-function on male fertility.

In addition to the above experiments, we also examined sterile mutant males using antibody markers to determine if there were defects in testicular morphology. In these experiments we used phalloidin to visualize the muscle cells that coat the testis. Of two sterile individuals examined, testicular morphology was disrupted in one of these sterile hemizygous mutant males (figure 3-4). This is shown by the inability of the testis to take on its typical coiled shape. This bulbous, rather than coiled, testis is reminiscent of the RNAi knock-down phenotype seen in a few of the escaper individuals previously described in chapter 1. A second sterile hemizygous mutant male did not have as severe of a phenotype. Additionally, a single sterile wild-type male from the control cross did not have obvious defects in morphogenesis. Fertile males from the above assay were not examined for testicular defects.

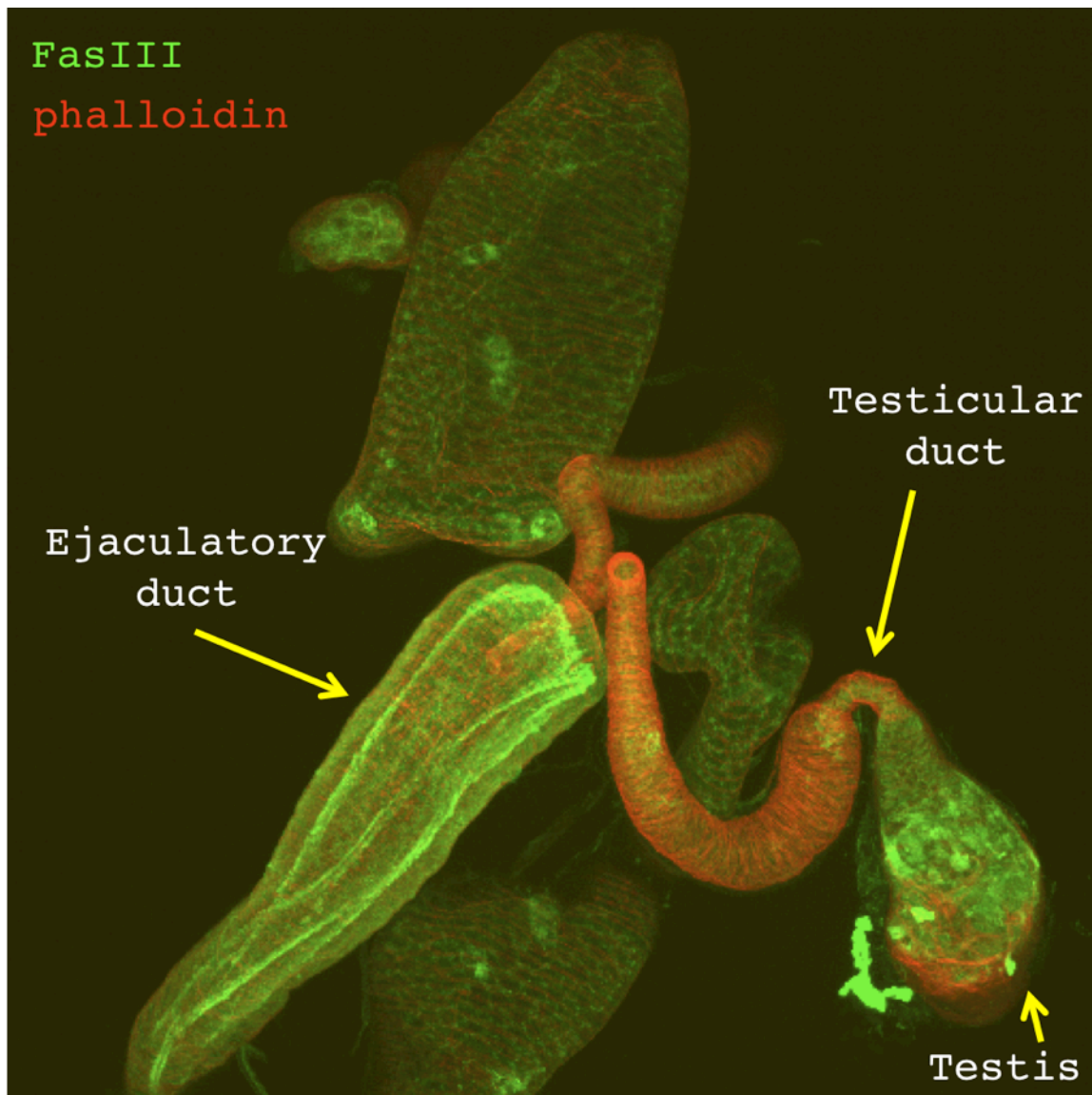


Figure 3-4: Testicular defect of a *dmrt93B*^{GAL4KI} hemizygous mutant. Only one of the sterile *dmrt93B*^{GAL4KI} /Df-*dmrt93B*-delta9 hemizygous mutants had visible defects in testis morphology. Phalloidin (red) is used to stain muscle cells. Fasciclin III (FasIII) is shown in green.

Since the testicular phenotype appears mild with this particular allele, we checked if any visible defects could be found in randomly selected adult males that are deficient for *dmrt93B* with the genotype $w^*; +; Df-dmrt93B-delta9 / Df(3R)ED10845$. In this blind experiment done in collaboration with Sam Su, 20 adult males were examined for characteristically coiled testes as an indication of normal testis morphogenesis. All males examined had normal testis shape indicating that the complete loss of *dmrt93B* had no effect on the development of the male reproductive system.

In the analysis of female fertility, the double deficiency null animals were completely sterile. This is not surprising as the *rudimentary-like* gene has previously been shown to be female sterile and this gene is affected by the deficiency. The hemizygous and homozygous *dmrt93B^{GAL4KI}* mutant females were found to be fertile. This result is consistent with previous observations that *dmrt93B* is not expressed in the female gonad. In summary, the *dmrt93B^{GAL4KI}* mutant only has minor defects in the development of the reproductive tissue and fertility.

3. The *dmrt93B* GAL4 driver forces UAS-GFP expression in the suboesophageal ganglion of the central brain.

Previous RT-PCR studies suggested *dmrt93B* might be expressed in several non-gonadal tissues, including the CNS. Since no specific antibodies were available for this factor, I took advantage of the GAL4 knock-in allele and the UAS based reporters to determine the tissue in which the promoter of *dmrt93B* is active. For this experiment, I crossed *dmrt93B*^{GAL4^{KI}} flies with a strain bearing the UAS-2X-eGFP reporter on chromosome 2. The progeny from this cross were observed as live larvae under a stereomicroscope. A distinct expression pattern of the UAS-GFP reporter can be seen in the anterior portion of the animal in both sexes. GFP-expressing structures can be seen in the central brain and the ventral ganglion as well as the proventricular ganglion. Additionally, third instar larvae express GFP in scattered cells within the midgut. During pupation these cells later appear to contract and change shape with a loss of signal within 24 hours of pupation.

To determine the specific tissues expressing the GFP reporter driven by the *dmrt93B*^{GAL4^{KI}} allele, I examined larval males and females by dissecting various organ systems intact (figure 3-5). The GFP signal was amplified in this analysis by staining with anti-GFP antibody. Since

Figure 3-5: Expression of *dmrt93B* in larvae.

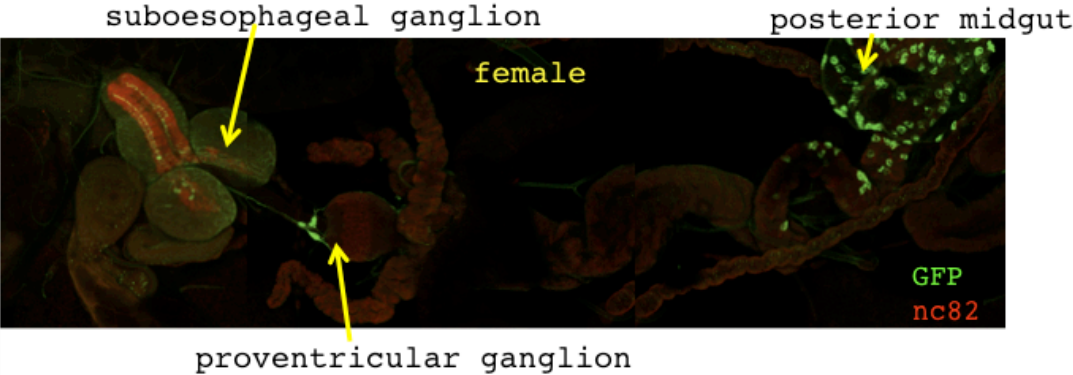
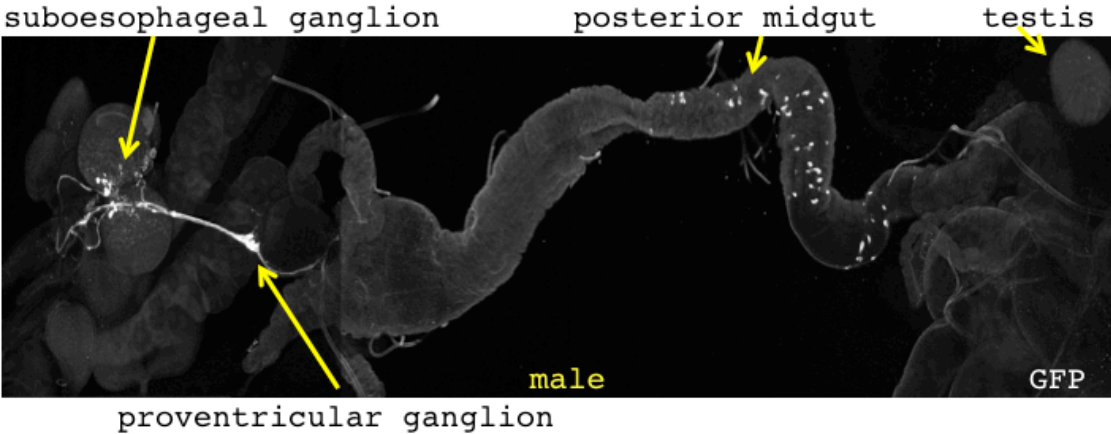


Figure 3-5: Expression of *dmrt93B* in larvae. 10X Z-stack image collected by confocal microscopy reveals tissues expressing reporter GFP enhanced with anti-GFP (shown in white for male and green for female). Both male and female larvae have similar expression patterns marking the suboesophageal ganglion, proventricular ganglion, and midgut. Reporter GFP is not seen in the testis although tissue specific RT-PCR indicates high levels of *dmrt93B* expression. Anti-nc82 (red) is used as a counterstain.

tissue specific RT-PCR analysis of *dmrt93B* transcripts indicated that the heads from adults of both sexes expressed the gene, I used the anti-nc82 antibody as a counter-stain to mark neuropil in the brain (figure 3-6).

The reporter GFP reveals a discrete expression pattern of *dmrt93B* in the suboesophageal ganglion, a tissue associated with feeding behavior (Mitchell, 1992). Interestingly, expression is also seen in the proventricular ganglion, which is located on the anterior portion of the *Drosophila* proventriculus. It is thought that the proventricular ganglion regulates the contraction of the proventriculus for the passage of food into the digestive tract from the esophagus. Previous reports of the proventricular ganglion indicate a branching pattern of the ganglia with neurons located at the most anterior portion.

To determine if the entire proventricular ganglion expressed *dmrt93B*, I used the anti-22c10 antibody to mark this tissue. This antibody was previously used to label cells in the ganglion and to demonstrate that there are on average 3-5 distinct nerves radiating from it. However, the incomplete overlap of the anti-22c10 staining and GFP reporter indicated *dmrt93B* is expressed only in a subset of

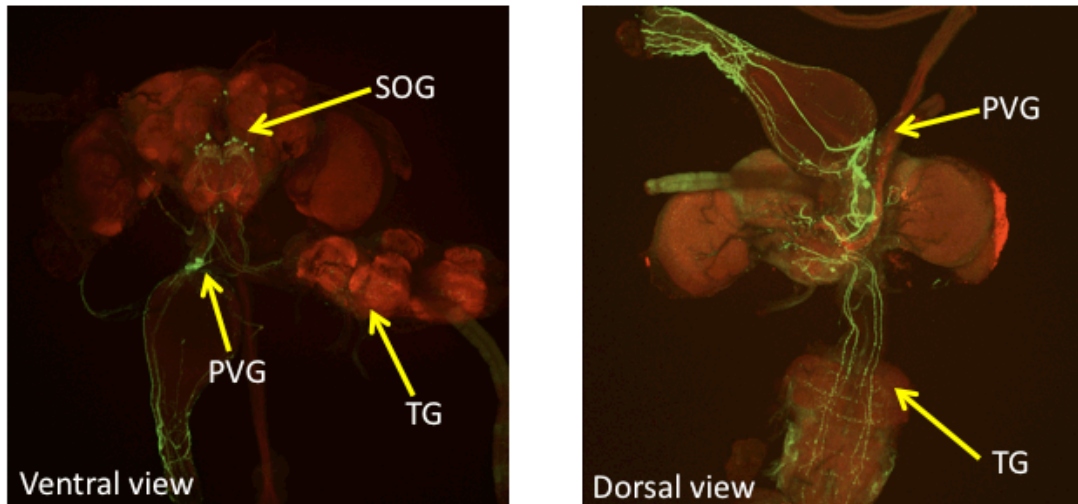


Figure 3-6: Expression of *dmrt93B* in adults. 10X Z-stack image collected by confocal microscopy of adult female tissue stained with anti-GFP in green for reporter expression and anti-nc82 in red as a neuropil counterstain. Both ventral and dorsal views are shown. Abbreviations: suboesophageal ganglion (SOG), proventricular ganglion (PVG), and thoracic ganglion (TG). Reporter GFP was not detected in adult gut.

the neurons associated with the proventriculus (figure 3-7).

To further characterize the proventricular ganglion expression, I ablated cells expressing *dmrt93B* and asked if any 22c10 positive neurons remain on the proventriculus. Males carrying the UAS-2X-eGFP transgene and *dmrt93B*^{GAL4KI} were crossed to females with the UAS-hid transgene and their progeny were stained with anti-GFP and anti-22c10. While GFP staining cells on the proventriculus were eliminated, a number of 22c10 positive neurons remained. This further confirms that *dmrt93B* is expressed within a subset of the proventricular ganglia.

4. Expression of the reporter GFP is altered in hemizygous mutant brains in comparison to heterozygous wild-type.

For this experiment I used a strain containing the second chromosome UAS-2X-eGFP enhanced GFP reporter (Bloomington stock #6874) and the third chromosome *dmrt93B*^{GAL4KI}. This combined UAS-GAL4 stock is used as a read-out of how the knock-in allele affects expression of the gene and maintenance of the cells in *dmrt93B*^{GAL4KI} mutants over deficiency. The deficiency Df-*dmrt93B*-delta9 used for this mutant analysis was established in our lab by

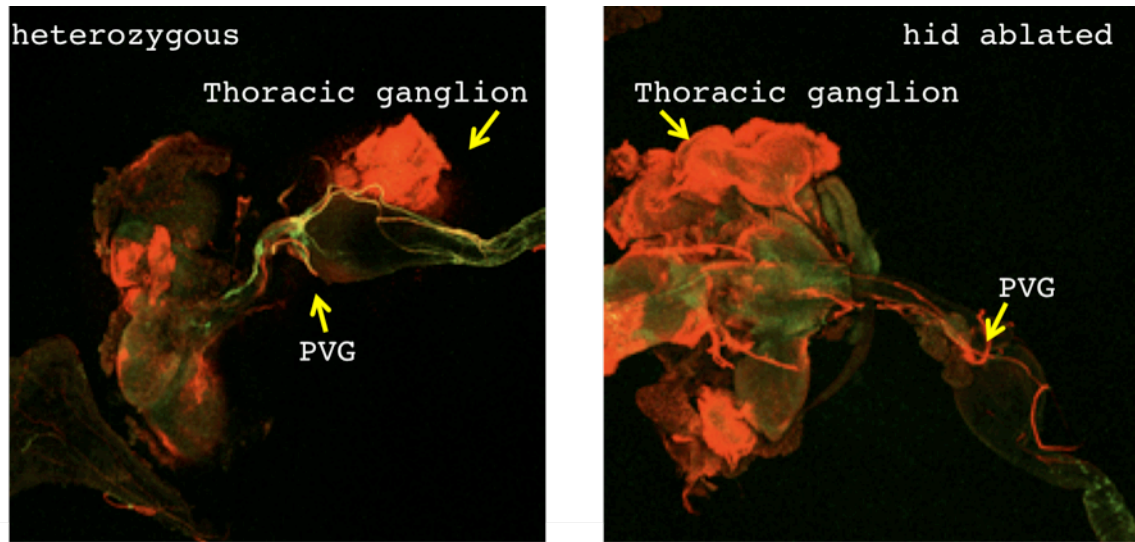


Figure 3-7: Reporter GFP not detected in the proventricular ganglion of ablated animals. Heterozygous is $+/+$; $UAS-2X-eGFP/+$; $dmrt93B^{GAL4KI}/+$. Hid ablated is $UAS-hid/+$; $UAS-2XeGFP/+$; $dmrt93B^{GAL4KI}/+$. Anti-GFP (green) represents GAL4 expression from the $dmrt93B^{GAL4KI}$ allele. Anti-22C10 (red) is used to stain the proventricular ganglion (PVG). As seen in the heterozygous panel, not all reporter neurons are marked with this antibody. Overlapping signal is seen as yellow. This marker also strongly stains the thoracic ganglion.

recombining P-element insertions, PBac{RB}r-l^{e01755} and PBac{RB}CG7056^{e01316}, in the neighboring genes *rudimentary-like* and CG7056. As previously mentioned, this deficiency placed over the larger deficiency Df(3R)ED10845 has an obvious *rudimentary-like* wing phenotype. These individuals are also female sterile, a phenotype previously reported in *rudimentary-like* mutants. Thus, the deficiency *dmrt93B*-delta9 cannot be used independently from the *dmrt93B*^{GAL4KI} allele to specifically study *dmrt93B* function because it affects the neighboring genes.

In a comparison of adult female brains, I found that the suboesophageal ganglion had fewer GAL4 expressing projections in the knock-in hemizygous mutant flies than it did in heterozygotes for *dmrt93B*^{GAL4KI}. To determine how consistent this observation of fewer projections was, I compared three females from each genotype. The wild-type females appear to have more projections from the suboesophageal ganglion than the hemizygous mutant females (figure 3-8, C and D).

Given the differences observed above in the adult brains, I then analyzed larval brains to see if there is a detectable difference in the cells expressing the GFP reporter under the control of the GAL4 knock-in allele. For this stage in development the same *dmrt93B* genotypes

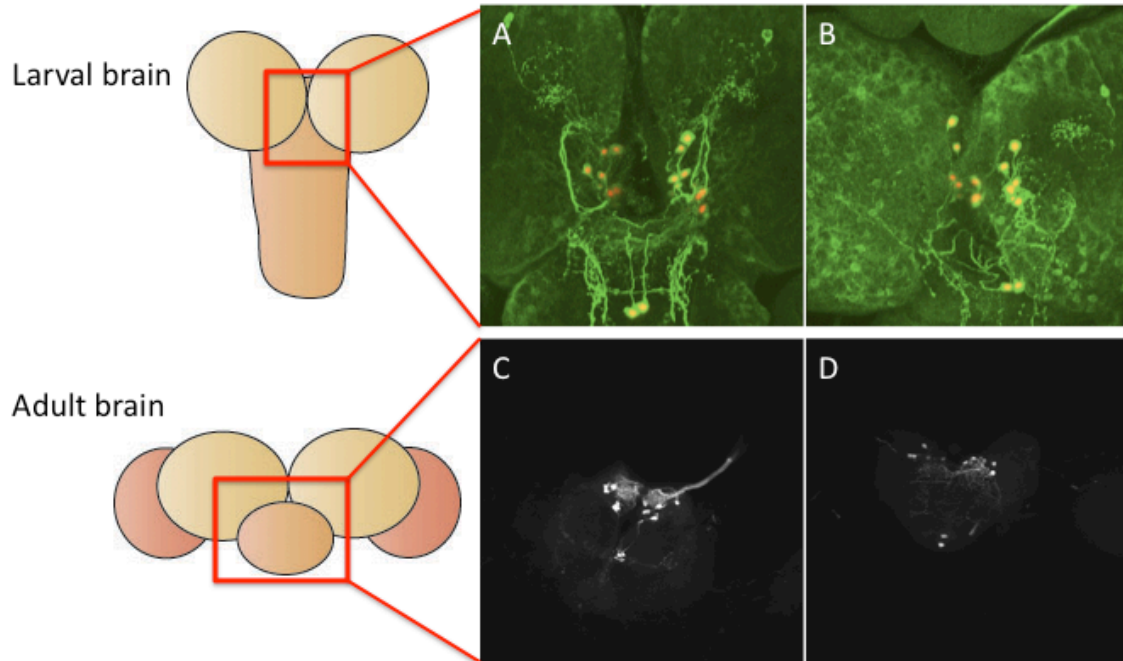


Figure 3-8: Comparison of wild-type and mutant reporter GFP. The subesophageal ganglion is examined in both larval (A-B) and adult (C-D) female brains. While expression is seen in both heterozygous *dmrt93B^{GAL4KI}* over wild-type and hemizygous *dmrt93B^{GAL4KI}* over deficiency, the mutant brains (B and D) have a reduction in GFP reporter and appear to have few nerves projecting from the region.

from the adult analysis were used, but the fluorescent reporter was a combination of UAS-2X-eGFP to mark the nucleus and cytoplasm of the cell and UAS-StingerRFP (Bloomington stock #5964) to mark nuclei. The fixed tissues were stained with anti-GFP to enhance the signal from the GFP reporter and RFP was detected directly by live fluorescence. This double staining provides a clearer image of the effects on the mutant allele described below.

The genotypes compared in this larval analysis were w^* ; UAS-2XeGFP:UAS-StingerRFP/ +; $dmrt93B^{GAL4KI}$ / Df- $dmrt93B$ -delta9 for hemizygotes and w^* ; UAS-2XeGFP:UAS-StingerRFP/ +; $dmrt93B^{GAL4KI}$ / + for heterozygotes. Imaging of the suboesophageal ganglion region of the CNS revealed that nerve projections in the mutants are both fewer and less organized relative to heterozygous larvae, but number of nuclei is similar (figure 3-8, A and D).

5. CAFÉ assay indicates the $dmrt93B^{GAL4KI}$ allele results in increased consumption of food.

The expression of $dmrt93B$ in regions associated with taste and digestive tract function led me to test feeding behavior in adult flies. To test the $dmrt93B^{GAL4KI}$ allele for effects on feeding, we examined adult male feeding

behavior with the Capillary Feeder (CAFÉ) assay (Ja et al., 2008). The *dmrt93B^{GAL4KI}* allele does not carry a mini-w+ eye-color marker and carries a w¹¹¹⁸ X-chromosome, thus these flies have white colored eyes. Without eye color, flies are essentially blind and may overcompensate with other senses such as smell and taste (personal communication Gregg Roman). To address this issue, we use the mini-w+ gene from the UAS-2X-eGFP transgene on the second chromosome to equally provide all individuals with a similar eye color background. In this section I will describe CAFÉ assay results that suggest an over eating phenotype in adult males.

CAFÉ assays were performed in collaboration with Shiyu Xu in the lab of Gregg Roman at the University of Houston. In the initial experiments, we examined individual daily intake of liquid food (5% sucrose/ 5% yeast in water) by ten adult males from four different genotypes (figure 3-9). The control w¹¹¹⁸; UAS-2X-eGFP/ +; +/ + adult males were compared to heterozygous w¹¹¹⁸; UAS-2X-eGFP/ +; +/ *dmrt93B^{GAL4KI}*. Additional classes examined were homozygous for the knock-in allele w¹¹¹⁸; UAS-2X-eGFP/ +; *dmrt93B^{GAL4KI}* / *dmrt93B^{GAL4KI}* and hemizygous over deficiency w¹¹¹⁸; UAS-2X-eGFP/ +; Df-*dmrt93B*-delta9 / *dmrt93B^{GAL4KI}*. In our initial study using 10 adult males, we found both the homozygous

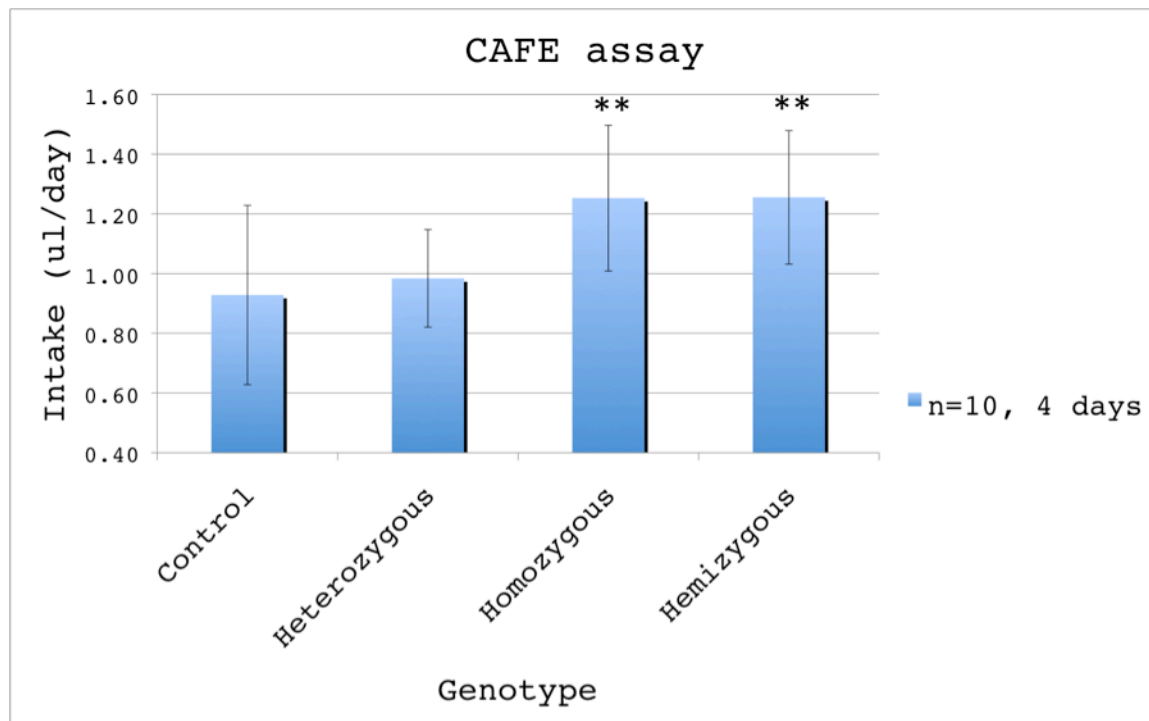


Figure 3-9: CAFE assay indicates *dmrt93B^{GAL4KI}* mutants consume more food than control. This Capillary Feeder (CAFE) assay measures the volume of liquid food consumed by 10 individual adult males over 4 days. Larvae were raised at 25C on a 12 hour light/dark cycle, changed to a new vial at eclosion, and set up in the CAFE apparatus 2 days later. Food is changed daily and measured on days 4-7. Error is +/- one standard deviation. Asterisk indicates p-value <0.0001 by Student's t-test.

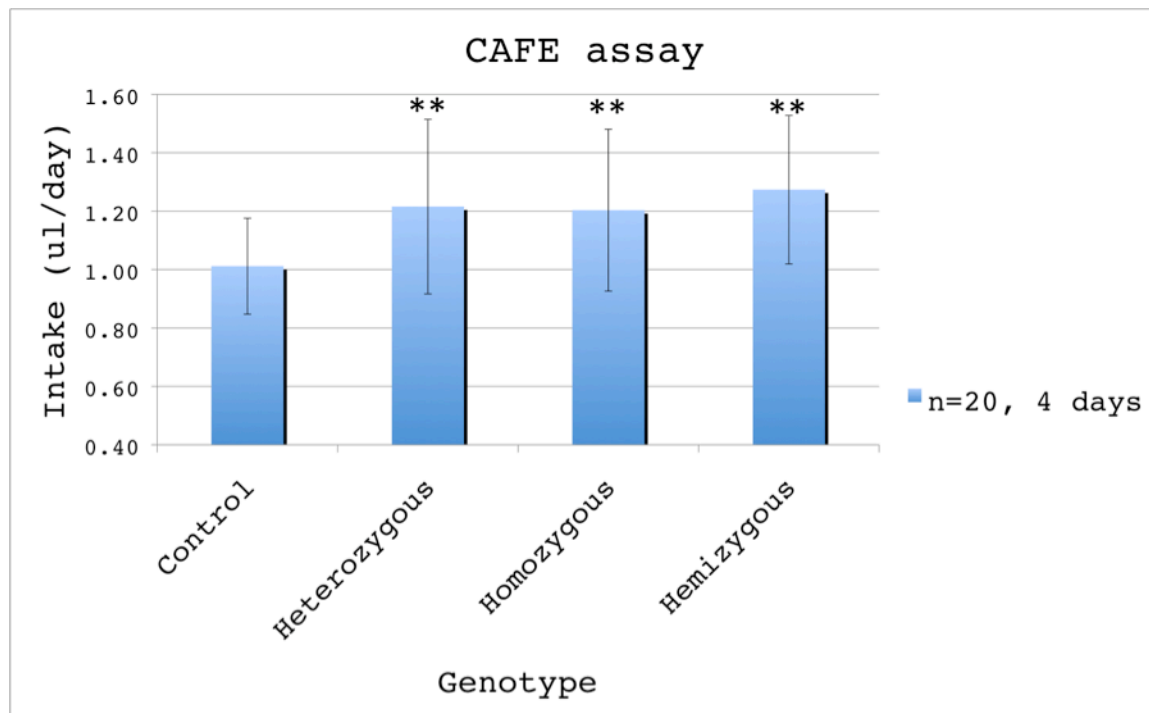


Figure 3-10: CAFÉ assay indicates *dmrt93B*^{GAL4KI} has a dominant effect on food intake. This Capillary Feeder (CAFÉ) assay measures the volume of liquid food consumed by 20 individual adult males over 4 days. Larvae were raised at 25C on a 12 hour light/dark cycle, changed to a new vial at eclosion, and set up in the CAFÉ apparatus 2 days later. Food is changed daily and measured on days 4-7. Error is +/- one standard deviation. Asterisk indicates p-value <0.000001 by Student's t-test.

and hemizygous mutant classes consumed more liquid food than the control and heterozygous classes. Based on these results we decided to repeat the assay using twenty adults males for each genotype and measure the before and after pooled weights of these examined flies.

In the second CAFÉ assay using a larger sample of flies with the genotypes previously described, we again observed that homozygous mutants displayed increased food consumption. However, in this experiment, heterozygous mutants also displayed increase food consumption relative to the control class (figure 3-10). However, we do not see a significant gain in weight for these flies that consume more than the control class. This result indicates a dominant effect of *dmrt93B* loss-of-function on feeding behavior.

Discussion

Characterization of *dmrt93B*^{GAL4KI} mutants reveals that a large number die prior to eclosion. Since the mutant pupae appear to frequently reach to stage of wing pigmentation, a developmental landmark before eclosion, it is possible that the pharate mutants are unable to break through the pupal case to eclose. Since these mutants that are unable to

emerge from the pupal case may have a more severe phenotype than the ones that are able to eclose, analysis of these mutants may reveal more dramatic defects in morphology.

Extensive fertility tests on the eclosed mutants indicated they do not have a significant reduction in fertility. Analyzing the morphology of the testes from two rare sterile mutant males selected from these experiments, I found one with a phenotype similar to the RNAi knockdown defects. This finding suggests the mutants with this *dmrt93B*^{GAL4KI} allele may indeed have the testicular defects associated with the RNAi experiment, although not consistently. In a blind experiment, however, testicular defects were not seen in the twenty randomly selected w*; +; Df-*dmrt93B*-delta9/ Df(3R)ED10845 null males. Since only minor defects in the male reproductive tissue is seen in *dmrt93B* mutants, the original hypothesis that *dmrt93B* is required for the formation and maintenance of the male reproductive tract is not supported by these findings.

It is possible that the RNAi knock-down had off-target effects that may have resulted in the testicular defects observed in earlier experiments. However, a search of the fly genome using an algorithm to check if there were any strong secondary targets revealed no obvious candidate. As mentioned in the previous chapter, this *dmrt93B*^{GAL4KI} allele

was designed with several point mutations in the conserved DNA binding domain of the gene to ensure elimination of its function. The most likely explanation for the lack for testis expression with this GAL4 knock-in allele is that these mutations eliminated enhancer elements needed for testicular expression.

Although reporter expression was not seen in the testis with this allele, there was GFP detected in the brains of both males and females. This non-sex specific expression in the head is consistent with tissue specific RT-PCR analysis of *dmrt93B* transcripts. Since antibodies for this gene were unavailable and transcripts of *dmrt93B* were hard to detect by RNA *in situ*, this *dmrt93B*^{GAL4KI} allele provides the first data at the cellular level for the neuronal expression of the gene. This finding enhances what we previously knew from tissue-specific RT-PCR analysis of the gene in both male and female heads.

Based on the location at the anterior portion of the proventriculus, the cells expressing GFP were either the proventricular ganglion (Spieß, 2008) or wreath cells (Aggarwal and King, 1967). The wreath cells, which play the equivalent role of the kidney in insects by filtering the hemolymph, also coat the anterior portion of the proventriculus (Mehta, 2009). However, the shape of the

cells expressing the GFP reporter indicates they are neuronal. Additionally, the use of a known antibody, anti-22c10, to mark the proventricular ganglion shows an overlap of the GFP reporter thus supporting the neuronal identity of the cells. The role of the proventricular ganglion in feeding is not well understood. In the blowfly (*Calliphora vicina*), the proventricular ganglion is not essential for organ function but it may affect the frequency of the peristalsis (Schoofs, 2007).

Another common occurrence in some, but not all, homozygous mutant individuals are black spots randomly found in pairs at various positions in the abdomen of the larvae and adults. These melanotic capsules may possibly form in response to an "immune challenge" such as infection (Qui et al., 1998). Though the *dmrt93B*^{GAL4^{KI}} allele is backcrossed several generations to the wild-type parental strain to remove exogenous mutations, there may be a few unexpected mutations left. For this reason, the use of the heteroallelic deficiency background may provide a more gene specific mutant analysis of *dmrt93B*.

The expression of the *dmrt93B*^{GAL4^{KI}} allele in the suboesophageal ganglion and proventricular ganglion of both larvae and adults implicated a possible role in feeding behavior. In collaboration with Gregg Roman's lab at the

University of Houston, we found that both heterozygous and homozygous mutant males consume more food daily in a Capillary Feeder (CAFÉ) assay than do controls. This finding indicates the normal role of *dmrt93B* expressing neurons is to inhibit food intake. The expression of the GFP reporter in the proventriculus is similar to serotonin, 5-Hydroxytryptamine (5-HT), secreting neurons that coat the proventriculus (Valles and White, 1988). The over-eating behavior seen in the *dmrt93B*^{GAL4^{KI}} mutants is consistent with the loss of serotonin, a regulator of feeding behavior.

Recently, the cellular expression pattern of *doublesex* in the CNS has been revealed through the use of both antibodies and GAL4 insertions (Sanders and Arbeitman, 2008; Rideout et al., 2010; Robinett et al., 2010). The expression of *doublesex* is apparent in a variety of tissues without *dmrt93B* expression in the CNS, although both have some expression in the suboesophageal ganglion (Robinett et al., 2010). Colocalization of *doublesex* GAL4 with another sex pathway gene, *fruitless*, shows overlap in some regions of the CNS (Rideout et al., 2010). Additionally, *fruitless* appears to have more expression in the suboesophageal ganglion than *doublesex*. In the analysis of these neurons in feeding behavior, *fruitless* silenced virgin females

demonstrate increased feeding frequency reflective of mated females (Barnes et al., 2008).

Chapter 4: Conclusion and Future Directions

The GAL4 knock-in allele generated in this work provides the first information on the cellular localization of *dmrt93B* in the brain and digestive system of both larvae and adults. Additionally, the use of this knock-in allele in mutant analysis demonstrates affects in feeding behavior for adult males. Since we see an increase in overall food intake in these mutant animals, our next step will be to test if transgenic expression of *dmrt93B* is able to rescue the mutant phenotype. This rescue experiment could also be used to evaluate the effects on viability. The use of a genomic transgene including all *dmrt93B* sequence is preferable for this experiment as a UAS linked *dmrt93B* cDNA presents only one isoform of the gene.

Generally, females require more nutrients than males for egg production. To understand the role of *dmrt93B* in feeding, we should also look the feeding behavior of female *dmrt93B^{GAL4KI}* mutants using the CAFÉ assay. Previous reports have shown that silenced *fruitless (fru)* females consume food at an increased frequency than control females (Barnes et al., 2008). Additionally, expression of FRU-M in the CNS may overlap with *dmrt93B* (Goldman and Arbeitman, 2007; Rideout et al., 2010). An antibody colocalization

experiment with anti-FRU-M and the *dmrt93B*^{GAL4KI} would be useful in determining if these neurons are the same. This may provide additional evidence of a common role for these neurons in feeding behavior. In a separate report, interestingly, the overall food intake by adult males with silenced *fru* is decreased. The *fru*-silenced adults have increased fat stores compared to control animals (Al-Anzi et al., 2009). These findings support the need to measure multiple factors in feeding assays.

Based on data published on BioGRID, *dmrt93B* has three potential protein interacting partners with only one being a characterized protein, Huntingtin-interacting protein 14 (HIP14). HIP14 may play a role in synaptic efficacy in neuronal transport pathways (Singaraja et al., 2002; Stowers and Isacoff, 2007). Future experiments can be carried out to determine the mutant effects of *dmrt93B* on *hip14* expression by introducing the GFP-HIP14 or the *dHIP14YFP* transgene into the *dmrt93B* mutant background (Ohshima, et al., 2007; Stowers and Isacoff, 2007).

Bibliography

Aggarwal, S.K., and King, R.C. (1967). The ultrastructure of the wreath cells of *Drosophila melanogaster* larvae. *Protoplasma* 63, 343-352.

Al-Anzi, B., Armand, E., Nagamei, P., Olszewski, M., Sapin, V., Waters, C., Zinn, K., Wyman, R.J., and Benzer, S. (2010). The Leucokinin Pathway and Its Neurons Regulate Meal Size in *Drosophila*. *Curr Biol*.

Al-Anzi, B., Sapin, V., Waters, C., Zinn, K., Wyman, R.J., and Benzer, S. (2009). Obesity-blocking neurons in *Drosophila*. *Neuron* 63, 329-341.

An, W., and Wensink, P.C. (1995). Three protein binding sites form an enhancer that regulates sex- and fat body-specific transcription of *Drosophila* yolk protein genes. *EMBO J* 14, 1221-1230.

Baker, B.S., and Ridge, K.A. (1980). Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* 94, 383-423.

Ballinger, D.G., and Benzer, S. (1989). Targeted gene mutations in *Drosophila*. *Proc Natl Acad Sci U S A* 86, 9402-9406.

Bao, X., Wang, B., Zhang, J., Yan, T., Yang, W., Jiao, F., Liu, J., and Wang, S. Localization of serotonin/tryptophan-hydroxylase-immunoreactive cells in the brain and suboesophageal ganglion of *Drosophila melanogaster*. *Cell Tissue Res* 340, 51-59.

Barnes, A.I., Wigby, S., Boone, J.M., Partridge, L., and Chapman, T. (2008). Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proc Biol Sci* 275, 1675-1683.

Bell, L.R., Maine, E.M., Schedl, P., and Cline, T.W. (1988). Sex-lethal, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. *Cell* 55, 1037-1046.

Bodenstein, D. (1994). The postembryonic development of *Drosophila*. In *Biology of Drosophila*, M. Demerec, ed. (New York, CSH Laboratory press), pp. 275-367.

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

Budnik, V., Wu, C.F., and White, K. (1989). Altered branching of serotonin-containing neurons in *Drosophila* mutants unable to synthesize serotonin and dopamine. *J Neurosci* 9, 2866-2877.

Burtis, K.C., and Baker, B.S. (1989). *Drosophila* doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* 56, 997-1010.

Burtis, K.C., Coschigano, K.T., Baker, B.S., and Wensink, P.C. (1991). The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. *EMBO J* 10, 2577-2582.

Chen, H., Ma, Z., Liu, Z., Tian, Y., Xiang, Y., Wang, C., Scott, M.P., and Huang, X. (2009). Case studies of ends-out gene targeting in *Drosophila*. *Genesis* 47, 305-308.

Coschigano, K.T., and Wensink, P.C. (1993). Sex-specific transcriptional regulation by the male and female doublesex proteins of *Drosophila*. *Genes Dev* 7, 42-54.

Cumberledge, S., Szabad, J., and Sakonju, S. (1992). Gonad formation and development requires the abd-A domain of the bithorax complex in *Drosophila melanogaster*. *Development* 115, 395-402.

DeFalco, T., Le Bras, S., and Van Doren, M. (2004). Abdominal-B is essential for proper sexually dimorphic development of the *Drosophila* gonad. *Mech Dev* 121, 1323-1333.

DeFalco, T.J., Verney, G., Jenkins, A.B., McCaffery, J.M., Russell, S., and Van Doren, M. (2003). Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* embryonic gonad. *Dev Cell* 5, 205-216.

Duffy, J.B. (2002). GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 34, 1-15.

Erdman, S.E., and Burtis, K.C. (1993). The Drosophila doublesex proteins share a novel zinc finger related DNA binding domain. EMBO J 12, 527-535.

Funk, N., Becker, S., Huber, S., Brunner, M., and Buchner, E. (2004). Targeted mutagenesis of the Sap47 gene of Drosophila: flies lacking the synapse associated protein of 47 kDa are viable and fertile. BMC Neurosci 5, 16.

Goldman, T.D., and Arbeitman, M.N. (2007). Genomic and functional studies of Drosophila sex hierarchy regulated gene expression in adult head and nervous system tissues. PLoS Genet 3, e216.

Golic, K.G., and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell 59, 499-509.

Gong, W.J., and Golic, K.G. (2003). Ends-out, or replacement, gene targeting in Drosophila. Proc Natl Acad Sci U S A 100, 2556-2561.

Gorfinkiel, N., Sanchez, L., and Guerrero, I. (2003). Development of the Drosophila genital disc requires

interactions between its segmental primordia. Development 130, 295-305.

Groth, A.C., Fish, M., Nusse, R., and Calos, M.P. (2004). Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. Genetics 166, 1775-1782.

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

Hazelrigg, T., Levis, R., and Rubin, G.M. (1984). Transformation of white locus DNA in *drosophila*: dosage compensation, zeste interaction, and position effects. Cell 36, 469-481.

Horn, C., Jaunich, B., and Wimmer, E.A. (2000). Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. Dev Genes Evol 210, 623-629.

Horn, C., Offen, N., Nystedt, S., Hacker, U., and Wimmer, E.A. (2003). piggyBac-based insertional mutagenesis

and enhancer detection as a tool for functional insect genomics. *Genetics* 163, 647-661.

Huang, J., Zhou, W., Dong, W., Watson, A.M., and Hong, Y. (2009). From the Cover: Directed, efficient, and versatile modifications of the *Drosophila* genome by genomic engineering. *Proc Natl Acad Sci U S A* 106, 8284-8289.

Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., and Benzer, S. (2007). Prandiology of *Drosophila* and the CAFE assay. *Proc Natl Acad Sci U S A* 104, 8253-8256.

Jaglarz, M.K., and Howard, K.R. (1994). Primordial germ cell migration in *Drosophila melanogaster* is controlled by somatic tissue. *Development* 120, 83-89.

Jursnich, V.A., and Burtis, K.C. (1993). A positive role in differentiation for the male doublesex protein of *Drosophila*. *Dev Biol* 155, 235-249.

Kato, Y., Kobayashi, K., Oda, S., Colbourn, J.K., Tatarazako, N., Watanabe, H., and Iguchi, T. (2008).

Molecular cloning and sexually dimorphic expression of DM-domain genes in *Daphnia magna*. *Genomics* 91, 94-101.

Keisman, E.L., and Baker, B.S. (2001). The *Drosophila* sex determination hierarchy modulates wingless and decapentaplegic signaling to deploy dachshund sex-specifically in the genital imaginal disc. *Development* 128, 1643-1656.

Keisman, E.L., Christiansen, A.E., and Baker, B.S. (2001). The sex determination gene doublesex regulates the A/P organizer to direct sex-specific patterns of growth in the *Drosophila* genital imaginal disc. *Dev Cell* 1, 215-225.

Kerkis, J. (1931). The Growth of the Gonads in *DROSOPHILA MELANOGASTER*. *Genetics* 16, 212-224.

Lebo, M.S., Sanders, L.E., Sun, F., and Arbeitman, M.N. (2009). Somatic, germline and sex hierarchy regulated gene expression during *Drosophila* metamorphosis. *BMC Genomics* 10, 80.

Lei, N., Hornbaker, K.I., Rice, D.A., Karpova, T., Agbor, V.A., and Heckert, L.L. (2007). Sex-specific

differences in mouse DMRT1 expression are both cell type- and stage-dependent during gonad development. Biol Reprod 77, 466-475.

MacDougall, C., Harbison, D., and Bownes, M. (1995). The developmental consequences of alternate splicing in sex determination and differentiation in *Drosophila*. Dev Biol 172, 353-376.

Manoli, D.S., Foss, M., Villella, A., Taylor, B.J., Hall, J.C., and Baker, B.S. (2005). Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour. Nature 436, 395-400.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., et al. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417, 559-563.

Mehta, A., Deshpande, A., Bettedi, L., and Missirlis, F. (2009). Ferritin accumulation under iron scarcity in *Drosophila* iron cells. Biochimie 91, 1331-1334.

Miller, A. (1941). Position of Adult Testes in *Drosophila Melanogaster* Meigen. *Proc Natl Acad Sci U S A* 27, 35-41.

Mitchell, B.K., and Itagaki, H. (1992). Interneurons of the subesophageal ganglion of *Sarcophaga bullata* responding to gustatory and mechanosensory stimuli. *J Comp Physiol A* 171, 213-230.

Murphy, M.W., Zarkower, D., and Bardwell, V.J. (2007). Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. *BMC Mol Biol* 8, 58.

Nagoshi, R.N., McKeown, M., Burtis, K.C., Belote, J.M., and Baker, B.S. (1988). The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*. *Cell* 53, 229-236.

Narendra, U., Zhu, L., Li, B., Wilken, J., and Weiss, M.A. (2002). Sex-specific gene regulation. The Doublesex DM motif is a bipartite DNA-binding domain. *J Biol Chem* 277, 43463-43473.

O'Keefe, L.V., Smibert, P., Colella, A., Chataway, T.K., Saint, R., and Richards, R.I. (2007). Know thy fly. Trends Genet 23, 238-242.

Ohshima, T., Verstreken, P., Ly, C.V., Rosenmund, T., Rajan, A., Tien, A.C., Haueeter, C., Schulze, K.L., and Bellen, H.J. (2007). Huntingtin-interacting protein 14, a palmitoyl transferase required for exocytosis and targeting of CSP to synaptic vesicles. J Cell Biol 179, 1481-1496.

Qiu, P., Pan, P.C., and Govind, S. (1998). A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. Development 125, 1909-1920.

Radford, S.J., Goley, E., Baxter, K., McMahan, S., and Sekelsky, J. (2005). Drosophila ERCC1 is required for a subset of MEI-9-dependent meiotic crossovers. Genetics 170, 1737-1745.

Raymond, C.S., Murphy, M.W., O'Sullivan, M.G., Bardwell, V.J., and Zarkower, D. (2000). Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev 14, 2587-2595.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., and Zarkower, D. (1998). Evidence for evolutionary conservation of sex-determining genes. *Nature* 391, 691-695.

Rideout, E.J., Dornan, A.J., Neville, M.C., Eadie, S., and Goodwin, S.F. (2010). Control of sexual differentiation and behavior by the doublesex gene in *Drosophila melanogaster*. *Nat Neurosci* 13, 458-466.

Robinett, C.C., Vaughan, A.G., Knapp, J.M., and Baker, B.S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biol* 8, e1000365.

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

Ross, J.M., Kalis, A.K., Murphy, M.W., and Zarkower, D. (2005). The DM domain protein MAB-3 promotes sex-specific neurogenesis in *C. elegans* by regulating bHLH proteins. *Dev Cell* 8, 881-892.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218, 348-353.

Sanders, L.E., and Arbeitman, M.N. (2008). Doublesex establishes sexual dimorphism in the *Drosophila* central nervous system in an isoform-dependent manner by directing cell number. *Dev Biol* 320, 378-390.

Schoofs, A., Niederegger, S., van Ooyen, A., Heinzel, H.G., and Spiess, R. (2010). The brain can eat: Establishing the existence of a central pattern generator for feeding in third instar larvae of *Drosophila virilis* and *Drosophila melanogaster*. *J Insect Physiol*.

Schoofs, A., and Spiess, R. (2007). Anatomical and functional characterisation of the stomatogastric nervous system of blowfly (*Calliphora vicina*) larvae. *J Insect Physiol* 53, 349-360.

Seum, C., Pauli, D., Delattre, M., Jaquet, Y., Spierer, A., and Spierer, P. (2002). Isolation of Su(var)3-7 mutations by homologous recombination in *Drosophila melanogaster*. *Genetics* 161, 1125-1136.

Shen, J., Ford, D., Landis, G.N., and Tower, J. (2009). Identifying sexual differentiation genes that affect *Drosophila* life span. *BMC Geriatr* 9, 56.

Sheng, X.R., Posenau, T., Gumulak-Smith, J.J., Matunis, E., Van Doren, M., and Wawersik, M. (2009). Jak-STAT regulation of male germline stem cell establishment during *Drosophila* embryogenesis. *Dev Biol* 334, 335-344.

Shirangi, T.R., Dufour, H.D., Williams, T.M., and Carroll, S.B. (2009). Rapid evolution of sex pheromone-producing enzyme expression in *Drosophila*. *PLoS Biol* 7, e1000168.

Singaraja, R.R., Hadano, S., Metzler, M., Givan, S., Wellington, C.L., Warby, S., Yanai, A., Gutekunst, C.A., Leavitt, B.R., Yi, H., et al. (2002). HIP14, a novel ankyrin domain-containing protein, links huntingtin to intracellular trafficking and endocytosis. *Hum Mol Genet* 11, 2815-2828.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., and Sinclair, A.H. (2009).

The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature* 461, 267-271.

Sonnenblick, B.P. (1941). Germ Cell Movements and Sex Differentiation of the Gonads in the *Drosophila* Embryo. *Proc Natl Acad Sci U S A* 27, 484-489.

Spiess, R., Schoofs, A., and Heinzl, H.G. (2008). Anatomy of the stomatogastric nervous system associated with the foregut in *Drosophila melanogaster* and *Calliphora vicina* third instar larvae. *J Morphol* 269, 272-282.

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

Stowers, R.S., and Isacoff, E.Y. (2007). *Drosophila* huntingtin-interacting protein 14 is a presynaptic protein required for photoreceptor synaptic transmission and expression of the palmitoylated proteins synaptosome-associated protein 25 and cysteine string protein. *J Neurosci* 27, 12874-12883.

Teleman, A.A., Maitra, S., and Cohen, S.M. (2006). *Drosophila* lacking microRNA miR-278 are defective in energy homeostasis. *Genes Dev* 20, 417-422.

Theodosiou, N.A., and Xu, T. (1998). Use of FLP/FRT system to study *Drosophila* development. *Methods* 14, 355-365.

Timmons, L., Becker, J., Barthmaier, P., Fyrberg, C., Shearn, A., and Fyrberg, E. (1997). Green fluorescent protein/beta-galactosidase double reporters for visualizing *Drosophila* gene expression patterns. *Dev Genet* 20, 338-347.

Valles, A.M., and White, K. (1988). Serotonin-containing neurons in *Drosophila melanogaster*: development and distribution. *J Comp Neurol* 268, 414-428.

Veith, A.M., Klattig, J., Dettai, A., Schmidt, C., Englert, C., and Volff, J.N. (2006). Male-biased expression of X-chromosomal DM domain-less *Dmrt8* genes in the mouse. *Genomics* 88, 185-195.

Volff, J.N., Zarkower, D., Bardwell, V.J., and Schartl, M. (2003). Evolutionary dynamics of the DM domain gene family in metazoans. *J Mol Evol* 57 Suppl 1, S241-249.

Vomel, M., and Wegener, C. (2008). Neuroarchitecture of aminergic systems in the larval ventral ganglion of *Drosophila melanogaster*. *PLoS One* 3, e1848.

Wen, S. (2002). Analysis of *dmrt93B*, a *doublesex* related gene, suggests that it plays a role in sexual differentiation (Houston, The University of Texas Health Science Center at Houston).

Williams, T.M., Selegue, J.E., Werner, T., Gompel, N., Kopp, A., and Carroll, S.B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* 134, 610-623.

Yang, Y., Zhang, W., Bayrer, J.R., and Weiss, M.A. (2008). Doublesex and the regulation of sexual dimorphism in *Drosophila melanogaster*: structure, function, and mutagenesis of a female-specific domain. *J Biol Chem* 283, 7280-7292.

Vita

Diana O'Day, a native Houstonian, graduated from the Michael E. DeBakey High School for Health Professions, Houston, Texas in 1997. She received the degree of Bachelor of Science with honors with a major in Biology and a minor in Chemistry from the University of Houston in May, 2001. For the next two years, she worked as a research technician in the Department of Genetics at Baylor College of Medicine with Dr. Patricia Ducey. In the Fall of 2003 she entered The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In the Spring of 2004 she joined the lab of Dr. William Mattox in the Department of Genetics at the University of Texas M. D. Anderson Cancer Center.

Permanent address:

11403 Hillcroft St.

Houston, Texas 77035