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The role of fruitless P3 and P4 transcripts in Drosophila melanogaster

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Abstract

The *fruitless* gene is highly conserved across many insect species, and its role in sex determination and sexual behaviour in *Drosophila melanogaster* males has been well characterized. The *fruitless* gene is alternatively spliced to produce at least 15 transcripts, but little is known about the alternative transcripts not involved in sexual traits. Transcripts beginning with the P3 and P4 first exons have previously been shown to be more heavily expressed during developmental stages of the *D. melanogaster* life cycle and have been implicated in developmental and fitness traits. Yet, these transcripts have never been assessed for their involvement in developmental traits. To study the role of these transcripts individually, the expression of *fruitless* P3 and P4 transcripts were reduced through RNA interference (RNAi) in combination with the Gal4/UAS binary system. Subsequent verification through quantitative PCR (qPCR) found that P3 transcript was successfully knocked down but P4 transcript was not. Assays scoring the influence of *fruitless* P3 on development found that knockdown of P3 transcript expression causes lethality at the pupal stage of development, where larvae form pupae but do not progress past this stage of development to form adult flies. This demonstrates that *fruitless* P3 plays a critical role in development, and that knockdown of P3 alone is sufficient to induce lethality.

Keywords: Drosophila melanogaster, alternative splicing, development, viability, *fruitless*, gene complexity, Gal4-UAS, RNAi

Summary for Lay Audience

What makes a genome more complex than another has been debated extensively. Interestingly, eukaryotes have processes that lead to increased genome complexity without heavily altering the size of their genome or the number of protein coding genes in their genome. One of these processes is called alternative splicing, where parts of a gene can be arranged and come together in different ways to make similar, but differing proteins. A gene that produces alternatively spliced transcripts and has high complexity is the *fruitless* gene in fruit flies. While we know a lot about the *fruitless* gene's role in the development of sexual characteristics and male behaviour in fruit fly species, there are components of the gene that haven't been explored. The *fruitless* gene produces multiple different transcripts, RNA intermediates of transcription/translation, that aren't related to these traits. Two of these lesser-known transcripts are the *fruitless* P3 and P4 transcripts, which are active during key development periods and may be heavily tied to the survival of these flies. Determining the function of *fru* P3 and P4 in development and survival could help shed light on the complexity of this alternatively spliced gene.

In this project, I introduced a tool that significantly reduces the amount of P3 and P4 transcripts throughout the whole fly. Unfortunately, after quantifying the expression of P4 transcript, the results demonstrated that the P4 transcript was not being reduced, and therefore could not be tested further. I tested the flies expressing reduced *fru* P3 to determine if the transcript had an effect on development, whether its reduction influenced survival and developmental time. With reduced *fru* P3, flies couldn't progress past the developmental stage to become adult flies and progressed slower through the developmental phase compared to controls. This finding highlights the crucial role of *fruitless* P3 transcripts in development and its effect on viability. It also demonstrates the importance of *fruitless'* role outside of the development of sexual characteristics and male sexual behaviour.

Authorship Statement

Drosophila stocks obtained from the Bloomington Drosophila Stock Center were used in this project. William Yeung performed the microinjections of transgenic constructs into embryos. Joshua Isaacson verified the specificity and efficiency of Alpha-tubulin for qPCR.

All remaining laboratory work described in this project was conducted by me.

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List of Abbreviations

BDSC: Bloomington Drosophila Stock Center

BTB: Broad-Complex, Tramtrack and bric á brac

cDNA: complimentary DNA

CNS: central nervous system

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

dsRNA: double-stranded RNA

dsx: doublesex

fru: fruitless

fru^M: male specific *fruitless* transcript

LB: lysogeny broth

MOL: Muscle of Lawrence

PCR: polymerase chain reaction

qPCR: quantitative PCR

RISC: RNA-induced silencing complex

RNAi: RNA interference

RpL: Ribosomal protein L

shRNA: short hairpin RNA

siRNA: small-interfering RNA

Sxl: Sex-lethal

tra: transformer

tra2: transformer-2

UAS: Upstream activating sequence

1 Introduction

1.1 Organismal complexity

1.1.1 The sources of phenotypic complexity

The source of organismal complexity has been debated for decades. Many scientists initially believed that organismal complexity was correlated with genome size, only to discover that genome size ranges greatly across living organisms, with some protists and plants having genomes order of magnitudes larger than mammals. One of the largest genomes reported is found in the plant *Paris japonica* at 148.8 Gb, almost 50 times large than the human genome (Pellicer et al., 2010). It is energetically expensive to maintain a larger genome, and therefore isn't always advantageous for organisms (Akashi & Gojobori, 2002; Lynch & Marinov, 2015; Wagner, 2005). If increasing genome size doesn't equate to increased complexity, another potential explanation is that the number of protein-coding-genes is correlated with complexity. The more protein coding genes present in a genome should equate to greater diversity of proteins being coded for and produced, but as with genome size, this relationship did not prove to be present. For example, humans (~20,000 protein coding genes) have lower than expected protein coding genes, while some species like *Oryza sativa* (rice) and *Mus musculus* (laboratory mouse) have many more coding genes that humans, approximately 32,000 and 30,000, respectively (Breschi et al., 2017; Itoh et al., 2007; Piovesan et al., 2019; Venter et al., 2001). Like genome size, the number of protein coding genes present in a genome doesn't necessarily correlate to greater complexity. To generate high complexity in eukaryotic organisms, the eukaryotic genome has evolved in other ways. A lot of that complexity stems from how the genome is regulated and the ability of one gene to encode multiple distinct transcripts that can code for multiple protein products, a process referred to as alternative splicing.

1.1.2 Alternatively splicing– diversifying function

Gene expression is regulated in many ways in eukaryotic organisms that lead to cell specificity and organismal complexity. Organismal complexity requires protein diversity, and one way to get greater protein diversity with a relatively smaller increase in genome size is through a process called alternative splicing. Alternative splicing is one mechanism that eukaryotic organisms use to increase organismal complexity without increasing the number of protein-coding genes it has and without greatly affecting the size of its genome. Genes are made up of both exons and introns. Exons come together to make transcripts which are then translated into proteins. Exons can be arranged in differing ways, leading to the generation of more than one transcript (family of transcripts) being generated from one gene, potentially leading to different proteins being made (Breitbart et al., 1987; Chen & Manley, 2009). Alternative splicing can also lead to a gene developing multiple functions as different exons can evolve over time to have divergent functions, leading to a species potentially adapting to or have an advantage in their environment. The new functions gained through alternative splicing can potentially be conserved over millions of years or continue to evolve and take on new functions, diverging between closely-related species, with the conservation or divergence of function depending on how important the function of the transcript is to the survival of the organism. Gene complexity through alternative splicing has been studied extensively in the model organism *Drosophila melanogaster*. There are many genes, such as *paralytic* or *Dscam*, that can be spliced into thousands of transcripts (reviewed in Park & Graveley, 2007), or *foraging*, a protein kinase, involved in many behaviours such as foraging, sleep, learning and memory and social behaviour (reviewed in Anreiter & Sokolowski, 2019). One well-studied gene that has alternatively-spliced transcripts involved in both development and complex behaviour is the *fruitless* (*fru*) gene in *Drosophila*, which was the focus of this study (Ito et al., 1996; Ryner et al., 1996).

1.2 The *fruitless* gene

1.2.1 Conservation and background

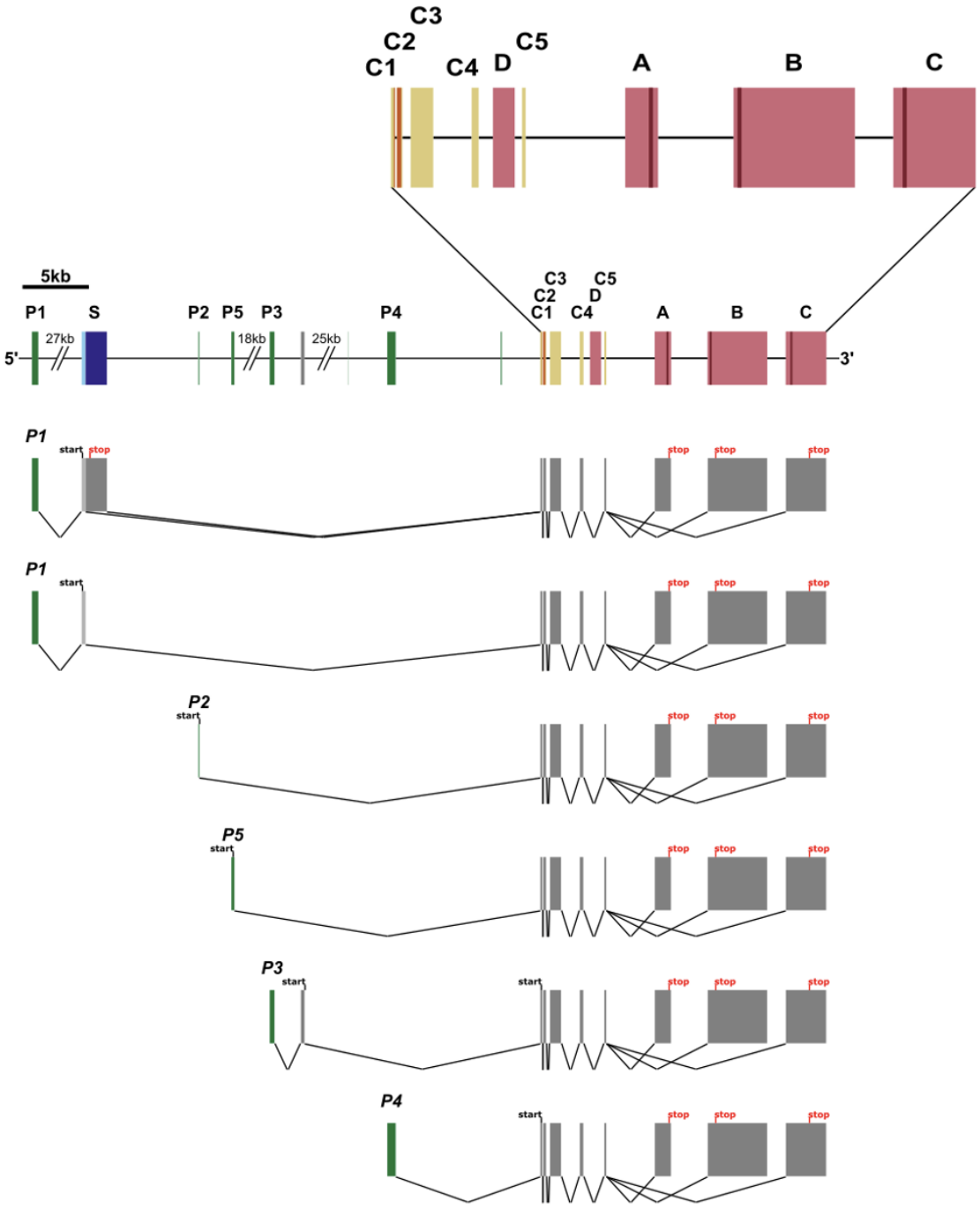
The *fruitless* (*fru*) gene has been well characterized for its role in sexual determination and differentiation across many different insect species. Its function has mainly been studied in Drosophila but has been shown to be conserved across the Dipteran order with its evolutionary conservation stretching as far as hemimetabolous insects (Bertossa et al., 2009; Fujii & Shimada, 2007; Gailey et al., 2006; Ohbayashi et al., 2001; Salvemini et al., 2009; Ustinova & Mayer, 2006).

1.2.2 Architecture and alternative splicing in *D. melanogaster*

When studying *fruitless* across different species, homologues/orthologues of the gene are almost always compared back to *Drosophila melanogaster fru*, which is the most studied/annotated version of the gene. *Fruitless* was first characterized in *D. melanogaster* over 50 years ago by a male-courtship-deficient mutant, that later was identified to have come from disruption of the gene *fru* (Gill, 1963; Ito et al., 1996; Ryner et al., 1996). *Fruitless* is found on the right arm of the 3rd chromosome and spans approximately 130 kb. It is a very complex gene, composed of multiple exons that are combined in multiple ways to form at least 15 different transcripts. These transcripts contain an alternative first exon, called P1 through P5, a series of common exons found in all transcripts, and 4 variable final exons, called A through D (Figure 1; Ito et al., 1996; Ryner et al., 1996).

Almost all isoforms that come from *fru* contain two functional domains: the Broad-Complex, Tramtrack and bric á brac (BTB) binding domain (~120 amino acids in length) which is found within the common exons, and two C2H2 zinc finger binding domains found in the variable end exons A, B, and C (Ito et al., 1996; Ryner et al., 1996; Usui-Aoki et al., 2000). Variable exon D seems to have a pair of cysteine and histidine residues separated by a motif of 28 amino acids, which differs substantially from the sequences found in variable ends A, B, and C. Variable end D may code for an uncharacteristic, a non-functional or novel zinc-finger domain (Parker et al., 2014; Wolfe

et al., 2000). The BTB binding domain is a homodimerization domain found at the N terminus of proteins and appears in proteins that contain either C2H2 zinc fingers or Kelch repeats (Zollman et al., 1994). C2H2 zinc fingers contain a short β hairpin and an α helix, where a zinc atom is held in place by Cys(2)His(2) (C2H2) residues (Iuchi, 2001). The structure of the products of *fru*, especially the BTB domain and zinc finger motifs, suggest that *fru* is a transcription factor (Ito et al., 1996). Many BTB proteins are transcriptional regulators that potentially act by altering chromatin structure, and C2H2 zinc fingers are the most common DNA-binding motifs found in eukaryotic transcription factors (Wolfe et al., 2000; Zollman et al., 1994). Additionally, BTB domains are found in many genes that are involved in development in *Drosophila*, potentially implicating *fru* in development (e.g., Zollman et al., 1994).


C3
C2
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D
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P1
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P3
P4
27kb
18kb
25kb
5'
3'
start
stop
P1
P1
P2
P5
P3
P4

Figure 1. Schematic of the *fruitless* gene and alternatively spliced transcripts

Schematics of the *fruitless* genomic locus (top) and its alternatively spliced sex-specific and sex-non-specific transcripts (bottom). Exons are categorized by colour: green (P1-P5) represents the variable first exon(s); blue (S) represents the sex-specifically spliced exon, where the dark blue exon is spliced out in males; yellow (C1-C5) represents the common exons (C1-C2 encodes for the BTB domain in orange); and red (A-D) represents the variable last exon, encoding for zinc finger domains in burgundy. An expansion of the common and variable last exons (top right) gives a better view of the locations of the BTB domain (orange) in the C1 exon of the common region and the variable zinc finger domains (burgundy) in the variable last exons. Translational start and stop site codons are indicated in black and red above exons in the transcript schematics. Lines connecting exons in the transcript schematics represent possible splice patterns. Schematic modified from Yeung, 2021.

1.2.3 The *fruitless* Pathway

In females, the expression of the X-linked gene *Sex-lethal* (*Sxl*) initiates female development and leads to the activation of the female splice variant of *doublesex* (*dsx*) and lack of activation of the *fru* branches of the sex determination pathway (reviewed in Yamamoto et al., 2014). *Sxl* has a feedback regulatory function that is dose dependent, where it autoregulates the splicing of its own pre-mRNA. If two X chromosomes are present, *Sxl* is activated, leading to the downstream regulation and splicing of *transformer (tra*) pre-mRNA and expression of the functional protein, TRA^F, in females. Subsequently, the activation of TRA^F in combination with the expression of the female splice variant of the *transformer-2* (*tra2*) protein (TRA-2) activates the expression of the female specific transcript of dsx^F and fru^F. The TRA/TRA-2 complex binds a binding site/splicing enhancer, activating a female specific weak 5' start site and a female specific region containing an early stop codon is included, leading to a non-functional female specific protein (Heinrichs et al., 1998).

In males on the other hand, the lack of *Sxl* activation leads to the expression of small, non-functional TRA protein, which means that the TRA/TRA-2 complex is not formed. The lack of *tra* expression allows for the expression of the male specific *fruitless* and *doublesex* transcripts (fru^M and dsx^M), leading to the production of male specific proteins (FRU^M and DSX^M) (Heinrichs et al., 1998; Ryner et al., 1996). FRU^M has a 101 amino

acids long N-terminus upstream of the BTB, unique to male *D. melanogaster* (Heinrichs et al., 1998; Ito et al., 1996; Ryner et al., 1996). In this pathway, both *fru* and *dsx* have fundamental, yet distinct roles in the development of sex-specific tissues and behavioural traits. Research that established that the expression of *fruitless* is controlled by *tra* and *tra2*, confirmed that *fru* plays a role in the *Drosophila* sex-determination hierarchy that controls male sexual behaviour and the development of the muscle of Lawrence (MOL; a muscle spanning the male *D. melanogaster* abdomen) (Lawrence & Johnston, 1984; Ryner et al., 1996), and directly controls the regulation of downstream genes associated with those traits. The sex-specific splicing of *fru*-P1 transcripts is the critical regulatory event that induces the emergence of male courtship behaviour (e.g., Demir & Dickson, 2005; Ito et al., 1996).

1.3 *fruitless* transcript functions in *D. melanogaster*

Drosophila fru transcripts start with one of five first exons: P1-P5. Transcripts starting with the P1 exon are sex-specifically spliced while the other *fru* transcripts are expressed the same in males and females.

1.3.1 Sex-specific *fru* P1 transcript function

Of all *fruitless* transcripts, P1 transcripts are the most highly studied and understood. P1 transcripts, containing the two most distal *fru* exons (P1 and P1-S), are spliced in a sex-specific manner, which is controlled by the sex determination hierarchy (Heinrichs et al., 1998; Ryner et al., 1996). In males, splicing removes the part of the P1-S exon that contains an early stop codon, while in females the sex-specific splicing mechanism is blocked through the binding of Transformer (TRA) and Transformer-2 (TRA-2) proteins at three tandem binding sites within the S exon (Heinrichs et al., 1998; Ryner et al., 1996).

1.3.1.1 Effect on neuronal patterning and tissues

When the *fru* P1 exon is disrupted, the male-specific FRU protein is not produced, and the neurocircuitry of males is altered (reviewed in Siwicki & Kravitz, 2009 and

Yamamoto et al., 2014). The main change observed is the feminization of a cluster of *fru*-expressing interneurons located medially just above the antennal lobe (Kimura et al., 2005; G. Lee et al., 2000). This male specific cluster plays a vital role in the activation of male courtship and may function as a decision-making center to initiate male courtship behaviour. In wildtype males, this cluster has three distinct features that differ from females. First, the cluster is made up differing number of neurons in males and females, 5 in females and 30 in males. Second, in males, these neurons have ipsilateral and contralateral neurites descending to the suboesophageal ganglion, while females have a single contralateral neurite descending to the suboesophageal ganglion. Lastly, in the suboesophageal ganglion, the neurite terminates in a horsetail-like structure in males, while dividing into two branches to form a Y-shape in females. In males with loss of-function *fru* mutations, all three of these features are feminized in the interneuron cluster, indicating the potential that *fru* controls all three of these characteristics (Goto et al., 2011; Kimura et al., 2005). In terms of the greater number of neurons found in the male interneuron cluster, it looks like there is active elimination of cells in females, suggesting the possibility that *fru* protects these neurons from cell death in males (Kimura et al., 2005). Extensive research has identified ~2,000 *fru* P1 expressing neurons, where approximately one-third exhibit structural sex differences (Cachero et al., 2010; Chiang et al., 2011; J. Y. Yu et al., 2010).

Another feature that is directly linked to the function of *fru* P1 transcripts is the development of the Muscle of Lawrence (MOL) (Ryner et al., 1996). The MOL is a large prominent muscle spanning the fifth segment of the dorsal abdomen of adult wildtype *D. melanogaster* males (Lawrence & Johnston, 1984), and is thought to potentially allow the male abdomen to be able to curl forward in successful copulation. The MOL formation depends on the sex of the contacting motoneuron: when the motoneurons are male, the MOL is formed, when the motoneuron is female, the MOL is not formed (Lawrence & Johnston, 1984). When looking at the evolutionary history of this muscle, researchers concluded that out of 95 species within the subfamily Drosophilidae, 67 had no MOL (Gailey et al., 1997). There have been many individual losses of the MOL over evolutionary history.

In addition to neuronal patterning and MOL formation, the *fru* gene may also contribute more directly to sensation of olfactory stimuli in *Drosophila*. There is evidence that antennal glomeruli, which connect to sensory neurons in olfactory receptors of the antennae, are sexually dimorphic (Kondoh et al., 2003). The size of the antennal glomeruli seems to be controlled by the expression of fru^M in the subset of olfactory receptor neurons in the antennae that the glomeruli are connected to (Manoli et al., 2005, 2006; Stockinger et al., 2005). Males expressing fru^M have larger glomeruli compared to females (Stockinger et al., 2005).

1.3.1.2 Effect on behaviour

The earliest study to identify *fruitless* and its effects on courtship behaviour did so through an X-ray induced recessive mutation (Gill, 1963). Courtship is a very intricate, multi-step process between male and female *D. melanogaster*, and homozygous *fru* mutant males showed many differences in courtship behaviour compared to their wildtype counterparts. The most noticeable differences were that these males didn't curl their abdomens towards females when attempting copulations (behaviourally sterile) and they would court both males and females. Homozygous females showed no phenotypic or behavioural differences compared to wildtype females (Hall, 1978). Another study induced fru^M in females to determine if fru^M is sufficient to induce male courtship behaviour. These females were masculinized and displayed male courtship-like behaviour towards other females, but not to the degree that is observed in wildtype males (Demir & Dickson, 2005). Removal of the function of fru^M through CRISPR-Cas9 revealed that males without sex specific P1 function had severe behavioural deficiencies when placed with a female: males took a much longer time to initiate courtship, and once initiated there was a significantly lower levels of courtship displayed in comparison to controls (Neville et al., 2021). Males also never copulated and were considered behaviourally sterile, and courted and were courted by other males, findings in line with the earliest study (Gill, 1963; Neville et al., 2021). Additionally, P1 disruption caused a change in male courtship song, where males produced lower levels, irregular, and shorter courtship songs. Lastly, it was observed that *fru* P1 derived products play a role in the

control of baseline locomotor activity, limiting the variation in the peaks and troughs of activity over a 24-h period when compared to controls (Neville et al., 2021).

Another behaviour that *fru* P1 has been implicated in is aggression. Aggression, just like courtship/mating behaviour, is a sexually dimorphic behaviour in *Drosophila* (Vrontou et al., 2006). Both males and females display patterns of aggression in same sex pairings (male-male, female-female), and several of the behavioural patterns observed are sex-specific (Nilsen et al., 2004). The expression of Fru^M is required for normal inter-male aggression (Vrontou et al., 2006), and Fru^M in females is necessary and sufficient to transform patterns of aggressive behaviour from female-like to male-like (Nilsen et al., 2004).

As stated above, *fru* can be alternatively spliced to include differing 5' first exons (P1-P5), as well as different 3' end exons (A, B, C, or D) that include three DNA binding zinc finger domains. P1 transcripts containing different end exons (A, B, or C) are expressed in most male fru^M positive cells and contribute to male courtship but differ in their effects on behaviour and expression. The D isoform is not expressed in the *D. melanogaster* central nervous system (von Philipsborn et al., 2014). Each 3' isoform (A, B, or C) makes an important contribution to courtship behaviour, however, when individually eliminating, the resulting effects were comparatively mild to the complete loss of the male-specific *fru* transcripts (von Philipsborn et al., 2014). The strongest effect observed on courtship behaviour is from the *fru* transcript ending in exon C (*fruC*) (Billeter et al., 2006; Mellert et al., 2010; von Philipsborn et al., 2014). *FruC* is essential to the generation of sexual dimorphisms in neuron number and nerve branching. When *fruC* is knocked out, flies fail to copulate, have courtship song defects, lack a major component of song, no longer court in the dark, and don't form the MOL (Usui-Aoki et al., 2000; von Philipsborn et al., 2014; Vrontou et al., 2006).

1.3.2 Non-sex-specific *fru* transcript functions

Compared to the wealth of knowledge about *fru* P1 transcript function, very little is known about the roles of the sex-nonspecific transcripts (P2-P5) of *fruitless*. While the P5

transcript has not been studied to date, there is some research on the function of the other three transcript groups. Recent work on *fru* P2 discovered that it has a role in female mate rejection behaviour (Chowdhury et al., 2020). Female *D. melanogaster* that are homozygous for a *fru* P2 deletion exhibit lower mating with wildtype males. It is theorized that the loss of the P2 transcript significantly reduces female receptivity. Additionally, *D. melanogaster* and *D. simulans* hybrid females will mate with *D. melanogaster* males, but when those same females are missing the *D. melanogaster* allele of *fru* P2 – they only have the *D. simulans* allele – they reject *D. melanogaster* males. The effect of *fru* P2 on female receptivity is therefore observed both conspecifically and heterospecifically, implicating *fru* P2 in behavioural isolation (Chowdhury et al., 2020).

In another study, when P1 and P2 transcripts were simultaneously disrupted, the resulting flies were viable (Anand et al., 2001). However, when P3 transcripts were disrupted in combination with P1 and P2 transcripts, inviability was observed, indicating that P3 transcripts may be responsible for processes affecting viability. These mutants were able to reach the pharate adult stage but were unable to eclose from their pupal casings (Anand et al., 2001). If dissected out of their pupal casings, the adults were able to survive from 7-14 days (average *Drosophila* lifespan is 2-3 months; Helfand & Rogina, 2003; Ziehm et al., 2013) and had minor phenotypic defects (Anand et al., 2001). For example, flies were unable to fully extend their wings, their femur was frequently bent, and there were defective joints (enlarged/extra bristles) observed in one or more legs. Neurologically, there were fewer motoneuronal terminals, with fewer branches from the nerve terminal present on the lateral muscles of the abdomen. Additionally, the innervation of the lateral abdominal muscles was concentrated in the lateral and middle zones and did not penetrate more medial zones of these abdominal muscles (synaptic defect). More severe mutations which removed all *fru* coding sequence, as well as all or part of six genes flanking *fruitless*, resulted in flies that reached the pre-pupal stage, but then died either immediately after forming pre-pupa or after developing pigmentation but before forming any distinct structures within the puparium (Anand et al., 2001).

Therefore, one or more of these transcripts have implications in the proper development of *D. melanogaster* and play a role in specific developmental stages, potentially in the

pupal stage. However, due to these mutations simultaneously targeting multiple *fru* transcripts, it has not been determined whether P3 transcripts are directly implicated in viability, as these transcripts have never been knocked out or down on their own, but in combination with other upstream *fruitless* transcripts and potentially disrupting regulatory elements for downstream transcripts. Therefore, it is unknown whether P3 disruption alone affects viability, at what stage P3 disruption would affect viability, or if inviability is only caused by the combined disruption of multiple transcripts together.

1.4 Expression Data

As with the function and effect of *fru* P1 transcripts, the expression pattern of P1 transcripts has been more heavily studied than the other *fru* transcripts. Earlier studies that used immunocytochemistry, with probes specific for the common region of the FRU protein, detected FRU protein only in the central nervous system of males (G. Lee et al., 2000; Usui-Aoki et al., 2000). This is also observed in a genome-wide study of expression patterns (FlyAtlas 2: Leader et al., 2018). Using an RNA-Seq approach, the expression of individual *fruitless* transcripts were quantified. Expression of the sex specific P1 transcripts is highest in the head, brain, and ganglion in adult males and females. This is consistent with the results found in prior research.

Although there are very few studies exploring the functions of non-sex specific *fru* transcripts, the genome-wide study of expression patterns (FlyAtlas 2: Leader et al., 2018) can be used to observe the expression of these individual transcripts, therefore giving us the ability to infer the location and amount of the sex-non-specific transcript activity, and potentially help inform function. The *fru* P2 transcripts are most highly expressed in the head and eyes of adult males and females (FlyAtlas 2: Leader et al., 2018). Expression of *fru* P5 transcripts seems to be much lower relative to the other transcripts, its highest expression being in the head and brain of adult male and female flies.

The *fru* P3 and P4 transcripts both have their highest levels of expression in the larval stage. Within the larval stage, P3 transcripts are most highly expressed in the central nervous system, trachea, and carcass. There is a small amount of expression observed in

the salivary glands, midgut, hindgut, garland cells, and fat body, with zero expression observed in the Malpighian tubules (FlyAtlas 2: Leader et al., 2018). In contrast, P4 transcripts are expressed in all systems of the larvae, and most highly expressed in the hindgut, trachea, tubule, and carcass, with the lowest expression observed in the garland cells and fat body. Overall, it looks like there is more expression of P4 transcripts relative to P3 transcripts. An example of this is that the P3 transcript is most highly expressed in the central nervous system, while the P4 transcript is most highly expressed in the hindgut. The system with the 6th highest expression of P4 is the central nervous system, but its expression in the central nervous system is at a similar level to P3 transcripts expression levels in the central nervous system (FlyAtlas 2: Leader et al., 2018).

In the adult stage, *fru* P3 and P4 transcripts are expressed at much lower levels in nearly all systems, being most highly expressed in the brain. P4 transcripts also have slightly elevated expression patterns in the adult hindgut, which is consistent with its higher expression in the hindgut of larvae.

1.5 Comparing *fruitless* between *Drosophila* species

Like in *D. melanogaster*, *fruitless* homologs in other *Drosophila* species are implicated in the sex determination pathway (reviewed in Salvemini et al., 2010). Looking at *fruitless* across *Drosophila* species can help us understand how the complex gene has evolved in each of these closely related species over evolutionary time, and potentially provide insight as to which exons are ancestral. These homologs share sequence similarities and functional domains with the *fruitless* gene in *D. melanogaster*. When looking at the fruitless gene across many *Drosophila* species, the functional domains (BTB domain and zinc finger motifs) are most highly conserved (Parker et al., 2014). However, even with these highly conserved regions there can be a lot of variability in the sequences/transcripts, potentially leading to species-species sex differences. Variability is especially observed in the common exons between the BTB domain and the A-C exons and in the A and D exons themselves. In the most variable end/zinc finger exons, exon A is longer than the others, and has regions outside of the zinc finger motif which can be variable without disrupting the function of the motif. As previously mentioned above,

exon D isn't found in all transcripts, and deviates from the conserved zinc finger sequence found in exons A, B, and C. It is hypothesized that this exon may have evolved over time to have a different function (Parker et al., 2014).

While fru^M is expressed in the brain and ventral nerve chord of male *D. melanogaster*, and is not expressed in females, expression of fru^M in other *Drosophila* species differs (Yamamoto et al., 2004). fru^M is expressed similarly between *D. melanogaster* and the species *Drosophila simulans* and *Drosophila pseudoobscura*, in both males and females. The most interesting differences in expression out of those species that have been examined are in *Drosophila yakuba* and *Drosophila suzukii*, where *D. yakuba* expresses fru^M in retina of males, but males do not have a MOL. Surprisingly, there is also expression of fru^M in *D. yakuba* female retinas. In *D. suzukii*, fru^M is expressed similarly in males to *D. melanogaster*, except that it is also expressed in the lamina. In females on the other hand, there isn't female-specific repression of FRU^M translation, and it is expressed in the brain, lamina, and the ventral nerve cord. Although a number of cells express FRU^M protein in the female brain, these cells are different from male FRU^M expressing cells. Therefore, although females express FRU^M, the expression pattern is different. Additionally, the presence or absence of the MOL isn't always linked to sex-specific regulation of FRU translation (Yamamoto et al., 2004).

In the two Hawaiian species of the picture-winged group of subgenus *Drosophila*, *D. heteroneura* (Davis, Kurihara, & Yamamoto, 2000) and *D. silvestris* (Davis, Kurihara, Yoshino, et al., 2000), there is high conservation in exon-intron organization within *fru*, especially the exons containing the BTB-domain (80-100%), in respect to *D. melanogaster*. The highest variability in sequence is found in the seventh exon of *D. silvestris fru,* alternative exon A (*fruA*) specifically, which has less than 50% homology outside of the zinc finger domain. Additionally, these two species do not have the male-specific sequence of *fru* P1, meaning that sex specific splicing may not occur in these two species or may differ from *D. melanogaster* (Davis, Kurihara, Yoshino, et al., 2000). The *fru* homologue in *D. heteroneura* also does not include the female specific sequence upstream of the BTB domain (Davis, Kurihara, & Yamamoto, 2000). There may be a different sex-specific *fru* regulatory mechanism responsible, relying on sex-specific

alternative exons. These two species, amongst many *Drosophila* species, also lack the MOL. This muscle seems to have been lost repeatedly over the evolutionary history of *Drosophila* (Gailey et al., 1997). It is speculated that this loss may be linked to the potential loss of *fru* P1 sequence not present in these species, a necessary component in the formation of MOL in *D. melanogaster* (Davis, Kurihara, & Yamamoto, 2000; Davis, Kurihara, Yoshino, et al., 2000).

1.6 *fruitless* across different insect species

When a broader look is taken, *fruitless* is also found outside of the *Drosophila* genus. Studying *fruitless* across these evolutionarily distant species can give insight to its most critical and highly conserved functions (reviewed in Salvemini et al., 2010). The first conclusive evidence of *fru* conservation in Diptera, outside of the genus *Drosophila*, was in the mosquito, *Anopheles gambiae* (Gailey et al., 2006), which diverged 250 million years ago from *D. melanogaster* (Gaunt & Miles, 2002; Zdobnov et al., 2002). The genomic organization of *fru* and sequence of its BTB and zinc finger functional domains are highly conserved (amino acid identity ranging from 75 -100%) between *A. gambiae* and *Drosophila*, as well as its sex-specific splicing regulation and expression of P1-like male transcripts in male heads (Gailey et al., 2006; Salvemini et al., 2010). The *fru* gene is so well conserved between the two species that when ectopic *A. gambiae* male FRU P1 protein was expressed in transgenic *D. melanogaster*, development of MOL is rescued in mutant males and induced in mutant females (Gailey et al., 2006). Additionally, *A. gambiae* has five tandem TRA/TRA-2 binding sites near the regulated splice sites in female-specific transcripts, similar to the three present in *D. melanogaster* (Lam et al., 2003). The presence of these binding sites in *A. gambiae* indicates that sex-specific splicing may be conserved and under the control of co-evolved TRA/TRA-2 homologous. With *fru* so well conserved over millions of years of evolution between the two species, this led to the hypothesis that *fru* is a primitive gene of male sexual behaviour across the Dipteran order (Gailey et al., 2006). Alternatively, in another mosquito species, *Aedes aegypti*, although *fru* is responsible for sexual differentiation as in other Dipteran species,

its sequence and regulation seem to differ substantially from *A. gambiae* and *D. melanogaster* (Salvemini et al., 2013).

Similar to *A. gambiae*, a *fru* orthologue was isolated in the common housefly, *Musca domestica* (Meier et al., 2013). The structure of *fru* is highly conserved in *M. domestica*, where four zinc finger motifs were identified that correspond to four different transcript variants, found in both males and females. This species also demonstrates potential conservation of sex-specific splicing, also under the control of TRA/TRA-2 homologues. Similar finding to the conservation in the *A. gambiae fru* homologue, female-specific sequences in *M. domestica* contained four repeated TRA/TRA-2 binding sites. The proteins encoded from the male-specific transcripts have high sequence similarity to *fru* P1 male transcripts in *Drosophila* (Meier et al., 2013). Therefore, the *M. domestica* genome has an ortholog of *fru* that is most likely targeted by the sex determination pathway. As in *D. melanogaster*, *fru* is similarly involved in controlling male courtship behaviour in *M. domestica*. In males lacking *fru* function, copulation is severely reduced and multiple steps in the mating ritual are affected (Meier et al., 2013). This leads to behavioural sterility of male *M. domestica* lacking *fru* function. Therefore, male mating behaviour is controlled by a branch of the sex-determining pathway mediated by *fru* in *M. domestica* (Meier et al., 2013).

A closer relative to *D. melanogaster*, the Mediterranean fruit fly, *Ceratitis capitata*, has 120 million years of evolutionary distance separating the two species (Beverley & Wilson, 1984). Although evolutionarily closer to *D. melanogaster* than *A. gambiae*, the upstream initiator of sex determination (a gene called *Sex-lethal*) is not regulated in a sex-specific manner, yet the genes acting immediately downstream of *Sxl* in the pathway act similarly to genes in the *Drosophila* sex determination pathway (Pane et al., 2002; Saccone et al., 1998). Initially, only non-sex specific regions of *fru* cDNA, coding for a BTB domain, were found in *C. capitata* (Davis, Kurihara, & Yamamoto, 2000). Eventually, male-specific *fru* transcript was isolated and found to occur only in male fly heads, suggesting that the *C. capitata fru* gene is regulated by sex-specific alternative splicing as with *D. melanogaster fru*, and begins with a P1-like exon (Salvemini et al., 2009). The *fru* gene is most likely also involved in *C. capitata* male sexual behaviour

because when *tra* and *tra-2* functions are impaired, masculinized female adults exhibit male mating behaviours. Male-specific mRNAs of *fru* were detected in masculinized females, indicating that *Ceratitis* may produce sex-specific *fru* mRNA. It is therefore possible that the *fru* gene may be involved in *Ceratitis* courtship regulation, as in *Drosophila* (Salvemini et al., 2009).

Moving outside of the Dipteran order, *Nasonia vitripennis*, a wasp in the Hymenoptera order, was discovered to have a highly conserved *fru* locus. The transcript architecture and sex-specific transcript splicing pattern of *N. vitripennis* are almost identical compared to *Drosophila*. As with *Drosophila*, *N. vitripennis fru* transcripts begin with differing first exons, found in both sex specific and non-specific transcripts, which are then linked to the BTB coding, connecting, and alternative zinc finger domains coding exons. P1 transcripts in this species undergo sex-specific splicing, and *fru* sex-specific splicing in the head of adult gynandromorphs follows the sex of the tissue (Bertossa et al., 2009; Kamping et al., 2007). A unique quality of *fru* in Hymenoptera is a large female-specific *fru* transcript found in *N. vitripennis* and unique nucleotide repeats in both *N. vitripennis* and *Apis mellifera* (honeybee) are found in the female-specific P1-S exon that may correspond to recognition sites for regulatory factors involved in the activation of female-specific splicing (Bertossa et al., 2009). The findings that have come from studying *fru* in *N. vitripennis* suggest that *fru* sex-specific splicing either was acquired independently by Hymenoptera and Diptera or evolved prior to the split between the two orders (250–300Myr) (Bertossa et al., 2009).

Even more distant from *D. melanogaster*, is the Lepidopteran order. This order is extremely diverse, consisting of holometabolous insects that have a mechanism of sex determination very different from Dipterans (Suzuki, 2010). *Bombyx mori* (silkworm moth), a lepidopteran model species, uses a ZZ/WZ sex determination system, where the homogametic sex (ZZ) is male and heterogametic sex (WZ) is female (Hasimoto, 1933). *B. mori*'s sex determination pathway appears to use some of the same gene products involved in the first steps of the *Drosophila* sex-determination pathway, but implements them in different ways, specifically in how they affect alternative splicing (Ohbayashi et al., 2001; Suzuki, 2010; Suzuki et al., 2001). *B. mori* has a complex mating dance that

involves wing vibrations, and its *fru* homologue may be implicated in the development of the male-specific neural network involved in the control of this behaviour (Kanzaki, 1998; Sasaki et al., 2009). The *fru* homologue has a well conserved BTB-domain and differing first exons, and when alternatively spliced produce multiple FRU isoforms. Differences in expression of *fru* are observed between the sexes of *B. mori*, suggesting sex-specific functions (Fujii & Shimada, 2007; Ohbayashi et al., 2001). A recent study has also implicated *B. mori fru* in mating behaviour. When the gene is mutated, males have a reduced perception of certain pheromones, implicating *fru* in pathways that regulate olfactory-based sexual behaviour (Xu et al., 2020).

The *fruitless* gene was also identified in hemimetabolous insects, which undergo incomplete metamorphosis, suggesting that the gene arose prior to the divergence of hemi- and holometabolous insects. These insects are separated by about 350 million years of evolution from *Drosophila* (holometabolous), which undergo complete metamorphosis and have distinct larval – pupal – adult stages. In 2006, a partial *fru* gene was discovered in three closely-related grasshoppers species, *Chorthippus biguttulus*, *C. brunneus*, and *C. mollis* (Ustinova & Mayer, 2006). This partial *fru* gene has similar organization to *fru* in holometabolous insects, with conserved BTB and zinc finger domains separated by a non-conserved linker region (Salvemini et al., 2010; Ustinova & Mayer, 2006). Similarly, alternative splicing leads to the production of multiple transcripts of *fru* in grasshoppers, but unlike holometabolous species, the *fru* gene only generates non-sex-specific transcripts. Due to this, researchers are unsure whether the *fru* gene in grasshoppers is involved in the sex determination pathway or the control of male behaviour. Additionally, a unique feature of the *fru* gene was discovered in these grasshoppers, where many closely related *fru* paralogues exist in their genomes. Similar to alternative splicing regulating the production of different transcripts and functions of *fru* in *D. melanogaster*, these *fru* paralogues could potentially adopt different functions in grasshopper species (Salvemini et al., 2010; Ustinova & Mayer, 2006). In other hemimetabolous insects, such as the desert locust *Schistocerca gregaria* and the cockroach *Blatella germanica*, *fru* orthologues were isolated but no sex-specific transcripts were detected. Despite this, experiments have revealed that *fru* orthologues play important roles in the regulation of

successful copulation in the adult male and in male sexual behaviour in both species (Boerjan et al., 2011; Clynen et al., 2011).

The analysis of the *fru* gene across many insect species shows that although there are structural and functional differences between species, the sex-specific alternative splicing that defines male sexual behaviour in *Drosophila* hasn't changed drastically for the past 350 million years. Many studies on insects, including *Drosophila*, have established that the proteins coded by genes on sex chromosomes vary extensively across species; whereas proteins more downstream in the pathway, coded by autosomal genes, are more highly conserved (Reveiwed in Yang et al., 2021). Therefore, it is not surprising that the sex determination pathway is conserved in these insects over hundreds of millions of years. There is a theory that evolution of developmental pathways, such as the sex determination pathway, follows an "hourglass" pattern, where the most upstream and downstream components of developmental pathways diverge more rapidly, while the middle components, such as the *tra* gene, are more highly conserved (Hazkani-Covo et al., 2005). Although *fru* has maintained its integral function in male sexual behaviour over millions of years of evolution, it may have developed other functions in *D. melanogaster* such as in development and viability.

1.7 Using *Drosophila* to identify the function of *fru* proteins

Drosophila melanogaster is a highly dynamic model organism. There are many tools that have been discovered and made to manipulate their genome, which allows for identification of the function of the non-sex-specifically spliced transcripts of *fru*. Additionally, the developmental stages of *Drosophila* are very well characterized, allowing for a precise determination of when lethality may be occurring in flies with a specific *fru* transcript disrupted.

1.7.1 Developmental stages in *D. melanogaster*

Drosophila melanogaster begin as eggs, which hatch into larvae after approximately 16-24 hours (Ashburner, 2005). These larvae molt twice at approximately 24 and 48 hours,

remaining in their larval state for approximately 4 days. Around the 4-day mark, the larvae will form a puparium that encapsulates their body. For the next 4 days, the larva goes through metamorphosis, eventually forming the adult fly. This stage includes shortening of the larva's body, eversion of the anterior spiracles, and tanning of their skin. The puparium forms by developing a hard larval skin that encapsulates the third instar larvae within, making them immobile until they reach adult form. The puparium is soft and white when it is first formed, darkening and hardening over time. Around the 4 to 6 hour mark, apolysis, which is the retraction of the epidermis from the cuticle of the previous instar, occurring anteriorly to posteriorly (Jenkin & Hinton, 1966). A characteristic of this stage is the formation of a gas bubble in the abdomen. After apolysis, 12 hours after pupariation, the developing organism is technically a pupa. This stage is characterized by the dispersion of the gas bubble, the appearance of an air bubble under the puparium and head eversion. 24 hours after pupariation, pupal/adult ecdysis begins. A detailed summary of 15 stages (P1-P15) is reviewed in Ashburner (2005). At the end of their development, an adult fly will eclose from the puparium, forming an adult fly. There are many genetic tools that can be introduced in early developmental and adult stages to allow for molecular manipulation of *D. melanogaster*.

1.7.2 Balancer chromosomes

Balancer chromosomes, also referred to as balancers, are genetically engineered chromosomes that are used in laboratory setting in organisms to maintain recessively lethal mutations (reviewed in Miller et al., 2019). Under circumstances when not dealing with recessively lethal mutations, *Drosophila* can be bred to maintain a mutation with homozygosity. This is not possible with a recessively lethal mutation as homozygosity causes lethality in early developmental stages and these mutations are selected against. This is where the use of balancer chromosomes becomes integral to maintaining and studying recessively lethal mutations. Balancers are composed of a few key components that make this possible, including multiple inversions and rearrangements which constrain recombination from occurring near inversion break points and prevent recovery of recombinant offspring as recombinants are inviable. The presence of recessive lethal

or sterile mutations on balancers ensure that the homologous chromosomes paired with the balancer are never lost through homozygosity of the balancer, as homozygosity causes lethality. Therefore, mutations of interest can be maintained through heterozygosity in stock. Lastly, the presence of a dominant visible phenotypic marker allows for visual tracking of a balancer through generations (reviewed in Miller et al., 2019). These components come together to make it very difficult to lose mutations through selection when balanced. Although balancers can be extremely useful in maintaining stocks with transgenics, they can sometimes be detrimental to the health of *Drosophila* and cause effects that get in the way of observing results in behavioural or developmental assays. It is therefore best to remove them in experimental flies and only use them for maintenance of fly stocks.

1.7.3 RNA interference (RNAi)

Gene silencing is a gene regulatory mechanism, capable of knocking down the abundance of cellular transcripts. Achieving this outcome can take one of two pathways: the suppression of transcription or the activation of a sequence-specific RNA degradation process. Across many organismal kingdoms, different forms of RNA degradation have been identified, encompassing processes such as suppression or post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference (RNAi) in the animal kingdom. RNAi was first characterized in the nematode *C. elegans*, where it evolved as a response to small double-stranded RNA (dsRNA), resulting in the highly specific silencing of genes (Fire et al., 1998).

RNAi is an intricate, multi-step process (Figure 2). When dsRNA enters cells, it is initially recognized and processed into 21–23 base-pair small interfering RNAs (siRNA) by Dicer, a member of the RNase III family of ribonucleases. These short interfering RNAs are subsequently integrated into the RNA-induced silencing complex (RISC), a nuclease complex, which is tasked with targeting and ultimately destroying the corresponding RNA, thereby silencing the gene in question (Sontheimer, 2005). siRNA oligonucleotides, typically comprising 21–22 base pairs, can be synthesized chemically (Micura, 2002) or generated through in vitro transcription using T7 RNA polymerase (Yu

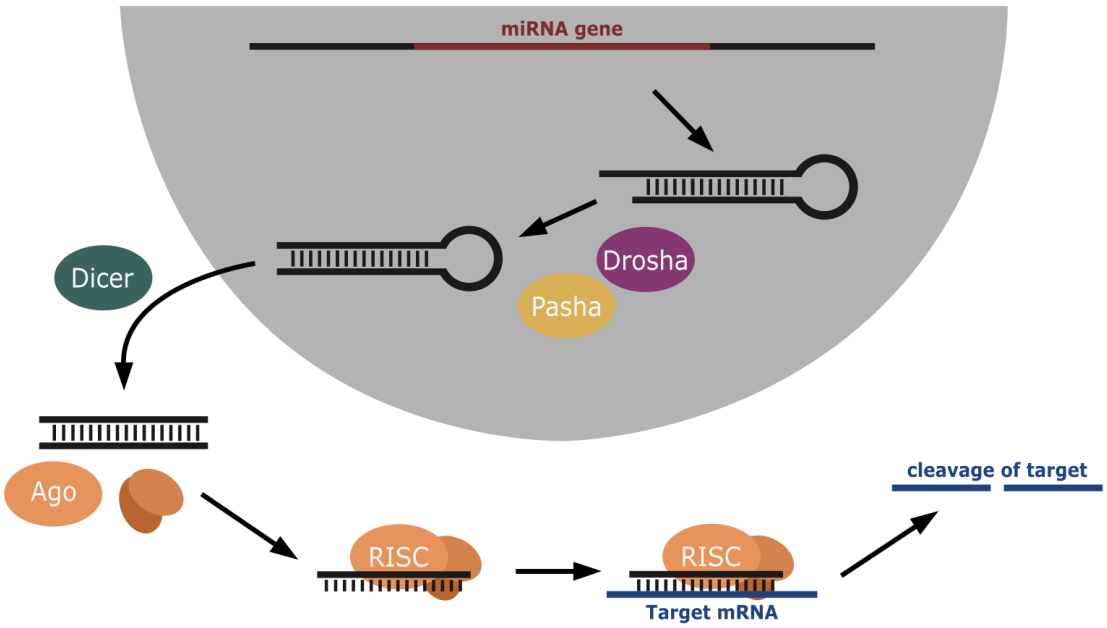

Figure 2. Processing of shRNA with RNAi using the RISC complex

An illustration of how short hairpin RNA (shRNA) molecules are processed, and RNAi is activated. The grey rounded region represents the nucleus. Micro-RNA (miRNA) genes are transcribed in the nucleus into primary miRNA. These molecules are processed by Drosha and Pasha into pre-miRNA and exported out of the nucleus. Dicer unwinds the pre-miRNA and separates the active antisense strand from the passenger strand. The antisense RNA is then incorporated into the RNA-induced silencing complex (RISC) which then finds and cleaves complementary mRNA.

et al., 2002). Alternatively, siRNAs can be endogenously expressed in the form of short hairpin RNA (shRNA) and introduced into cells using plasmids or viral/bacterial vectors (Brummelkamp et al., 2002). Synthesis of siRNAs in the laboratory is a fairly quick and simple process, making them easy to generate. In recent years, several commercial manufacturers have streamlined siRNA oligonucleotide synthesis, making them readily available and greatly simplifying use of synthetic siRNA in research (Han, 2018). Alternatively, numerous resources are available for designing shRNAs and developing corresponding plasmids or viral/bacterial vectors, allowing for easily replicable procedures (e.g., Chang et al., 2014).

Small-interfering RNA and the RISC complex can be combined with other genetic tools, such as the GAL4/UAS system, to create a knockdown of a specific transcript or gene in differing cells or tissues within *Drosophila*.

1.7.4 The Gal4/UAS system

P3 and P4 transcripts are predicted to affect development and have implications in viability (Anand et al., 2001). Therefore, removing the function encoded by these transcripts individually, even partially, would give insight into their individual implications in development and/or viability. A binary system, using a target gene and activator, like the Gal4/UAS system can be used to accomplish this goal (Figure 3). This tool is commonly used in *D. melanogaster* and can be used for targeted expression, repression, or knockdown of specific genes in tissues of interest. A transcriptional activator only found in *Saccharomyces*, Gal4, is used to avoid any endogenous targets in *Drosophila*. Gal4 binds an upstream activation site (UAS) enhancer to promote transcriptional initiation of the gene of interest. Only genes/activation sites that contain binding sites for Gal4 can be activated by this system (Fischer et al., 1988), making the activation of this system very specific.

Additionally, the binary nature of this tool ensures that the parental strains remain viable as it separates the target transgene from its transcriptional activator (Brand & Perrimon, 1993). Therefore, if the system you are testing causes inviability the pieces can be maintained separately and tested only when crossed together. One of the parental strains has a UAS construct containing the gene or sequence of interest (shRNA) downstream of the UAS enhancer integrated into its genome. The gene of interest remains silent without an activator protein to bind the UAS enhancer. In the other parental strain, the activator protein, Gal4, is inserted into its genome, where it remains present but does not have a target gene to activate. The Gal4 is linked to a protein-specific activator sequence, meaning it will only be expressed where the protein is present within the organism (Fischer et al., 1988). When the parental strains are crossed together, the target gene can be turned on and expressed in the tissues with both the activator protein and transgene

present. Within the individual GAL4 and UAS construct lines, the baseline expression remains minimal or absent, as neither GAL4 nor UAS elements are endogenous to *D. melanogaster* (Fischer et al., 1988).

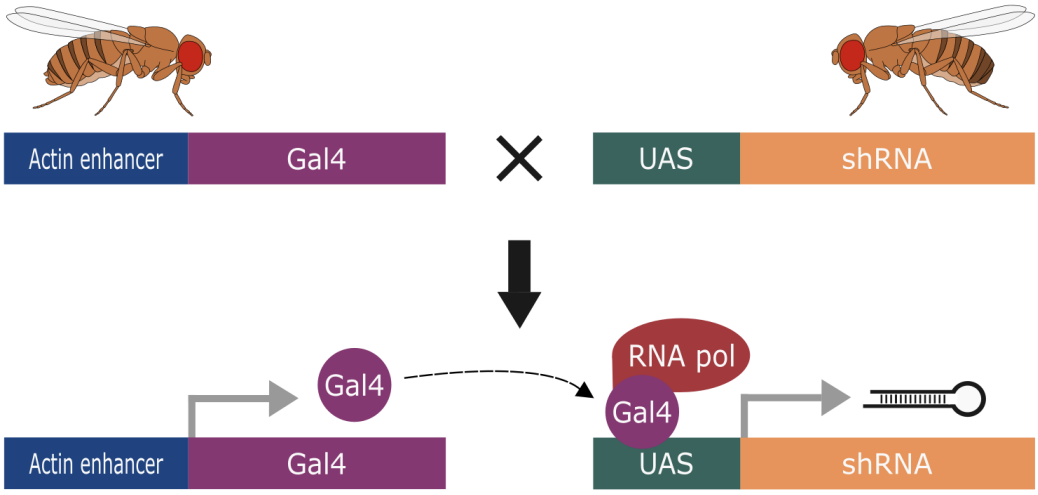

Figure 3. Driving expression of an shRNA using the Gal4/UAS system ubiquitously. The coloured bars represent transgenes within each fly line, with different transgenes in the female (top left) and male (top right) that when mated together are combined in the offspring (bottom). A driver line, which contains the Gal4 gene paired with a tissue or non-tissue-specific promoter (Actin enhancer), is crossed to an effector line that contains the gene of interest (shRNA) downstream of an upstream activating sequence (UAS). When the Gal4 protein (purple circle) binds to the UAS, it drives expression (via RNA polymerase) of the gene of interest. The gene of interest is not expressed in the parental lines because either the gene or driver is not present. The offspring of the cross contain both components however, so the gene of interest is expressed at high levels in the tissues dictated by the tissue-specific promoter.

Another advantage of this system lies in its flexibility, as the activator protein is custom-designed and introduced separately. Consequently, numerous activator-expressing lines (Gal4 lines) can be created and subsequently crossed with the line containing the gene or sequence of interest. This approach allows for the observation of gene expression, or its

absence, in various cell or tissue types, making it an exceedingly versatile tool. A multitude of transgenic lines containing GAL4 or UAS constructs have been generated and are readily available through Drosophila stock centers. For example, the Bloomington Drosophila Stock Center (BDSC) alone houses a repository of over 8000 GAL4 lines and more than 7000 UAS construct lines. Notably, GAL4 and UAS constructs can be integrated anywhere in the genome, and combinations of multiple GAL4 and UAS constructs can be used to simultaneously activate multiple effectors and/or reporters. One such effector that can be driven by this system is siRNA, which can be used in RNAi to selectively silence the expression of genes of interest.

1.8 Determining if *fru* P3 or P4 have an impact on development in *D. melanogaster*

In regards to *fru* P3 and P4 function, Anand et al. (2001) was able to indirectly determine that the P3 transcripts had implications in viability, but did not test a clean removal of P3 transcripts nor test *fru* P4 functions. There are no other studies that investigate the functions of these non-sex specific transcripts and therefore, there is very little indication or knowledge about the function of the P3 and P4 transcripts. Additionally, although P3 transcripts may be involved in viability, this cannot be confirmed due to the transcript not being independently targeted. The functions of P3 and P4 transcripts will only be able to be determined through their individual knockdown and assessment. My project's goal was to create independently targeted P3 or P4 transcript knockdown lines by utilizing RNA interference (RNAi) in combination with the Gal4/UAS system.

2 Methods

2.1 Fly husbandry/stocks

All transgenic *D. melanogaster* stocks were acquired from the BDSC (Bloomington, Indiana) or made within our laboratory. The wildtype strain, BJS, was obtained from B. Sinclair Laboratory, Western University. All stocks and genotypes are listed in Table 1. All strains, injected embryos, and assays (see below) were conducted on standard cornmeal-agar media (BDSC recipe) at 25°C, ~70% relative humidity, and a 14h:10h light-dark cycle.

2.2 Preparing shRNA constructs

2.2.1 DNA extraction/sequencing

P3 and P4 first exon regions in the two fly strains used to create the RNAi lines, the balancer stock (#3703) and the *Act5C*-Gal4 stock (#3954; Table 1), were sequenced following standard protocols. Sequences were obtained from these two strains to determine conserved regions of the P3 (F: 5'CACATGCGAAATTCACCGCT; R: 5'ACCGACATTCCTTTCTGTTTCT primers) and P4 (F: 5'GGACAGTGCTGCGTTCCTTA; R: 5'CTCCGGTGGGTCTTGTGATG primers) exons to subsequently design corresponding shRNA to knockdown P3 or P4 expression in the assay flies. DNA was purified using the Monarch DNA Gel Extraction Kit Protocol (NEB #T1020) and sequenced at Robarts Research Institute (London, ON).

2.2.2 Short hairpin RNA design

The targeting sequences of P3 and P4 shRNAs were designed using Invivogen siRNA Wizard Software. The P3 or P4 unique first exon regions of the transcripts were targeted to allow for RNAi knockdown of these transcripts without affecting other transcripts from the *fru* gene, lowering the chances of off-target effects. BLASTn was used to assess possible off-target binding. Any of the potential targeting sequences that shared 18 or more nucleotides with other transcripts in the *D. melanogaster* exome were not used due to potential off-target effects. P3 and P4 "Sham" targeting sequences were also designed

to use as a control: a scrambled version of their corresponding targeting sequence with no genomic or transcriptomic targets, using the same Invivogen software and assessed for off-targeted effects as above.

To complete the design of shRNA strands, targeting sequences of P3 and P4 shRNAs had additional sequence added to them, using an altered version of the protocol described by Chang *et al.* (2014). The shRNA hairpins were created by adding "tagttatattcaagcata" to the 5' end of the antisense strand, and then adding the complement of the antisense sequence to the 5' end of the added sequence. This is considered the "top" strand. The "bottom" strand was generated by taking the complimentary sequence of the "top" strand. To integrate the hairpin constructs into the selected vector, NheI and ExoRI sites were added to the 5' and 3' ends of the hairpins: 5'CTAGCAGT and 3'GCG added to the ends of the "top" strands, and 5'AATTCGC and 3'ACTG added to the ends of the "bottom" strand (Table 2).

2.2.3 Short hairpin RNA vectors

The P3, P4, P3-Sham, and P4-Sham shRNAs were integrated into the plasmid pVALIUM20, a gift from Dr. Norbert Perrimon and Dr. Jian-Quan Ni, using an altered version of the protocol described by Chang *et al.* (2014). Steps were followed in the "designing nucleotides" and "Cloning miR-1-Based shRNAs" sections, using different reagents for cloning. The pVALIUM20 vector contains a visible selectable marker (*vermilion* eye colour mutation rescue) making it easy to track, an *attB* site for targeted integration of the vector using ΦC31 integrase, UAS sequences, and an *AmpR* element (Groth et al., 2004; Isaacson, 2018).

The vector was initially double digested using restriction enzymes NheI and EcoRI, obtained from New England Biolabs (NEB), and optimized using the NEB double digest tool (https://nebcloner.neb.com/#!/redigest). New England BioLabs Ligation protocol with T4 DNA ligase and high efficiency transformation protocols, #M0202 and #C3019I respectively, were used to integrate the shRNA sequences into pVALIUM20 plasmids and transform the recombinant plasmid into 10-Beta *E. coli* cells following standard protocol. One hundred μL of the cell-media mixture was plated onto lysogeny broth (LB)

+ampicillin plates (100μL/mL) and grown for 16 hours at 37°C. Six colonies were then selected and grown in 4 mL of LB+ampicillin (100μL/mL) for 16 hours. Plasmid DNA was extracted using the Purelink Quick Plasmid Miniprep Kit (Invitrogen). Primers flanking the shRNA insertion region (F: 5'ACCAGCAACCAAGTAAATCAAC and R: 5'TAATCGTGTGTGATGCCTACC primers) were used to PCR amplify then visualize successful shRNA insertion on an agarose gel. A touchdown PCR with an annealing temperature beginning at 70°C and decreasing by 0.8°C for 15 cycles, ending with 25 cycles with an annealing temperature of 58°C. Successful ligation and transformation were visualized through a 350 bp RCR product, whereas unsuccessful transformants were observed as 900 bp PCR product (Figure 6). Isolated plasmids presenting ~350 pb PCR products were sequenced for verification using the forward primer.

2.3 Microinjections

2.3.1 Microinjection preparation

Protocol followed exactly as in Yeung (2021). In brief, plasmids were extracted from 3 mL of the P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, and P4 UAS-Sham ligated pVALIUM20 *E. coli* cultures using the Purelink Quick Plasmid Miniprep Kit (Invitrogen). The plasmids were concentrated using the same miniprep kit by binding >40 μg of purified plasmid to a spin column using W10 buffer, followed by wash steps as per manufacturer protocol and elution with Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water. Generic blue food dye was filter-sterilized using a Whatman Puradisc™ 0.2 μm filter then autoclave-sterilized within a glass syringe. A microinjection mix containing P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, or P4 UAS-Sham ligated pVALIUM20, Ultrapure Water, and filter-sterilized food dye was made (see Yeung, 2021 for full recipe). The mixes were lightly vortexed, centrifuged at 16000 RCF for 15 minutes to pellet any solids, and the supernatants were transferred, aliquoted, and stored at -20 °C until used for microinjection.

Loading needles were prepared by heating and hand stretching 1.0 mm inner diameter borosilicate capillary tubes (FHC) over a Bunsen burner. Injection needles were prepared

from 0.75 mm ID borosilicate capillary tubes (FHC) using a Sutter Instrument Micropipette Puller P-97 (courtesy of Greg Gloor). The unstretched end of the loading needle was inserted into a rubber bulb for aspiration of microinjection mix and subsequent transfer into injection needles.

To create the transgenic stock that produces shRNA targeting P3 or P4 exons, injections were done into embryos from a stock expressing phiC31 (ΦC31) integrase in the germ line, as well as an *attP* site in the left arm of the third chromosome (Stock #25709: y^1 v^1 *P{nos-phiC31\int.NLS}X; P{CaryP}attP40*). This system allows for a plasmid containing the shRNA of interest to be integrated into a compatible genomic site in the *Drosophila* embryo genome, using the actions of PhiC31 integrase (Chang et al., 2014). Approximately 400 injection line flies were transferred into an embryo collection cage (Genesee Scientific) on a petri dish with apple juice agar media smeared with active yeast paste (hereafter referred to as apple yeast plate). They were kept in the collection cage for three days to acclimate to their environment.

2.3.2 Microinjection protocol

Microinjections were conducted by William Yeung, following the protocol in Yeung (2021). Before beginning the first round of injection, the apple yeast plate in the embryo collection cage was replaced every 20 minutes for an hour to acclimate the flies to plate changing. This process also leads to the flushing of older eggs, limiting the number used in the injection process. Following this acclimation period, plates were changed 30 minutes before the next injection round. A fine synthetic paintbrush and deionized water were used to brush and remove embryos from the previous apple yeast plate. Eggs were aligned on a 22 x 22 mm glass coverslip, with embryos positioned laterally adjacent to one another and with posterior ends facing one edge of the coverslip. Embryos that had visibly reached cellularization, the developmental change from a single multinucleate cell into a multicellular blastoderm (Lecuit & Wieschaus, 2000) were excluded. The coverslip with aligned embryos was air desiccated for approximately 5 minutes to evaporate excess water and to allow adhesion of the embryos to the coverslip surface. During this time, an injection needle was filled with the prepared microinjection mix using a loading needle and installed into a Sutter Instrument ZenoWorks Digital Microinjector. Following

desiccation, the embryos were coated with generic extra-virgin olive oil and positioned for injection under a Nikon SMZ1500 stereoscopic microscope. The injection needle was opened and injection mix was injected into the posterior end of each embryo. Microinjector settings were adjusted to prevent backflow but varied for each round of injection due to variability in the gauge of injection needle tips.

Olive oil was gently rinsed off of the injected embryos using deionized water from a wash bottle, and excess water was wicked using Kimtech Science™ Kimwipes™ Delicate Task Wipers. The cover slip was placed into a standard cornmeal food vial with anterior ends of the embryos oriented towards the food and positioned 1-2 mm from the food surface. Vials containing embryos were incubated the same conditions as for stock maintenance, listed above, until reaching late-pupal stage. At late-pupal stage, the microinjection survivor (G0) pupae were sexed and separated into individual vials to ensure virginity.

2.4 Post-injection care and screening

2.4.1 Screening and balancing RNAi lines

Once eclosed, virgin injected *D. melanogaster* were crossed to other virgin adults that were injected with the same shRNA construct. This leads to an increased likelihood that the next generation (F1) would contain the shRNA construct. Adults were also crossed to the parental stock (#25709; Table 1) when no injected adults of the opposite sex were available. This was done to ensure proper screening for the construct in the next step (Figure 4).

The injection stock has the *vermilion* eye mutation while the injected plasmids contain functional copy of the *vermilion* gene. Offspring of injected flies were therefore screened for the construct through the presence of wildtype eyes, in comparison to the *vermilion* coloured eyes of non-transgenic flies. Fly stocks containing the visual marker were also confirmed via PCR, with PCR products of 350bp confirming the presence of the construct, as stated above. Once created, these lines were crossed to a stock containing multiple balancers (Stock #3703: w^{1118}/*Dp(1;Y)*y^{+}*; CyO/nub*^{1} *b*^{1} *sna*^{Sco} *lt*^{1} *stw*^{3}*;*

MKRS/TM6B, Tb^{1}) to prevent loss of and track the transgene over many generations (Figure 4). This created the stable P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, and P4 UAS-Sham stocks.

2.5 Creating experimental groups

To generate the flies used in experimental assays, the UAS-RNAi and UAS-Sham stocks were made homozygous and crossed to a stock with ubiquitous *Act5C*-Gal4 expression (Stock #3954: *y*^{1} *w*^{1}*; P{w*$^{+mC}$*=Act5CGAL4}17bFO1/TM6B, Tb*^{1}), ensuring that approximately 50% of the flies used in assays had both the Gal4 and UAS components of the Gal4-UAS system, and not a balancer chromosome. The groups created were P3 UAS-RNAix*Act5c*-Gal4, P3 UAS-shamx*Act5c*-Gal4, P4 UAS-RNAix*Act5c*-Gal4, and P4 UAS-shamx*Act5c*-Gal4 (Figure 5). The P3 UAS-RNAix*Act5c*-Gal4 and P4 UAS-RNAix*Act5c*-Gal4 were my test genotypes, while the P3 UAS-shamx*Act5c*-Gal4 and P4 UAS-shamx*Act5c*-Gal4 were used as controls. I was unable to generate stable stocks containing both the UAS-RNAi and Gal4 components due to inviability or loss of the transgene, therefore the UAS-RNAi and Gal4 lines were kept separately and crossed each time an assay was conducted.

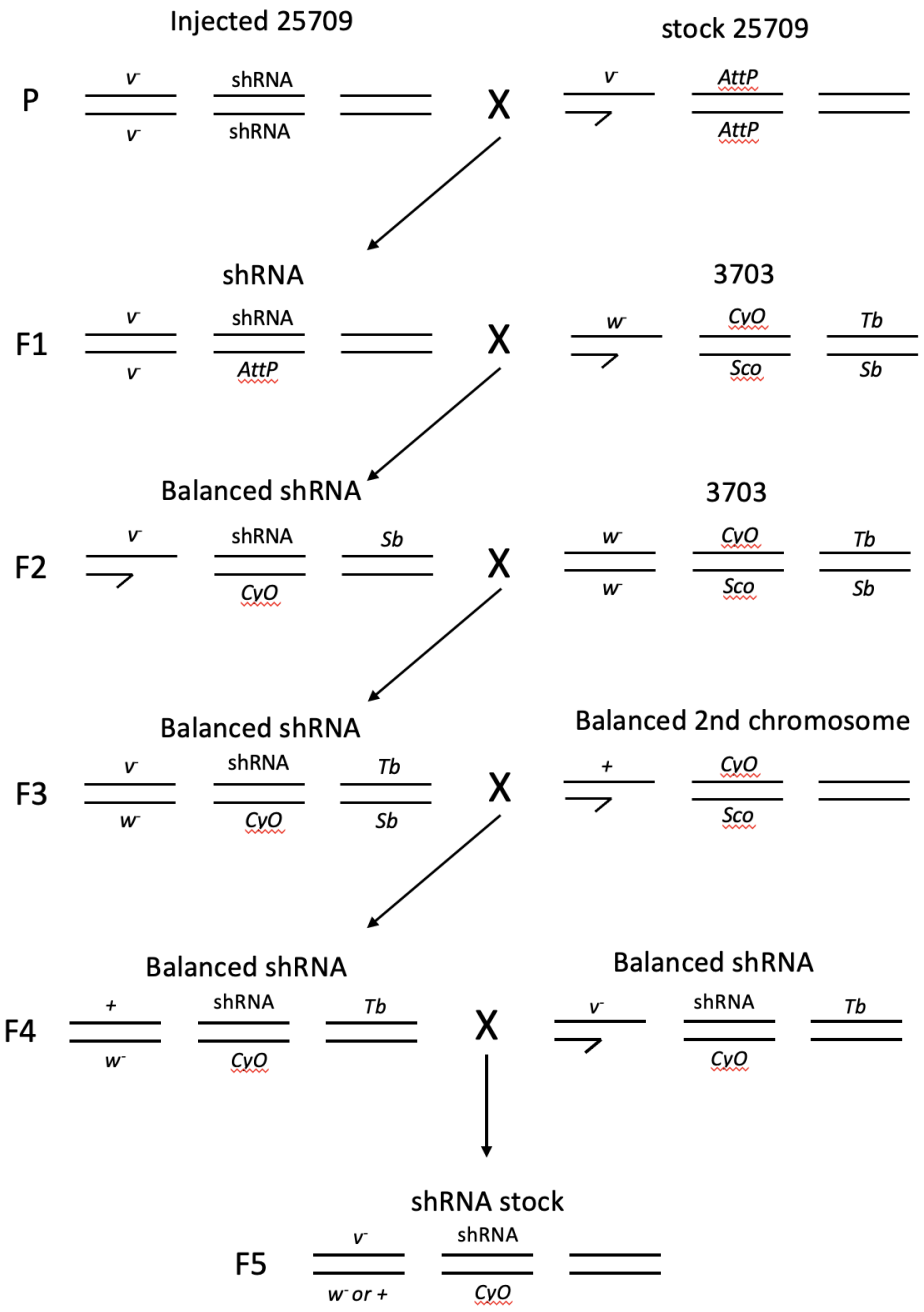

Figure 4. Crossing scheme used to make the stable shRNA stocks.

The three main chromosomes within the genomes of the flies being crossed are shown as horizontal lines, with both homologs shown for each chromosome (parallel lines). The Y chromosome is shown as a bent line. Mutations or genetic constructs are written above or below the corresponding homolog. The injected stock is crossed back to the injection stock (non-injected). Male progeny with the shRNA are selected for and crossed to a

balancer stock (#3703) to balance and maintain the shRNA. Balancer chromosomes are marked by their phenotypic marker; TM6B as Tb, MKRS as Sb, and CyO as CyO. Once fully balanced, the balanced shRNA is crossed to a 2nd chromosome balancer stock with a wildtype third chromosome to remove balancers from the 3rd chromosome. After two generations the final shRNA stock (P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, or P4 UAS-sham) is complete, balanced over CyO.

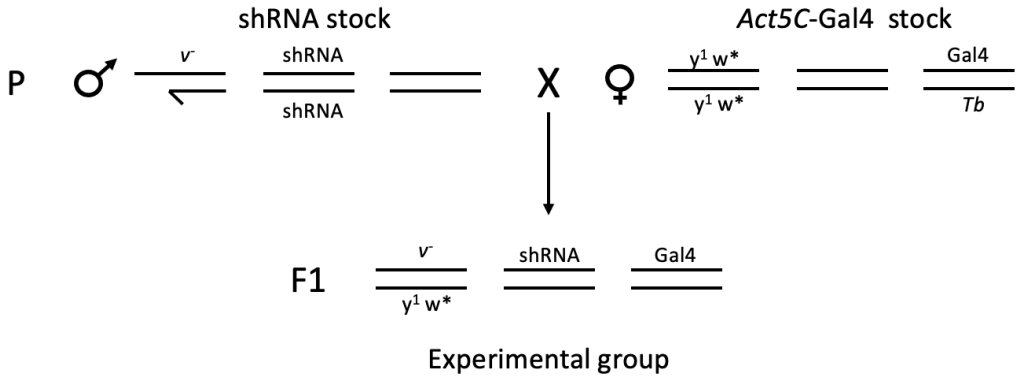

Figure 5. Crossing scheme for the cross used to make the experimental group.

Crossing scheme for the cross used to make the experimental group (Knockdown and sham) used for RNA extraction for qPCR analysis and developmental assays. The three main chromosomes within the genomes of the flies being crossed are shown as horizontal lines, with both homologs shown for each chromosome (parallel lines). The Y chromosome is shown as a bent line. Mutations or genetic constructs are written above or below the corresponding homolog. A shRNA stock (P3 UAS-RNAi, P3 UAS-sham, P4 UAS-RNAi, or P4 UAS-sham) crossed to the *Act5c*-Gal4 stock (Stock #3954), creating the experimental group at the bottom containing both the UAS-shRNA and Gal4. Crossing scheme depicts the two homologs the three chromosomes of *D. melanogaster* and any genetic markers/tools of note found on each chromosome. Balancer chromosomes are marked by their phenotypic marker; TM6B as Tb.

2.6 Quantifying *fru* P3 and P4 transcript expression

2.6.1 RNA extraction

RNA extractions were performed on samples of *D. melanogaster* at the early pupal stage, collected 24-36 hours after pupariation. Three biological replicates from each of the transgenic strains (P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, P4 UAS-Sham), the *Act5C-Gal4* strain, and the four transgenic UAS strains crossed to *Act5C*-Gal4 were extracted (N=12 pupae each), for a total of 27 samples. Samples were incubated at 25°C for an hour and then flash frozen by liquid nitrogen. An RNA extraction protocol by Elizabeth Allen, University of Massachusetts Medical School, adapted from the "Invitrogen Life Technologies Trizol manual" was used (Allen, 2016). RNA quality and quantity were evaluated using a NanoPhotometer P300 (Implen) spectrophotometer. RNA samples with a concentration greater than 1500 ng/μL, a 260/280 ratio of ~2.0, and 260/230 ratio above 1.8 were kept for future use. Complimentary DNA (cDNA) was synthesized using a Thermo Scientific Maxima H Minus cDNA Synthesis Kit with dsDNase (Thermofisher) in 40 μL total volume using 2 μg of RNA following the manufacturer's protocol. cDNA was visualized and assessed using primers for mRNA of the RpL housekeeping gene (Forward: 5'GGCATCAGATACTGTCCCTTG, Reverse: 5'CCAGTCGGATCGATATGCTAA primers), to observe consistency between samples.

2.6.2 Primer design and verification

Primers used for quantification of *fru*-P3 and *fru*-P4 mRNA expression in *D. melanogaster* were designed using Primer-BLAST (Table 3). They were further analyzed to ensure amplicon length fell between 100-150 bp, primers had GC content between 40 and 60% to increase binding stability and had a predicted melting temperature between 59°C and 68°C (Thornton & Basu, 2011). The reference gene primers that were selected correspond to Alpha-tubulin at 84B, spanning a 488bp intron (Table 3) (Ponton et al., 2011). Alpha-tubulin was verified for specificity and efficiency for quantitative PCR (qPCR) by Joshua Isaacson by assessing their standard curve and melt curve. Optimization of P3 and P4 primers was performed with a PCR thermal gradient (55–65°C) and annealing temperatures were assessed. Both P3 and P4 primers didn't have

visible off-target binding or primer dimers and had an optimal annealing temperature of 58-60°C. The efficiency and specificity of both primers were tested with a standard curve. Wildtype female *D. melanogaster* cDNA was diluted in a series of 10-fold dilutions and used in PCR reactions with P3 or P4 primers to build 5-log standard curves for each primer set. Each sample was loaded in triplicate, a no-template control added for each primer set. The PCR reaction ran for 40 cycles with an annealing temperature of 60°C using the CFX96™ Touch Real-Time PCR detection system. Efficiency, R^2 values and C_t values for melt curves were calculated using BioRad CFX Manager Software and evaluated prior to running experimental qPCRs.

2.6.3 Transcript quantification through qPCR

Quantitative PCR was performed to assess the expression levels of P3, P4, and the control gene *Alpha-tubulin*. Primer pairs were not multiplexed; amplifications were done separately but in parallel. Master mixes consisted of Thermo Fisher Scientific 10x PowerUp SYBR Green Master Mix, primer sets, and Nuclease free water. Five samples of cDNA were tested for each primer set: P3 UAS-RNAi x *Act5C*-Gal4, P3 UAS-RNAi, P3 UAS-Sham x *Act5C*-Gal4, P3 UAS-Sham, and *Act5C*-Gal4 and repeated for P4 cDNA samples. Each of the three biological replicates was repeated in two technical replicates. Samples were plated, sealed off with BIO RAD Microseal ‘B’ film and placed into the CFX96™ Touch Real-Time PCR detection system. A qPCR was run with an annealing temperature of 60°C for 39 cycles, and then one cycle with an annealing temperature of 65°C. Relative and normalized expression were assessed through BIO RAD CFX Manager 3.0 software (Version 3.0.1215.0601).

2.7 Developmental Assay

2.7.1 Assay overview

The time to transition between developmental stages and number of survivors to reach each stage of development was assayed. There were four groups tested in this assay, the P3 UAS-RNAi x *Act5C*-Gal4 line, and three control groups: P3 UAS-Sham x *Act5C*-Gal4 line, the P3 UAS-RNAi and *Act5C*-Gal4 lines on their own.

2.7.2 Survival and developmental time

For the P3 UAS-RNAi and *Act5C*-Gal4 control groups, 200+ flies were taken from each line and placed into their own separate embryo collection cage. For the P3 UAS-RNAi and P3 UAS-Sham lines that were crossed to the *Act5C*-Gal4 line, 200 female virgin flies were collected between the age of 1-6h from the *Act5C* -Gal4 line and put into an embryo collection cage with either 100 males from the UAS-RNAi or P3 UAS-Sham lines. The experimental group consists of the progeny retrieved from male P3 UAS-RNAi flies crossed to female *Act5C*-Gal4 flies, while the progeny from P3 UAS-RNAi, P3 UAS-Sham crossed to *Act5C*-Gal4, and *Act5C*-Gal4 strains were used as control groups.

After three days of acclimation in the embryo collection cage, an apple juice agar petri dish with active yeast paste was switched into the embryo collection cages and replaced every 30 minutes for two hours. After the 2-hour period, fresh plates were added and all groups were allowed to lay eggs for three hours. Two hundred to 250 eggs were collected from each plate and placed onto 22 x 22 mm glass cover slips, 40 eggs per cover slip. The cover slips were placed onto fresh food plates, separating each plate by treatment group. Plated eggs were incubated for 24 hours. The total number of eggs that hatched and reached the larval state was recorded for each treatment group. 288 test tubes (12x75mm) were filled with standard cornmeal food (1inch from base) 24 hours prior to loading and sorted into 4 tube racks. A total of 72 larvae were randomly chosen from each group and placed individually in a test tube filled with food. The test tube was marked with the genotype of the larva within, and using a random number generator, was given a randomized spot within the tube rack. After filling all 288 test tubes with larvae, the tube racks were placed in a 25°C incubator for the remainder of the assay. All test tubes were scored every 12 hours (11am/11pm) until all larvae reached the adult stage or died. The phenotype at death was recorded. The stage of development (larvae, pupa, or adult) was recorded for each individual every time they were scored. As expected, approximately 50% of the progeny from the crossed lines P3 UAS-RNAi or P3 UAS-Sham to *Act5C*-Gal4 did not possess the *Act5C*-Gal4 but a balancer chromosome instead and were eliminated from the assay. Mean time to reach each stage of development (larvae to pupae, pupae to adult), sex, and number of individuals that survived each developmental

stage were scored over the entire group of individuals and compared. This assay was repeated three times per treatment group, leading to 216 larvae per genotype being tested, and 864 individuals tested overall.

2.7.3 Comparing inviability between *fru* P3 knockdown and *fruitless* knockout groups

The phenotype observed at death in all *fru* P3 knockdown individuals (P3 UAS-RNAix*Act5C*-Gal4) was scored in the developmental assay. Once development arrested, the phenotype of the experimental group was recorded in as much detail as possible: axis that developed (anterior versus posterior), presence of pigmentation and basic anatomical features. The development of a *fruitless* knockout line (Stock #66692; Table 1) was also investigated to compare where development stopped between a complete knockout of *fru* and the knockdown of *fru* P3 transcript. This analysis was used to determine whether the removal of *fru* P3 transcript is sufficient to produce the phenotype observed when *fruitless* is null. Counts were conducted for both groups and the stage of death for the knockout flies was recorded and compared to the *fru* P3 knockdown line.

2.8 Statistics

2.8.1 Quantitative PCR

Expression data between the *fru* P3/P4 knockdown groups and control groups was assessed through the same BIO RAD CFX Manager 3.0 software used earlier. The *P*-value is calculated and statistical analysis is conducted with t-tests and one-way analysis of variance (ANOVA). *P*-values were set to a threshold of $\alpha = 0.05$. A Holm-Bonferroni analysis was conducted to correct for multiple comparisons (Holm, 1979). Relative normalized expression, comparing P3 or P4 transcript levels of the controls to the knockdown groups was plotted. The quantity of P3 or P4 transcript in the experimental groups was set to 1, the level of P3 or P4 transcript expression in the control groups demonstrating changes in transcript expression between groups. The relative expression of the transcripts are normalized to the reference gene (*Alpha-tubulin*).

2.8.2 Developmental time and survival analysis

For the developmental time analysis, individuals were scored every 12 hours, and these time measures were evaluated using a Wilcoxon rank sum test that was conducted between groups and within each developmental group ("Time to pupation" and "Time to adult" stages). For the survival analysis, assays were scores categorically for survival ("dead" or "alive"). The proportion survived was calculated for experimental and control groups and analyzed using a Fisher's exact test (2x2 contingency table) between each pair of genotypes. "Time-to-event" analysis is normally plotted through Kaplan-Meier curves. However, due to the nature of this data and the inability to know the exact point at which flies die during the pupal stage, Kaplan-Meier curves are not possible to plot for this data. *P*-values were corrected for multiple tests using Holm-Bonferroni analysis (Holm, 1979). All statistical analyses and graphical representation of development and survival data was performed using R 4.3.1 interfaced in RStudio 2023.06.0+421.

Table 1. Drosophila stocks.

Stock #	**Name**	**Genotype**
BJS	Wildtype	+; +; +
3954	*Act5C*-Gal4	$y^1\ w^*;\ P\{w^{+mC}=Act5C\text{-}GAL4\}17bFO1/TM6B,\ Tb^1$
3703	Balancer stock	$w^{1118}/Dp(1;Y)y^+;\ CyO/nub^1\ b^1\ sna^{Sco}\ lt^1\ stw^3;$ $MKRS/TM6B,\ Tb^1$
25709	phiC31 integrase	$y^1\ v^1\ P\{y^{+t7.7}=nanos\text{-}phiC31\backslash int.NLS\}X;$ $P\{y^{+t7.7}=CaryP\}Msp300^{attP40}$
P3 and P4	RNAi UAS lines for P3 or P4	$v^1;\ UAS\text{-}shRNA/CyO$
66692	*fruitless* knockout	$Df(3R)fru^{4\text{-}40},\ fru^{4\text{-}40}/TM6B,\ Tb^1$

Table 2. shRNA design. Capitalized bases correspond to targeting sequences. Lowercase bases between capitalized bases correspond to components that lead to the structure/formation of the hairpin. Bold lowercase bases correspond to restriction enzyme sites.

Arm	Sequence (5' to 3')
P3-RNAi shRNA (top strand)	**ctagcagt**GCTCGTCAAGCAACAACAAAtagttatattcaagcataTTTGTTGTTGCTTGACGAGC**gcg**
P3-RNAi shRNA (Bottom strand)	**aattcgc**GCTCGTCAAGCAACAACAAAtatgcttgaatataactaTTTGTTGTTGCTTGACGAGC**actg**
P3-sham shRNA (top strand)	**ctagcagt**ACTTAACCAGAGACCGAAACtagttatattcaagcataGTTTCGGTCTCTGGTTAAGT**gcg**
P3-sham shRNA (Bottom strand)	**aattcgc**ACTTAACCAGAGACCGAAACtatgcttgaatataactaGTTTCGGTCTCTGGTTAAGT**actg**
P4-RNAi shRNA (top strand)	**ctagcagt**GCGGCACTCTTAACCTATTTtagttatattcaagcataAAATAGGTTAAGAGTGCCGC**gcg**
P4-RNAi shRNA (Bottom strand)	**aattcgc**GCGGCACTCTTAACCTATTTtatgcttgaatataactaAAATAGGTTAAGAGTGCCGC**actg**
P4-sham shRNA (top strand)	**ctagcagt**ATAAGTAAGCGACTAACGTACtagttatattcaagcataGTACGTTAGTCGCTTACTTAT**gcg**
P4-sham shRNA (Bottom strand)	**aattcgc**ATAAGTAAGCGACTAACGTACtatgcttgaatataactaGTACGTTAGTCGCTTACTTAT**actg**

Table 3. qPCR primer sets with corresponding melting temperatures, and size of amplicon.

Target		Sequence	Tm °C	Size of amplicon (bs)
Alpha-tubulin	Forward	GCGTCGGTCAATTCAATCTT	60.08	138
	Reverse	AAGCTGCAACCTCTTCGTCA		
Fru-P3	Forward	TCATCAGCAAATGCCTCGTCG	62.3	198
	Reverse	CTCCTTGGTCAGTGTTGTACCTTG		
Fru-P4	Forward	TGGCTGCTGGAATCACTCTT	60.2	186
	Reverse	GGTCTGGTGAGCCTTGACTG		

3 Results

3.1 Creating *fru*-P3 and *fru*-P4 targeting vectors

All four shRNA constructs (knockdown and sham) were successfully cloned into the pVALIUM20 vector. The shRNA vectors were checked for size using PCR (Figure 6) and all constructs were verified through sequencing.

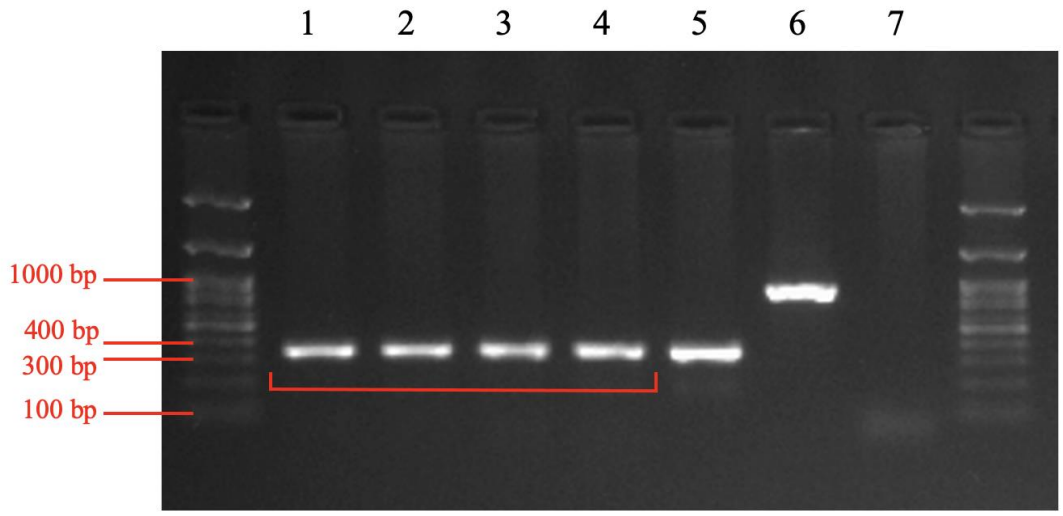

Figure 6. Confirming the accuracy of P3 and P4 targeting vectors

Agarose gel electrophoresis (1% agarose) of PCR amplified products using vector-specific PCR primer sets. **A)** pVALIUM20 vectors that contain the shRNA of interest produce a band ~350bp long, while untransformed vectors produce a band ~900bp long. Confirmation of P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, and P4 UAS-Sham are shown in this order (lanes 1, 2, 3, and 4). Experimental groups are compared to a positive control (lane 5), an undigested vector (Lane 6), and a no template control (Lane 7). Sample DNA was electrophoresed alongside 100 bp DNA ladder.

3.2 Verification of targeted vector insertion in *D. melanogaster* - Post microinjection

All four shRNA constructs (knockdown and sham) were successfully microinjected into *D. melanogaster* embryos and incorporated into their genome. The shRNA constructs were verified first using PCR to identify individual flies with the construct (Figure 7) and all constructs verified by PCR were subsequently verified through sequencing.

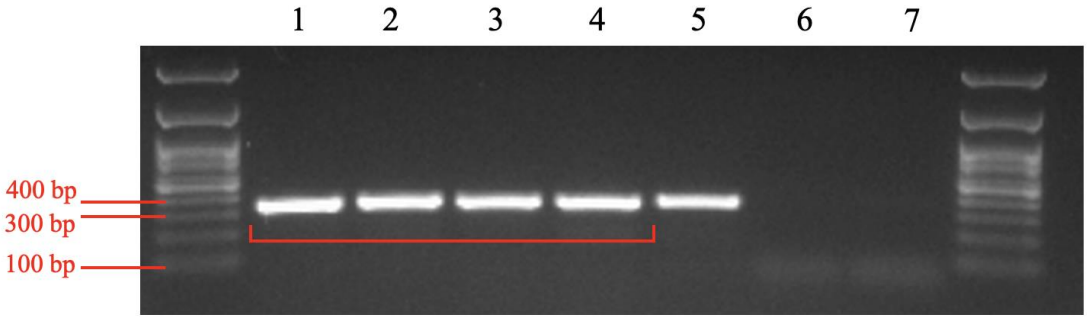

Figure 7. Confirming the integration of P3 and P4 targeting vectors in D. melanogaster genome
Agarose gel electrophoresis (1% agarose) of PCR amplified products using vector-specific PCR primer sets. **A)** *D. melanogaster* that have the vector integrated into their genome produce a band ~350bp long, whereas *D. melanogaster* without the vector integrated produce no band. Confirmation of P3 UAS-RNAi, P3 UAS-Sham, P4 UAS-RNAi, and P4 UAS-Sham are shown in this order (lanes 1, 2, 3, and 4). Experimental groups are compared to a positive control (lane 5), a control group without the vector integrated (Lane 6), and a no template control (Lane 7). Sample DNA was electrophoresed alongside 100 bp DNA ladder.

3.3 Quantitative real-time PCR results

3.3.1 Standard curve: Testing qPCR primers

After determining the optimal temperate through a temperate gradient and visualization of PCR of P3 and P4 primers (60 °C), a standard curve was conducted to further test the primers for efficiency and accuracy. Standard curve for P3 primers had an efficiency of

93.5% and an R^2 value of 0.995 (Appendix: Figure 1). Standard curve for P4 primers had an efficiency of 92.5% and an R^2 value of 0.995 (Appendix: Figure 2).

3.3.2 Expression of *fru* P3 or P4 transcripts in knockdown lines

Expression of P3 and P4 transcripts was quantified and demonstrated relative to the expression of transcript in the experimental groups (Figure 8 and 9). Normalized expression, relative normalized expression, and raw and adjusted *P*-values are listed in Table 4. Expression of P3 transcript was compared between P3 knockdown (P3 UAS-RNAi x *Act5c*-Gal4) and control groups (P3 UAS-Sham x *Act5c*-Gal4, P3 UAS-RNAi, P3 UAS-Sham, and *Act5c*-Gal4). P3 transcript levels were significantly lower in the knockdown group (P3 UAS-RNAi x *Act5c*-Gal4) compared to control groups (see adjusted *P*-values in table 4). The control groups had a range of ~3.5-8 times higher expression of P3 transcript compared to the knockdown group.

Expression of P4 transcript was compared between P4 knockdown (P4 UAS-RNAi x *Act5c*-Gal4) and control groups (P4 UAS-Sham x *Act5c*-Gal4, P4 UAS-RNAi, P4 UAS-Sham, and *Act5c*-Gal4). There was no significant difference between the experimental group and controls for the expression of P4 transcript, meaning that P4 transcript was expressed similarly between all groups (Table 11).

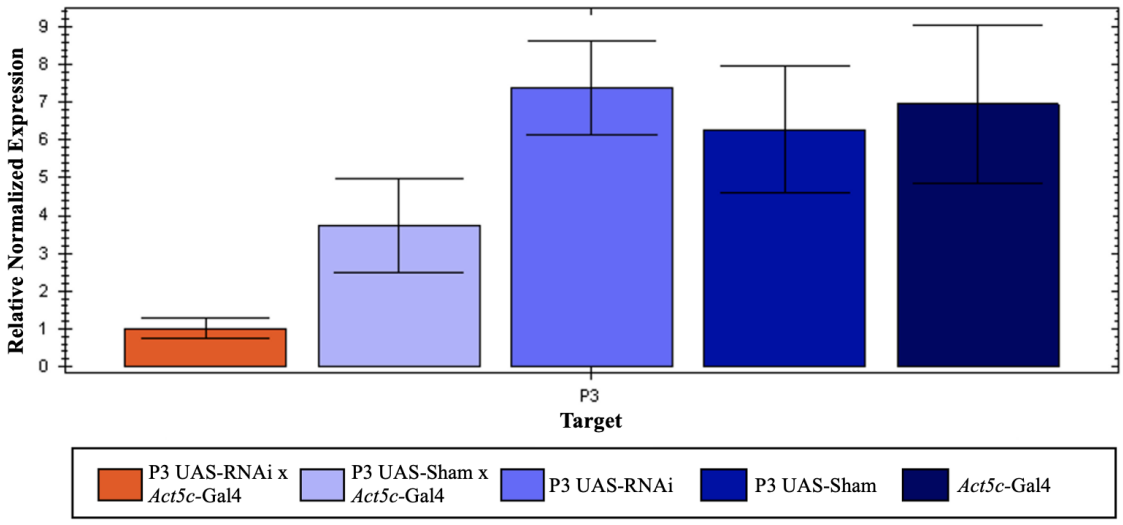

Figure 8. Expression data of *fruitless* P3 transcript in experimental/control groups.

Bar graph showing relative normalized expression of P3 transcript in the experimental group compared to control groups. Expression of P3 transcript in experimental and control groups is normalized to the reference gene, *Alpha-tubulin*. Each bar represents one of the 5 groups being tested in order: P3 UAS-RNAi x *Act5c*-Gal4 (1st), P3 UAS-Sham x *Act5c*-Gal4 (2nd), P3 UAS-RNAi (3rd), P3 UAS-Sham (4th), and *Act5c*-Gal4 (5th). Relative normalized expression of P3 transcript is represented through the y-axis. The experimental group (P3 UAS-RNAi x *Act5c*-Gal4) is set as the standard, and therefore expression is set to 1.0. The 4 control groups' expression of P3 transcript are compared to this group.

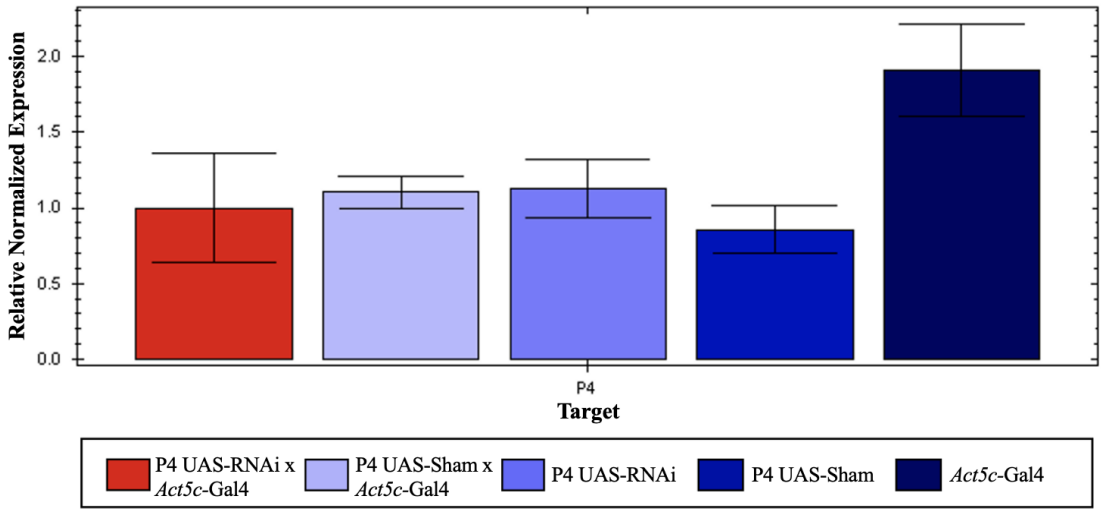

Figure 9. Expression data of *fruitless* P4 transcript in experimental/control groups.

Bar graph showing relative normalized expression of P4 transcript in the experimental group compared to control groups. Expression of P4 transcript in experimental and control groups is normalized to the reference gene, *Alpha-tubulin*. Each bar represents one of the 5 groups being tested in order: P4 UAS-RNAi x *Act5c*-Gal4 (1st), P4 UAS-Sham x *Act5c*-Gal4 (2nd), P4 UAS-RNAi (3rd), P4 UAS-Sham (4th), and *Act5c*-Gal4 (5th). Relative normalized expression of P4 transcript is represented through the y-axis. The experimental group (P4 UAS-RNAi x *Act5c*-Gal4) is set as the standard, and therefore expression is set to 1.0. The 4 control groups expression of P4 transcript are compared to this group.

3.4 Developmental assay

3.4.1 Comparing development time of *fru* P3 knockdown line

Developmental time was scored and compared between the *fru*-P3 knockdown line (P3 UAS-RNAi x *Act5c*-Gal4) and four controls (P3 UAS-Sham x *Act5c*-Gal4, P3 UAS-RNAi, P3 UAS-Sham, and *Act5c*-Gal4) from the larval to pupal stage and the pupal to adult stage (Figure 10; Table 5; table 7). The Wilcoxon statistical analysis was used to compare groups and is reported in Table 7. No *fru* P3 knockdown pupae reached the adult stage. The P3 knockdown group developed at a slower pace than the control groups from the larval to pupal stage (median=144 hours), which is significantly different from all

three control groups (median = P3 UAS-RNAi: 132 h, P3 UAS-Sham x *Act5c*-Gal4: 120 h, Act*5c*-Gal4: 132 h; $p < 0.001$). The sham control (P3 UAS-Sham x *Act5c*-Gal4) also developed significantly faster compared to the three other groups between the larval to pupal stage (median=120 h; $p < 0.001$) and the pupal to adult stage (median=228 h; $p < 0.001$). There is no significant difference between the developmental times of the control P3 UAS-RNAi (median pupal=132 h, median adult=240 h) and *Act5c*-Gal4 (median pupal=132 h, median adult=240 h) groups in either stage (Table 5).

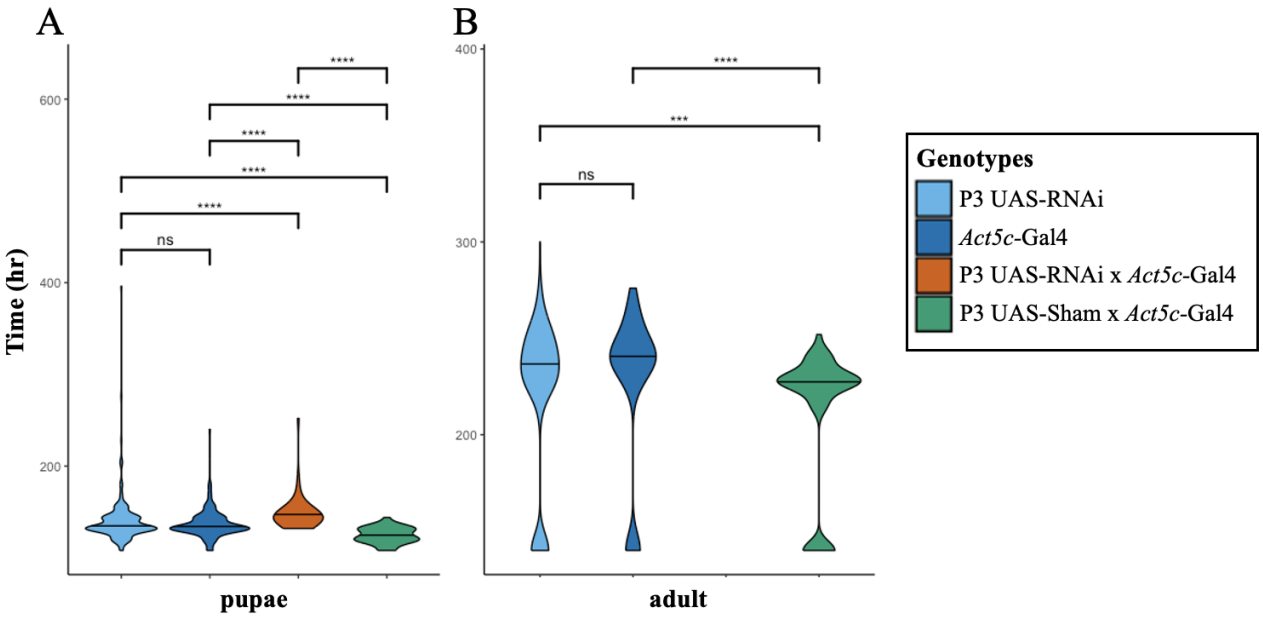

Figure 10. A violin plot demonstrating developmental time assay results.

(A) The time from egg hatching to the beginning of pupation and **(B)** from the beginning of pupation to eclosion of adults in hours between the experimental group and three controls, represented by four different colours: P3 knockdown (P3 UAS-RNAi x *Act5c*-Gal4) in orange, P3 UAS-RNAi (light blue), P3 UAS-Sham x *Act5c*-Gal4 (green), and *Act5c*-Gal4 (dark blue). The median of each group is indicated by the horizontal line through each of the sample plots. Note that there were no adult P3 UAS-RNAi x *Act5c*-Gal4 flies produced, hence no data is shown for that group in panel (B).

3.4.2 Survival Data

Survival between developmental stages (Figure 11; Table 6; Table 7) was scored and compared between the *fru*-P3 knockdown line (P3 UAS-RNAi x *Act5c*-Gal4) and the three controls (P3 UAS-RNAi, P3 UAS-Sham x *Act5c*-Gal4, and *Act5c*-Gal4). The proportion that survived from the larval to the pupal stage for all four groups was not significantly different between groups (P3 UAS-RNAi x *Act5c*-Gal4 = 0.92, P3 UAS-RNAi = 0.8981, P3 UAS-Sham x *Act5c*-Gal4 = 0.9151, and *Act5c*-Gal4 = 0.9136; Table 7). Survival between the initiation of pupation and eclosion as adults was significantly different between groups (P3 UAS-RNAi x *Act5c*-Gal4 = 0, P3 UAS-RNAi = 0.8814, P3 UAS-Sham x *Act5c*-Gal4 = 0.9794, and *Act5c*-Gal4 = 0.8716; Table 7), with no *fru*-P3 knockdown flies surviving to adulthood. There was also a significant difference in survival between the P3 UAS-RNAi or *Act5c*-Gal4 compared to the P3 UAS-sham x *Act5c*-Gal4 (*P*-values = 0.036 and 0.025, respectively).

3.5 Comparing *fru* P3 RNAi knockdown phenotype to *fruitless* knockout phenotype

There was a difference observed between the stage at which lethality occurs between the P3 RNAi knockdown and the *fruitless* null knockout line. The stage at which *fru* P3 knockdown flies died was observed. The highest death was observed in the earlier to mid stages of development, with most having no pigmentation, dying between 12-71h into pupation (43.67%), then in the brown puparium/prepupa stage, 1-12h after pupation initiation (33.33%), then the stage where pigment is developing (71-90h; 17.25%), and lastly 5.75% died in the stages after developing within puparium (>90h; Table 8). In comparison, zero larvae reached the pupal stage in the *fru* null stock (Stock#66692, Table 1).

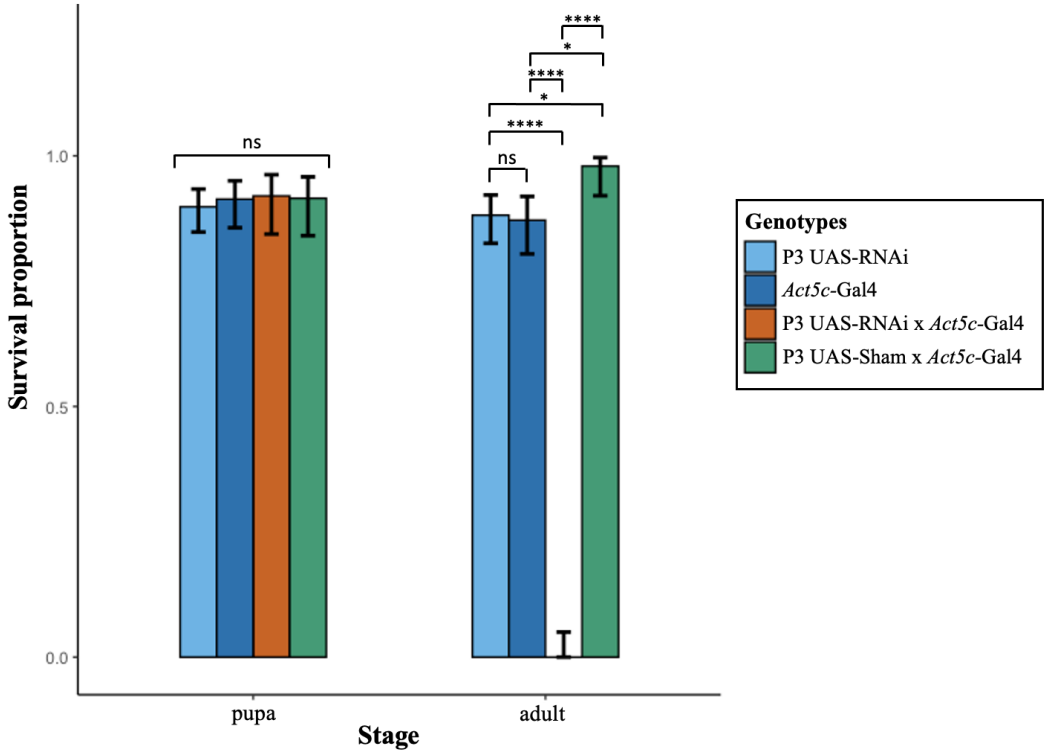

Figure 11. Bar graph representing survival data.

Survival data was compared between the experimental group and three controls, represented by four different colours: P3 UAS-RNAi x *Act5c*-Gal4 (orange), P3 UAS-RNAi (light blue), P3 UAS-Sham x *Act5c*-Gal4 (green), and *Act5c*-Gal4 (dark blue). Data separated by proportion that survived from larval to pupal stage (pupa), and pupal to adult stage (adult). Error bars represent a 95% confidence interval.

Table 4. Summarized data of P3 or P4 transcript normalized expression from qPCR for both P3 and P4 knockdown groups and relevant controls. Relative expression, raw P-values, and corrected (Holm-Bonferroni) P-values from expression data where expression of either *fru* P3 or P4 was compared between the *fru* P3 or P4 knockdown groups and controls.

Group	**Genotype**	**Normalized expression**	**Normalized Relative expression**	***P*-value**	**Corrected *P*-value**
P3 knockdown	P3 UAS-RNAi x *Act5c*-Gal4	0.00117	1.00000	n/a	n/a
	P3 UAS-RNAi	0.00862	7.39252	< 0.001	< 0.001
	P3 UAS-Sham x *Act5c*-Gal4	0.00437	3.75133	0.0336	0.0336
	P3 UAS-Sham	0.00733	6.28431	0.0063	0.0125
	Act5c-Gal4	0.00810	6.95184	0.0045	0.0136
P4 knockdown	P4 UAS-RNAi x *Act5c*-Gal4	0.00214	1.00000	n/a	n/a
	P4 UAS-RNAi	0.00241	1.12643	0.7962	1.2311
	P4 UAS-Sham x *Act5c*-Gal4	0.00236	1.10465	0.6155	1.2311
	P4 UAS-Sham	0.00183	0.85750	0.3830	1.1489
	Act5c-Gal4	0.00408	1.90780	0.1870	0.7479

Table 5. Summarized data for developmental assay showing median time to move from larval to pupal (pupa) and pupal to adult (adult) stage for each group and the range of times within each group, in hours.

Stage	Genotype	Median (h)	Range (h)
Pupa	P3 UAS-RNAi x *Act5c*-Gal4	144	132-252
	P3 UAS-RNAi	132	108-396
	P3 UAS-Sham x *Act5c*-Gal4	120	108-144
	Act5c-Gal4	132	108-240
Adult	P3 UAS-RNAi	240	140-300
	P3 UAS-Sham x *Act5c*-Gal4	228	140-252
	Act5c-Gal4	240	140-276

Table 6. Raw survival data from larval to pupal (pupa) and pupal to adult (adult) stages. Demonstrates the # or alive or dead, # dead in previous stage, proportion alive, proportion alive from previous stage and # of individuals in that stage.

Genotype	N	Alive	Dead	Stage	Proportion alive	Proportion of previous
P3 UAS-RNAi x *Act5c*-Gal4	100	92	8	pupa	0.92	0.92
P3 UAS-RNAi	216	194	22	pupa	0.8981	0.8981
P3 UAS-Sham x *Act5c*-Gal4	106	97	9	pupa	0.9151	0.9151
Act5c-Gal4	162	148	14	pupa	0.9136	0.9138
P3 UAS-RNAi x *Act5c*-Gal4	92	0	92	adult	0	0
P3 UAS-RNAi	194	171	23	adult	0.7917	0.8814
P3 UAS-Sham x *Act5c*-Gal4	97	95	2	adult	0.8962	0.9794
Act5c-Gal4	148	129	19	adult	0.7963	0.8716

Table 7. Raw and corrected (Holm-Bonferroni) *P*-values from developmental assays where developmental time and survival were scored. Groups separated by assay type (developmental time vs. survival) and by stage (pupa vs. adult).

Analysis	Stage	Comparison	*P*-value	Corrected *P*-value
Developmental Time – Wilcoxon	Pupa	(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-Sham x *Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-RNAi)	< 0.0001	< 0.0001
		(P3 UAS-RNAi x *Act5c*-Gal4) to (*Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-Sham x *Act5c*-Gal4) to (P3 UAS-RNAi)	< 0.0001	< 0.0001
		(P3 UAS-Sham x *Act5c*-Gal4) to (*Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-RNAi) to (*Act5c*-Gal4)	0.394	1.00
	Adult	(P3 UAS-Sham x *Act5c*-Gal4) to (P3 UAS-RNAi)	< 0.0001	< 0.001

		(P3 UAS-Sham x *Act5c*-Gal4) to (*Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-RNAi) to (*Act5c*-Gal4)	0.032	0.283
Survival – Fisher's exact	Pupa	(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-Sham x *Act5c*-Gal4)	1.00	1.00
		(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-RNAi)	0.681	1.00
		(P3 UAS-RNAi x *Act5c*-Gal4) to (*Act5c*-Gal4)	1.00	1.00
		(P3 UAS-Sham x *Act5c*-Gal4) to (P3 UAS-RNAi)	0.692	1.00
		(P3 UAS-Sham x *Act5c*-Gal4) to (*Act5c*-Gal4)	1.00	1.00
		(P3 UAS-RNAi) to (*Act5c*-Gal4)	0.724	1.00
	Adult	(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-Sham x *Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-RNAi x *Act5c*-Gal4) to (P3 UAS-RNAi)	< 0.0001	< 0.0001
		(P3 UAS-RNAi x *Act5c*-Gal4) to (*Act5c*-Gal4)	< 0.0001	< 0.0001
		(P3 UAS-Sham x *Act5c*-Gal4) to (P3 UAS-RNAi)	0.004	0.036
		(P3 UAS-Sham x *Act5c*-Gal4) to (*Act5c*-Gal4)	0.002	0.025
		(P3 UAS-RNAi) to (*Act5c*-Gal4)	0.868	1.000

Table 8. Phenotypes displayed at time of recorded death of P3 knockdown group. Phenotypes range from brown puparium/prepupa (1-12h after pupation initiation), developing structures (no pigment; 12-71h), developing pigment (71-90h), developed within puparium (>90h). Note that no P3 knockdown flies eclosed as adults.

Stage	**Counts**	**% of Total**
brown puparium/prepupa	29	33.33%
developing structures	38	43.67%
developing pigment	15	17.25%
developed	5	5.75%

4 Discussion

In this project, RNAi and the Gal4-UAS system were used to induce knockdown of the *fruitless* P3 or P4 transcripts in *D. melanogaster*. Quantitative real-time PCR was used to quantify the extent of knockdown of P3 or P4 transcript in transgenic lines. I successfully produced a functional transgenic P3 knockdown *D. melanogaster* line that was used in assays to determine the effect of *fruitless* P3 transcripts on development and viability. Unfortunately, I was unsuccessful in producing a functional transgenic *D. melanogaster* RNAi knockdown line to test the effects of *fruitless* P4 transcripts on development and viability.

4.1 Expression Data

To test the expression of P3 and P4 transcripts in my groups of interest, I first optimized my primers and generated a standard curve using cDNA from adult flies, as these samples were easiest to obtain. One limitation of this approach was that P3 and P4 transcript levels were quite low in adults. To compare the expression between the knockdown groups and controls, P3 and P4 transcript expression levels were instead assessed on pupae because that is when inviability has been observed in the past and when P3 and P4 transcript activity has been observed to be at its highest (development vs. adult) (Leader et al., 2018).

4.1.1 *fru* P3 knockdown

I was able to demonstrate through qPCR that the P3 transcript is being knocked down in my transgenic flies. When comparing the expression of P3 transcript in the experimental group to the control groups, there was 4-7 times less expression of P3 transcript in the knockdown experimental group. Although P3 transcript was still present at low levels, as not all transcript was degraded by the RNAi mechanism, there was a significant difference in transcript activity in the P3 knockdown group. Later, it becomes clear that enough transcript was removed to observe a phenotypic effect during the developmental assays. Although the expression levels of P3 transcript were statistically different compared to all of the controls, there was some variability observed between the control

groups. This is likely due to the strong shifts in expression levels across pupation. There was some variability in age of the pupa chosen (all picked between 24-30 hours), which may have contributed to the variability within the sample groups. Gene expression can vary substantially over development, especially if implicated in the development of specific tissues or structures as is the case with *fru* (Anand et al., 2001). The expression of the P3 transcript is highest during pupation, from 24 hours to 96 hours, compared to the other stages of development where there is a consistent, but lower level of P3 transcript activity ("FlyBase," 1996). I thought that the 6-hour window would have been sufficiently narrow enough, but if this assay was repeated in the future, an even smaller window would be beneficial to reduce variation.

4.1.2 *fru* P4 knockdown

After designing a P4 shRNA, microinjection, and subsequent verification, it was observed through qPCR that the expression of *fruitless* P4 transcript was not knocked down through RNAi and the Gal4-UAS system. While these flies were tested for development time, these assays cannot inform the effects of P4 knockdown, and can only serve as additional controls.

Almost all of the steps leading up to the qPCR quantification were identical between the P3 and P4 RNAi constructs except the shRNA targeting sequences, which were specific to the respective first exons of the two transcripts. There were many factors that went into choosing the right shRNA targeting sequence such as length, GC content and absence of off-target binding. There may be positional effects that have to do with the location within the transcript that I chose for my shRNA targeting sequence that is leading to very low P4 RNAi efficiency. Also, there is evidence that RNAi efficiency decreases when targeting transcripts with low expression, and P4 has lower expression during pupation than P3 ("FlyBase," 1996).

Another aspect that could affect the efficiency of RNAi is the accumulation of mutations in the targeting sequence over generations. When the shRNA is expressed, modified and incorporated into the RISC complex, it degrades mRNA that corresponds to the targeting sequence. Nucleotide mismatches, and the position of those mismatches, can have

varying effects on the specificity of target recognition (Holen et al., 2002). If the P4 targeting sequence has been mutated since being incorporated into the *D. melanogaster* genome, especially in the center of the targeting sequence in the siRNA duplex that forms, then target RNA cleavage is prevented (Elbashir et al., 2001). Sequencing of transgenic flies could help determine whether mutation of the targeting or target sequence may have occurred.

4.2 Developmental Assays

4.2.1 *fru* P3 knockdown

After confirmation that the P3 transcripts were being knocked down through qPCR, I was able to conduct developmental assays on my knockdown and control groups to determine if there was an observable phenotypic effect of the knockdown. Due to the original reporting of lethality during pupation when P3 and P4 were knocked down simultaneously (Anand et al., 2001), I was interested in investigating viability and developmental time between developmental stages when P3 alone was knocked down. Inviability was observed at the pupal stage, where there was no survival past the pupal stage and into the adult stage in the knockdown group. This falls in line with the predictions made by Anand et al. (2001) for the involvement of *fruitless* P3 transcripts in development and viability. In their study, they determined that removing the functions of P1, P2 and P3 transcripts with knockout by insertion of a *P*-element lead to inviability at the pupal stage where *D. melanogaster* reached the final stages of pupal development but were unable to eclose from their puparium without help. If helped out of their puparium, they could survive as adults for 7-14 days but had external phenotypic defects (Anand et al., 2001). In my developmental assays P3 transcript function was significantly reduced, and function of the P1 and P2 transcripts was retained, yet the effect of P3 knockdown seemed to have much more severe results than what was observed in the earlier study. Most of my knockdown flies didn't progress developmentally to the final stages of pupation, with approximately 30% dying in the earliest stages (12h). My results of earlier pupal lethality with P3 knockdown are difficult to reconcile with the later lethality observed after P1, P2, and P3 combined knockdown in Anand et al. (2001). It is possible

that my RNAi has an off-target effect that was not predicted by sequence similarity assessment, and that this additional transcript affected by my RNAi also influences pupal development (Seinen et al., 2011). It is also possible that the flies tested in Anand et al. (2001) did not have complete knockout of P3, with greater expression of P3 than in my RNAi flies, leading to lesser effect on the phenotype. Lastly, it is possible that Anand et al. (2001) only reported the greatest degree of survival, which I also observed in a small number of cases, and that the majority of their flies also died at earlier stages of pupal development.

When comparing the knockdown of P3 alone to knockout of the entire *fru* gene, the phenotype is much less severe when only P3 is absent. As in Anand et al. (2001), when removing the function of *fru* entirely, individuals only reach the initial stages of pupariation, where the puparium began to brown, but did not progress any further. When the function of both *fru* and the genes flanking *fru* are removed, the organism arrested at the developmental stage. The phenotype observed in my knockdown group were less severe than these two, where individuals progressed past the pupariation stage into the pupal stage. My P3 knockdown results were also compared to a fruitless disruption line (stock #66692, RRID:BDSC_66692; Table 1), where *fruitless*' function is fully removed. The disruption line demonstrates that when there is full removal of fru function, there is inviability observed in development. Not a single larva lacking *fru* function reached the pupal stage (Anand et al., 2001). The phenotype is more severe than when P3 transcripts are knocked down. This may be due to the presence of some *fru* transcripts in a knockdown driven by RNAi/lack of full removal of P3 transcripts or multiple transcripts of *fruitless* being involved in development and inviability, causing a much more severe reaction when function of all involved in viability being removed.

Interestingly, although inviability was observed in all individuals in the knockdown group, there was variability in the place that development stopped between individuals, with lethality occurring at all stages of pupal development (Ashburner, 2005; Table 8). In many cases it was also extremely difficult to pinpoint the developmental stage of lethality because the anterior and posterior ends of the animal appeared to be at different stages, with the anterior end more developed than the posterior. The variability that was

observed in developmental arrest may potentially have to do with the RNAi system being used to knock down the transcripts. Although a knockdown of P3 transcript doesn't remove all transcript, it removed enough P3 transcript for there to be a developmental effect. The quantitative data shows very little variability in P3 transcript expression in the knockdown group, but because it seems like this system may be dose dependent, any fluctuation of transcript level between individuals, especially in development, could cascade to visible differences in phenotype. Additionally, if there was also some spatial variation in the knockdown, this could have led to the variation I observed in anterior *vs.* posterior development.

The assay results demonstrated that the P3 UAS-RNAix*Act5c*-Gal4 knockdown group had a significant delay entering the pupal stage compared to the controls. Developmental delays have been tied to changes in environment or stress for *D. melanogaster* (Brown et al., 2019). Since most aspects of the environment had been controlled for and consistent between all groups, this change in developmental time was likely caused by the lack of *fru* P3 transcripts in the larval stage. P3 is known to be most highly expressed in the central nervous system and trachea (FlyAtlas 2: Leader et al., 2018). Due to its expression in the central nervous system, the knockdown of P3 transcript could have had a negative effect on locomotion, smell, taste, or cognitive function, all functions controlled by the central nervous system (Heimbeck et al., 1999; Huser et al., 2012). This may have caused stress on the organism or affected its ability to eat, which is a main factor for movement through developmental stages (Brown et al., 2019). On the other hand, the P3 UAS-shamxGal4 control group moved through development at a faster rate from larva to pupa and pupa to adult compared to all other groups. These effects may have been caused by off target effects of the shRNA or background genetic effects (Sudbery et al., 2010). The shRNA has a randomized targeting sequence and should not correspond to any transcript in the transcriptome of *D. melanogaster*, making it more likely that the decreased development time of the sham controls is due to background genetic effects.

The results of this study demonstrate that significantly lowering *fru* P3 transcript expression is sufficient to induce inviability. Therefore, the alternatively spliced P3

transcripts have an important role in development, a role distinct from that of the well-studied P1 transcripts in *D. melanogaster*, speaking to the complexity of the *fruitless* gene and its alternatively spliced products. This leads to more questions regarding the evolution of *fruitless* across closely and distantly related species. Since the focus of *fru* evolution has been solely on *fruitless*' involvement in the sex determination pathway, there is very little know on whether these findings could extend outside of *D. melanogaster*. If we look at another gene that's been conserved across different species, *foraging*, we can see that food-related behaviour is conserved across all closely related species, while functions in sleep, learning and memory, and social behaviour are only found in subsets of species that have the gene (Anreiter & Sokolowski, 2019). This demonstrates that food related behaviour has been strongly conserved over evolutionary time in these species, while these other traits may have been acquired or lost over time. Similarly, *fru* may have gained different functions associated with the acquisition of additional 5' first exons in *D. melanogaster* over time. The conservation of the function of *fru*'s alternatively-spliced transcripts, and the potential variation in this gene's function over evolutionary time, warrants further exploration.

4.3 Limitations

Despite my success in creating a transgenic line and being able to determine that the transcript in question is involved in development and viability, my work had a couple limitations. While RNAi is a great tool to use to induce knockdown and manipulate the expression of transcripts throughout an organism, it does come with its limitations. A limitation of RNAi that I encountered was that the system does not remove all function of the transcript of interest. Unlike with a knockout, the RNAi system targets transcripts, and doesn't affect the gene sequence. Therefore, not all transcripts are degraded. In some circumstances this could lead to no effect being observed if there is still enough transcript present. Fortunately, with the P3 transcript knockdown this wasn't the case, but variability in the phenotype was observed for stage of death in development. This makes it more difficult to definitively state what the outcome of removing P3 function is. *Fruitless* codes for a family of transcription factors, which lead to the activation of genes,

in the case of P3, important in the developmental stage. Therefore, any variability in the amount of P3 transcript knocked down between individuals, can lead to differing levels of transcription factor activity, leading to an observable difference in time of death. With a complete knockout of transcript function, there would be zero transcript expressed and potentially less variability in phenotype. Another limitation of this system is that there are a lot of factors that can lower the efficiency of the system, or cause it not to work at all, as with my P4 knockdown line. Although this is a great system to be used to determine the function of transcripts of interest, it can be very difficult to optimally design the construct.

4.4 Future Work

Substantial progress was made in evaluating the functions of *fru* P3 transcripts, and future work can build upon these findings. One of the goals of my project was to explore the function of P4 transcripts in development compared to P3 transcripts, but due to the lack of knockdown of P4 transcripts, I was unable to test those stocks. In the future, P4 knockdown stocks could be remade with a new shRNA design and tested with the same developmental assays as for P3 knockdown. Although P3 transcripts affect viability, P4 transcripts may also be involved. Alternatively, CRISPR-mediated knockout of the P3 or P4 transcript could be performed to ensure complete removal of transcript activity, and then subsequently tested.

When testing the effects of P3 knockdown a ubiquitous Gal4 was used to degrade P3 transcripts throughout the entire organism during its entire lifespan. Now that P3 transcripts have been implicated in development and are known to affect viability through whole body knockdown, the expression of the RNAi system can be further manipulated. P3 transcript can be knocked down in different tissue types using different Gal4s, and we can explore where P3 transcript expression is necessary for viability. Expression of P3 transcripts is highest in the central nervous system, and we could test if we would see the same effects of whole-body knockdown if P3 transcripts where only knocked down in the central nervous system. This is also quite simple to test since the transgenic line has

already been designed. Gal4 stocks targeting different tissue types or systems can easily be ordered from the Bloomington Stock Center, crossed to the transgenic P3 UAS-RNAi line, and tested. If we wanted to test the effects of P3 transcripts on adult behaviour, we would have to bypass the inviability observed in the pupal stage. This could be accomplished through the introduction of a temperature-sensitive Gal80, a repressor of the Gal4 system, into the transgenic lines (T. Lee & Luo, 1999; Suster et al., 2004). The Gal80 could be placed at the temperature that would allow Gal80 expression, repressing the activity of the Gal4 system during development, and therefore P3 transcripts would not be degraded during development. Newly-emerged adult flies could then be placed at the temperature where Gal80 is not active, and therefore P3 would be knocked down in adults, to see if removing function of P3 transcripts in only the adult stage causes any change in behaviour.

4.5 Conclusions

Fruitless P3 transcripts are implicated in development and viability in *D. melanogaster*. I produced a transgenic *fruitless* P3 knockdown line and determined that P3 transcript expression during development is required to prevent inviability at the pupal stage. The P4 transcript may also be implicated in development and viability, compounding the effects when both P3 and P4 function are removed. The effects of P4 transcripts in development and viability were unfortunately not able to be assessed due to the P4 RNAi construct not being functional. Therefore, I can conclude that the P3 knockdown is sufficient to cause inviability, but not certain whether P3 is the primary transcript group responsible for the lethality of *fruitless* whole gene deletion since P4 may also be involved in viability. My P3 transgenic line can be used to further study the function of P3 transcripts during development and in adult *D. melanogaster* to enhance our understanding of the complexity of the *fruitless* gene.

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Appendix: Standard curve results

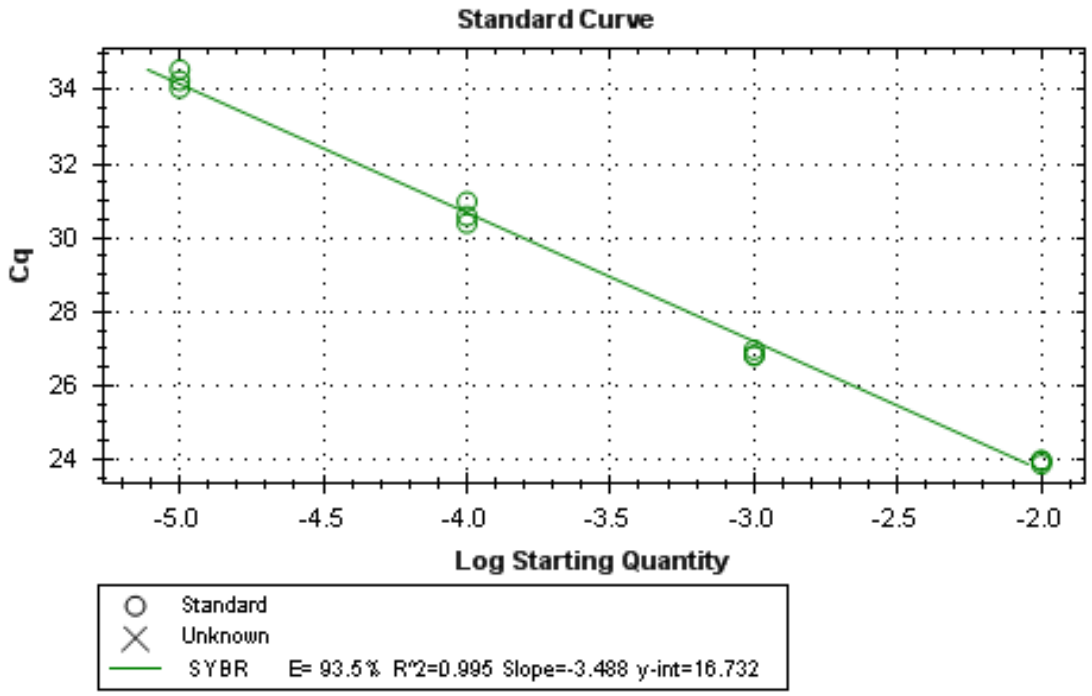

Figure A1. Standard curve for P3 qPCR primers.

The efficiency and specificity of primer set was tested using wildtype female *D. melanogaster* cDNA, which was diluted in a series of 10-fold dilutions and used in PCR reactions with P3 primers to build a 4-log standard curve, corresponding to the points on the graph. Each sample was loaded in triplicate, a no-template control. The PCR reaction ran for 40 cycles with an annealing temperature of 60°C using the CFX96™ Touch Real-Time PCR detection system. Efficiency = 93.5%, R^2 = 0.995, slope = -3.488, and y-intercept = 16.732 for melt curve was calculated using BioRad CFX Manager Software.

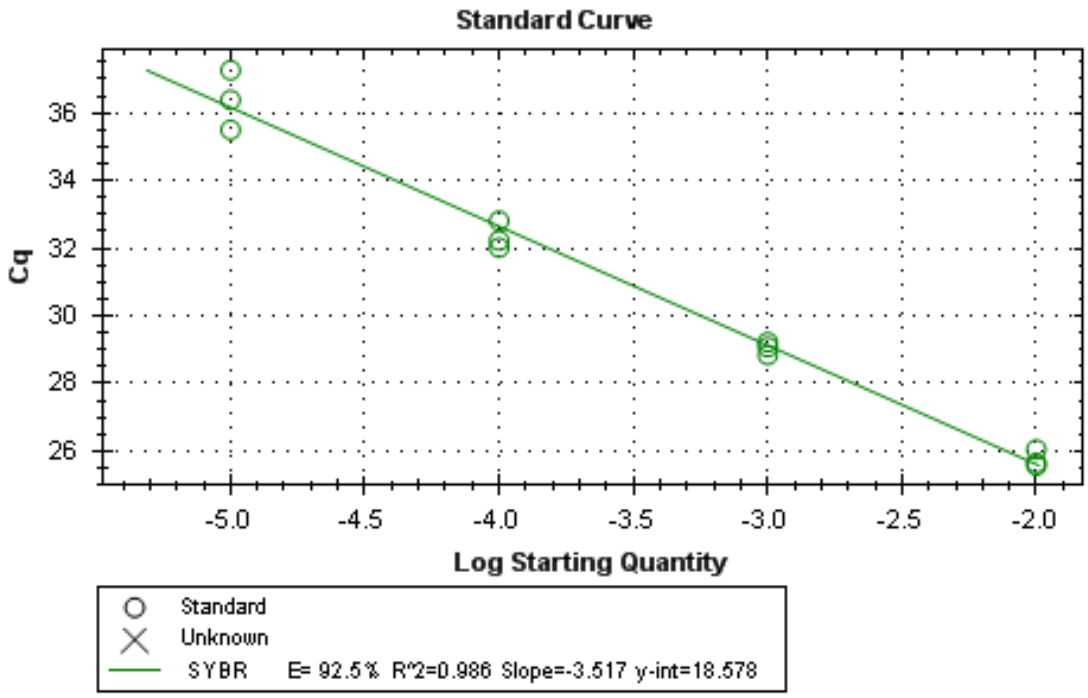

Figure A2. Standard curve for P4 qPCR primers.

The efficiency and specificity of primer set was tested using wildtype female *D. melanogaster* cDNA, which was diluted in a series of 10-fold dilutions and used in PCR reactions with P4 primers to build a 4-log standard curve, corresponding to the points on the graph. Each sample was loaded in triplicate, a no-template control. The PCR reaction ran for 40 cycles with an annealing temperature of 60°C using the CFX96™ Touch Real-Time PCR detection system. Efficiency = 92.5%, R^2 = 0.986, slope = -3.517, and y-intercept = 18.578 for melt curve was calculated using BioRad CFX Manager Software.

Curriculum Vitae

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Related Work Experience

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Presentations

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The role of *fruitless* P3 and P4 transcripts in *Drosophila melanogaster*.
Award recipient: Best Presentation

Ontario Biology Day, London, ON (April 2019)
The role of *fruitless* P3 and P4 transcripts in *Drosophila melanogaster*.

Western Biology Day, London, ON (April 2019)
The role of *fruitless* P3 and P4 transcripts in *Drosophila melanogaster*.