THE PENNSYLVANIA STATE UNIVERSITY
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THE REGULATION OF LIPID STORAGE BY THE SPLICING FACTOR TRANSFORMER2 (TRA2) IN *DROSOPHILA*

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ABSTRACT

Excess nutrients are stored as triglycerides mainly in the adipose tissue of an animal. These triglycerides are located in structures called lipid droplets within adipose cells. Previous genome-wide RNAi screens in *Drosophila* cells identified splicing factors as playing a role in lipid droplet formation. Dr. DiAngelo identified the SR protein, 9G8, as an important factor in fat storage as decreasing its levels results in augmented triglyceride storage in the fat body. However, whether 9G8 interacts with other proteins to affect lipid metabolism is unclear. Previous *in vitro* studies have implicated 9G8 in the control of sex determination by binding to transformer (tra) and transformer2 (tra2) to regulate *doublesex* (*dsx*) splicing; any function of these proteins in regulating metabolism is unknown. The goal of this study is to determine whether tra2 regulates fat storage *in vivo*. To test this hypothesis, we measured triglyceride and glycogen levels in flies with tra2^{dsRNA} induced in the adult fat body. Decreasing the expression of this sex determination gene resulted in an increase in triglyceride levels but no effect on glycogen storage, a phenotype similar to the 9G8 knockdown flies. Consistent with the triglyceride phenotypes, tra2 knockdown flies lived longer under starvation conditions. In addition, this increase in triglycerides is due to more storage of these molecules per cell and not an increase in the number of fat cells as DNA levels are unchanged in tra2^{dsRNA} flies compared to controls. This triglyceride accumulation phenotype also does not seem to be due to more feeding as food consumption was not increased in the tra2^{dsRNA} flies as measured by CAFÉ assay. We next wanted to determine whether the nutrient storage phenotype observed here is due to altered expression of the genes coding for important metabolic enzymes. While the levels of major lipid metabolic enzymes were mostly unchanged, the splicing of *CPT1*, an enzyme involved in the

breakdown of lipids, was altered in flies with decreased *tra2*. The less-catalytically active isoform of CPT1 which accumulated in tra2^{dsRNA} flies suggests a decrease in lipid breakdown, which is consistent with the increased triglyceride levels observed in these flies. Together, these results suggest a link between mRNA splicing, sex determination and lipid metabolism and may provide insight into the mechanisms underlying tissue-specific splicing and nutrient storage in the fat body.

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Chapter 1

Introduction

Human obesity

As animals consume food, excess nutrients are stored as triglycerides mainly within adipose tissue in structures known as lipid droplets. Such storage is the result of an evolutionary adaptation that allowed species to endure famines and plagues. As food becomes easily accessible in today's society and people lead sedentary lifestyles, negative consequences of overconsumption and excess nutrient storage are becoming increasingly apparent. The incidence of obesity, defined in an individual as a Body Mass Index (BMI) of 30 or higher, throughout the world has been growing (Ogden et al., 2014). In the United States, excessive fat storage has resulted in complications that account for some of the leading causes of death such as heart disease and stroke (Scully, 2012), with rates of childhood and adult obesity on the rise (Ogden et al., 2014). Understanding the biology that controls how our bodies metabolize and store excess nutrients can help both combat the increasing rates of obesity and treat obesity-related diseases such as diabetes and cardiovascular disease.

Much research has focused on attempting to identify the genes important for developing obesity. Some groups have performed genome-wide association studies (GWAS), which scan sets of single nucleotide polymorphisms (SNPs) to study relationships between disease phenotypes and variation in the genome (Xia and Grant, 2013). The GWAS approach has helped

identify genes that associate with the obesity phenotype and include many hormones, neuropeptides, metabolic enzymes and transcription factors (Xia and Grant, 2013). A large number of hormones identified in GWAS such as insulin, leptin and ghrelin have tissue-specific metabolic functions. Interestingly, these hormones were previously shown to act on specific neuronal populations resulting in metabolic consequences (Coll et al., 2007), highlighting the role of inter-organ communication in the development of obesity. In addition to the GWAS, there have been transcriptome-wide analyses performed on adipose tissue and muscle from obese patients. These studies have identified genes important for transcription and RNA processing as being involved in obesity (Pihlajamäki et al., 2011). While variations in many genes have been associated with obese individuals, whether a causal relationship exists between many of these molecules and obesity is unknown.

Using *Drosophila* as a model system to study obesity

Drosophila are organisms with modest dietary and spatial needs, they have a short generation time, and their use allows for significant results at a fairly low cost (Stocker and Gallant, 2008). *Drosophila* have a simple nervous system, and store triglycerides and glycogen in a liver and adipose-like organ (called the fat body), both of which share functional similarities to those of humans. The genes regulating glycogen and lipid storage in these organs within *Drosophila* are highly analogous to those of humans, coding for proteins such as insulin, glucagon, and lipases (Baker and Thummel, 2007). Due to all of these similarities, *Drosophila* provide a perfect system to study the molecular control of energy metabolism, more specifically how fat storage is regulated within their fat body.

In order to better understand genes important for triglyceride storage in *Drosophila*, genetic tools can be utilized to determine genes important in obesity. Previous RNA interference (RNAi) screens identified genes which, when disrupted, lead to obesity (Guo et al., 2008; Beller et al., 2008). One interesting family of genes, which resulted in smaller, more dispersed lipid droplets when decreased, included genes involved in RNA processing and these genes serve as the focus of my thesis.

Transcription and RNA processing

Transcription, the first step in gene expression, involves generating a strand of ribonucleotides complementary to a DNA template. In eukaryotes, this RNA molecule undergoes post-transcriptional processing, including the addition of both a guanine 5' cap and a 3' poly-adenine tail, and RNA splicing. The pre-mRNA transcript for most genes is encoded discontinuously, separated by many non-coding introns. RNA splicing allows for the removal of unwanted introns from an RNA strand in order to form mature transcripts that can be translated into protein. While some self-splicing introns exist, in eukaryotes, most introns are removed by a large ribonucleoprotein machine called the spliceosome. The spliceosome is composed of five small nuclear ribonucleoprotein complexes (snRNPs), consisting of both RNA and protein components: U1, U2, U4, U5, and U6. The recruitment of these snRNPs depends upon the presence of SR proteins, named because they contain domains high in serine and arginine residues. SR proteins are responsible for both promoting the binding of U1 to the 5' splice site and the binding of U2 to the intron branch site at the 3' end of the intron on the RNA transcript

which begins the formation of the spliceosome. Later in the spliceosome formation process, U4/U6 and U5 snRNPs are recruited to form a complete complex. The functional spliceosome is then capable of removing introns from the transcript (Matera and Wang, 2014).

Cells can be selective in choosing which introns and exons should be included in a mature transcript by recognizing different intron-exon junctions. Intron and exon enhancer and silencer sequences are capable of promoting or masking splice sites from the spliceosome. SR proteins and other heterogeneous ribonucleoproteins, known as hnRNPs, are responsible for identifying these sequences and selecting for various exon and intron junctions, leading to spliceosome assembly. Masking of some splice sites from the spliceosome can lead to the omission of exons, resulting in alternative splicing in which different combinations of exons in a gene are spliced together to produce varying mRNA molecules (Fig. 1). The presence and function of SR proteins in RNA splicing allows for the formation of a large variety of functional proteins from a single strand of DNA (Fu and Ares Jr, 2014). Additional variability is introduced by the expression of SR proteins in various tissues, allowing for different transcripts to be produced in different parts of the body.

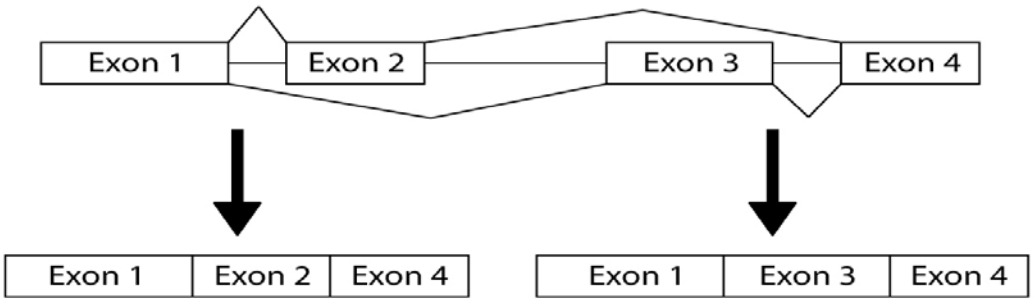


Figure 1. The alternative splicing of genes.

The inclusion and exclusion of specific splice sites results in different combinations of exons in a gene spliced together to produce varying mRNA molecules.

The function of splicing factors in lipid metabolism

To further understand the function of genes important for lipid storage, the DiAngelo lab has followed up on the previously described RNAi screens, focusing specifically on genes important for mRNA splicing. In addition to identifying members of the U1 and U2 snRNP as being important for lipid storage, Dr. Justin DiAngelo, my thesis supervisor, identified an SR protein called 9G8 that is important for triglyceride storage in the *Drosophila* fat body (Gingras et al., 2014). This phenotype can be explained by altered splicing of a lipid metabolic gene important for β-oxidation of lipids known as *CPT1*. In *Drosophila*, the *CPT1* gene has two isoforms resulting from an alternate sixth exon (exon 6A or 6B). The isoforms containing exon 6A exhibit higher enzyme activity than isoforms containing exon 6B, leading to increased lipid breakdown (Price et al., 2010). Interestingly, while wildtype flies had more *CPT1* that included exon 6A, flies with decreased fat body 9G8 expressed higher levels of the *CPT1* isoform containing exon 6B (Gingras et al., 2014). The presence of more exon 6B-containing *CPT1* isoforms leads to decreased lipid breakdown, resulting in augmented triglyceride stores. Identifying the metabolic functions of 9G8 raised questions in our lab as to whether other SR proteins were involved in the regulation of fat metabolism or if multiple SR proteins worked together in order to alter lipid storage. Previous studies have shown that 9G8 binds to two RNA-binding proteins called transformer (tra) and transformer2 (tra2) to correctly process the *doublesex* (*dsx*) gene, which is important for controlling whether a fly develops into a male or female (Sciabica and Hertel, 2006). While *tra* and *tra2* have been well characterized in the regulation of sex determination, any function of these proteins in controlling metabolism is unknown.

The main focus of my thesis is the investigation of tra2 function in the *Drosophila* fat body. Similar to the 9G8 phenotype, decreasing *tra2* levels in the *Drosophila* fat body using RNAi resulted in increased starvation resistance and a large increase in triglycerides. This increased storage of triglycerides seemed to be due to an increase in the amount of fat stored per cell and not due to increased food consumption or increased expression of genes coding for lipid metabolic enzymes. Similar to what was seen in previous 9G8 studies, the splicing of *CPT1* was altered in flies with decreased *tra2*, resulting in a higher amount of *CPT1* isoforms containing exon 6B, explaining the increased triglyceride levels. Together, the results in this study provide *in vivo* evidence for a connection between tra2-regulated splicing and lipid metabolism.

Chapter 2

Materials and Methods

Fly Genetics

The following fly lines were used in this study: yolk-Gal4 (Georgel et al., 2001), y[1] v[1]; P{y[+t7.7]=CaryP}attP2 (referred to as attp2; BL#36303), and y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02852}attP2 (referred to as $tra2^{dsRNA}$; BL#28018). Flies were grown at 25°C on a 12 hour:12 hour light:dark cycle on a standard cornmeal-sugar yeast medium (9 g *Drosophila* agar (Genesee Scientific), 100 mL Karo Lite Corn Syrup, 65 g cornmeal, 40 g sucrose, and 25 g whole yeast in 1.25 L water).

Macromolecule Assays

Two approximately 1 week-old whole females or three fat bodies dissected from these animals were homogenized in lysis buffer containing 140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X, and 1× protease inhibitor cocktail (Roche). Triglycerides were measured using the Infinity Triglyceride Reagent (ThermoFisher Scientific), proteins were measured using the Pierce BCA Assay kit (ThermoFisher Scientific), and DNA content was measured using the Quant-iT DNA Assay kit (Invitrogen) according to manufacturer's instructions. To measure glycogen levels, homogenized samples were treated with 8 mg/mL amyloglucosidase (Sigma-Aldrich) in 0.2M citrate buffer, pH 5.0 for 2 hours at 37°C. Total glucose was then measured

using the Pointe Scientific Glucose Oxidase Kit (ThermoFisher Scientific). A similar procedure was performed for samples that were not treated with amyloglucosidase in order to measure free glucose. Final glycogen levels were determined by subtracting free glucose from total glucose.

Feeding Assay

Food consumption was measured over a 24-hour period by using a modified version of the Capillary Feeder (CAFÉ) Assay as described previously (Ja et al., 2007). Three 1-week old adult female flies were placed in each vial containing 1% agar. A 5% sucrose solution was provided via a glass capillary tube as the sole food source. The amount of sucrose left was measured after 24 hours elapsed. Identical vials, with no flies in them, were used in order to correct for any evaporation that may have occurred during the experiment.

Starvation Resistance

Approximately 1 week-old female flies were placed in vials containing only a water source in the form of 1% agar. The number of surviving flies was measured every 6 hours until all flies were dead. The collected data was analyzed via Online application for survival analysis (OASIS (https://sbi.postech.ac.kr/oasis2/)).

RNA Isolation

Fat bodies dissected from approximately fifteen 1-week old adult females were homogenized in 1 ml Ribozol (AMRESCO). The sample was incubated at room temperature for 5 minutes (min)

and was then centrifuged at 12,000 rpm for 15 min at 4°C. The sample was chloroform extracted, incubated at room temperature again for 4.5 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. RNA was then precipitated by adding an equal volume of isopropanol to the sample and centrifuging at 12,000 rpm for 15 min at 4°C. The pellet was then washed with 70% ethanol. Ethanol was removed from the sample which then was left to air dry for 5 min. The pellet was re-suspended in RNase free water.

DNase Treatment, cDNA Synthesis and qPCR

5 µg of total RNA was DNase treated using the DNA-Free kit according to manufacturer's protocol (Life Technologies). 0.25 µg of DNase-treated RNA was reverse transcribed using qScript XLT cDNA SuperMix (Quanta Biosciences) according to manufacturer's protocol. cDNA product was amplified via qPCR using 1 µL cDNA, 1X PowerUp SYBR Green Master Mix (Applied Biosystems), and 0.2 µM primers in a 25 µL reaction. Samples were placed in a Step-One Plus qPCR machine starting with an incubation for 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 15 sec at 95°C. After the cycling conditions, a melt curve was performed. The primers used to amplify total CPT1 were: (Forward:5'GCAAGTGCAAATTGAGGAAA3', Reverse:5'AAGTGCTCCTCACCTTCCAC3'). Primers used for exon 6A-containing CPT1 were: (Forward:5'CCGCTGGTTTGACAAGTG3', Reverse: 5'TCATCGACGATCAGGTTCTC3'). Primers used for exon 6B-containing CPT1 were: (Forward: 5'AATGGTCGCGTTGGCTTC3', Reverse: 5'TCCCAAAACCGGTGCATC3'). Primers used for dFAS were: (Forward: 5'CTGGCTGAGCAAGATTGTGTG3', Reverse:

5’TCGCACAACCAGAGCGTAGTA3’). Primers used for dACC were: (Forward: 5’AGATGCAGAACGATGTCCGC3’, Reverse: 5’CTCTTTGTGAAGCAGCTCCG3’). Primers used for bmm were: (Forward: 5’ACGTGATCATCTCGGAGTTTG3’, Reverse: 5’ATGGTGTTCTCGTCCAGAATG3’). Primers used for rp49 were: (Forward: 5’GACGCTTCAAGGGACAGTATCTG3’, Reverse: 5’AAACGCGGTTCTGCATGAG3’). Primers used for tra2 were: (Forward: 5’GAACATCCACAAGCAAGCCG3’, Reverse: 5’ATACGGCGACCATCCACTTC3’). The relative expression of all of these genes were normalized by dividing by rp49 expression levels.

Statistics

A Student’s t-test was performed to compare the yolk-Gal4>tra2^{dsRNA} genotype to the yolk-Gal4>attp2 controls for all assays performed in this thesis except starvation resistance. The Kaplan-Meier Estimator was used in OASIS to analyze survival data during starvation and the Log Rank test was used to determine differences in mean lifespan while on starvation medium. P-values <0.05 were considered significant for all statistical analyses.

Chapter 3

Results

To examine the potential metabolic functions of the splicing factor tra2, we decreased *tra2* levels specifically in the fat body by using the Gal4-UAS system to induce RNAi knockdown of *tra2*. This approach was effective because relative *tra2* mRNA levels in yolk-Gal4>tra2^{dsRNA} flies were lower than that of yolk-Gal4>attp2 control flies (Fig. 2).

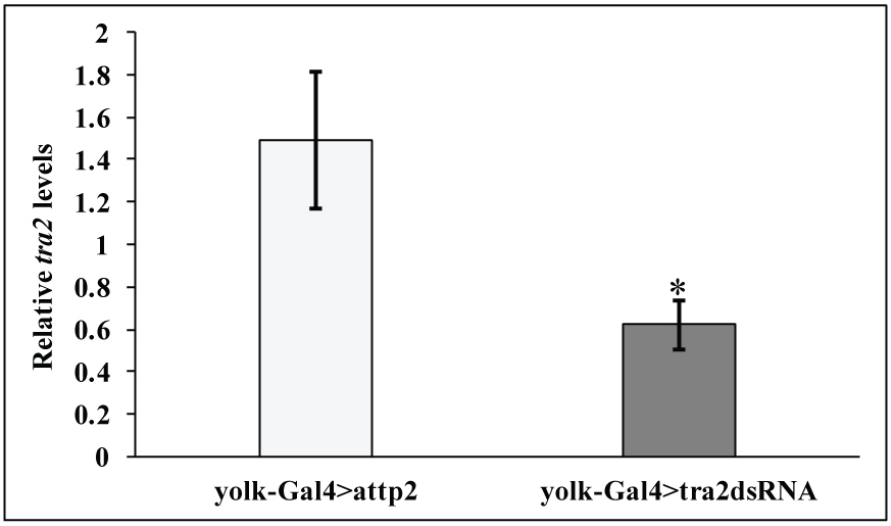


Figure 2. *tra2* levels are decreased in yolk-Gal4>tra2^{dsRNA} fat bodies.

***tra2* RNA levels were measured in adult, female yolk-Gal4>tra2^{dsRNA} fat bodies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error. *p<0.05 using Student's t-test.**

As a first step to determine any metabolic roles of *tra2*, yolk-Gal4>tra2^{dsRNA} and yolk-Gal4>attp2 flies were placed on only a water source (1% agar) to determine their starvation resistance. Flies with decreased *tra2* exhibited significantly longer survival rates on starvation media when compared to the yolk-Gal4>attp2 control flies (Fig. 3).

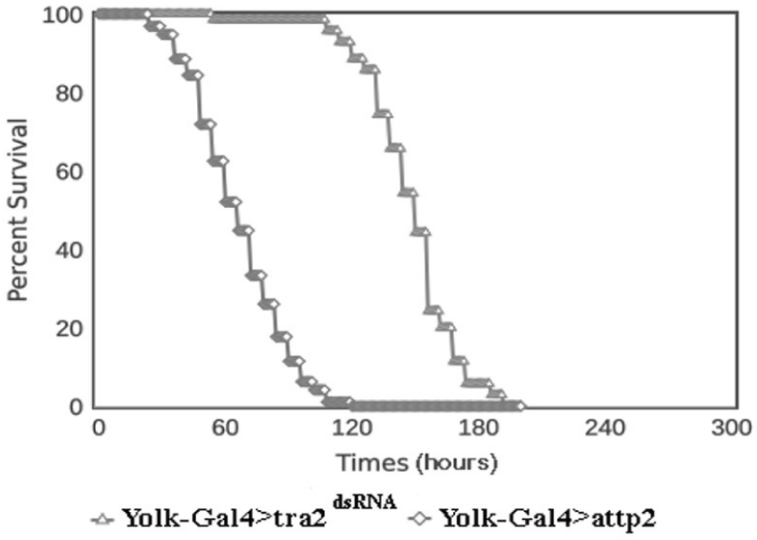


Figure 3. Decreasing *tra2* levels in the fat body results in enhanced starvation resistance.

Female yolk-Gal4>tra2^{dsRNA} and yolk-Gal4>attp2 adult flies were put on starvation media and the number of dead flies was measured every 6 hours. Survival curves were analyzed using the Kaplan-Meier Estimator on OASIS. $p<0.05$ using Log Rank test when comparing mean lifespan on starvation medium of both groups of flies.

Consistent with enhanced starvation resistance, decreasing the expression of *tra2* in the fat body also results in increased triglyceride storage in female adult flies compared to the *attp2* controls (Fig. 4A). However, glycogen storage was not altered in tra2-RNAi flies (Fig. 4B). Together, these findings indicate that the SR protein tra2 has metabolic functions in *D. melanogaster*.

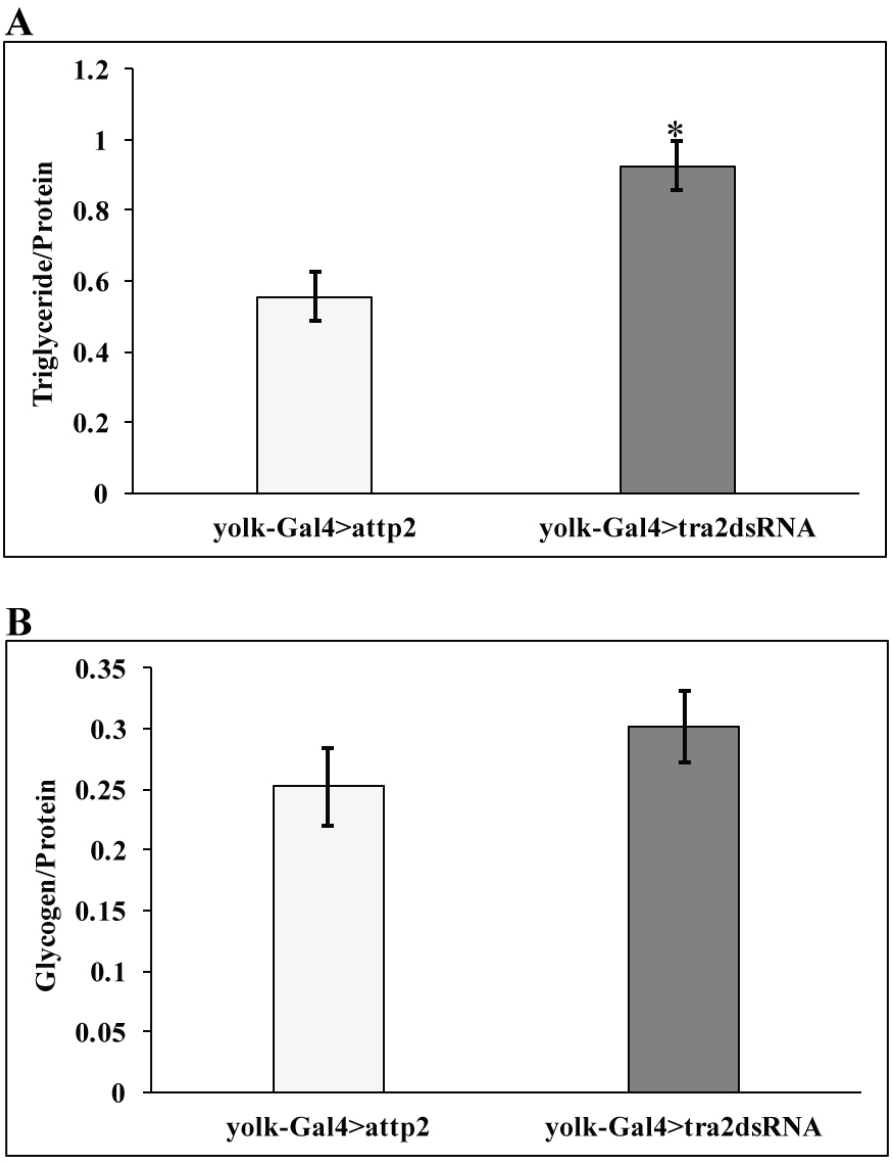


Figure 4. Decreasing *tra2* in the fly fat body results in increased triglyceride storage.

Triglyceride (A) and glycogen (B) levels were measured and normalized by total protein content for yolk-Gal4>tra2^{dsRNA} flies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error. *p<0.05 using Student's t-test.

One way in which triglyceride storage could be augmented is by increasing food intake. In order to test this hypothesis, food consumption was measured over a 24-hour period using capillary feeding (CAFÉ) assays (Ja et al., 2007). Monitoring food intake showed that yolk-

Gal4>tra2^{dsRNA} adult female flies were not consuming more food than the yolk-Gal4>attp2 control flies (Fig. 5). These results indicate that the augmented fat storage in tra2-RNAi flies was not caused by increasing feeding.

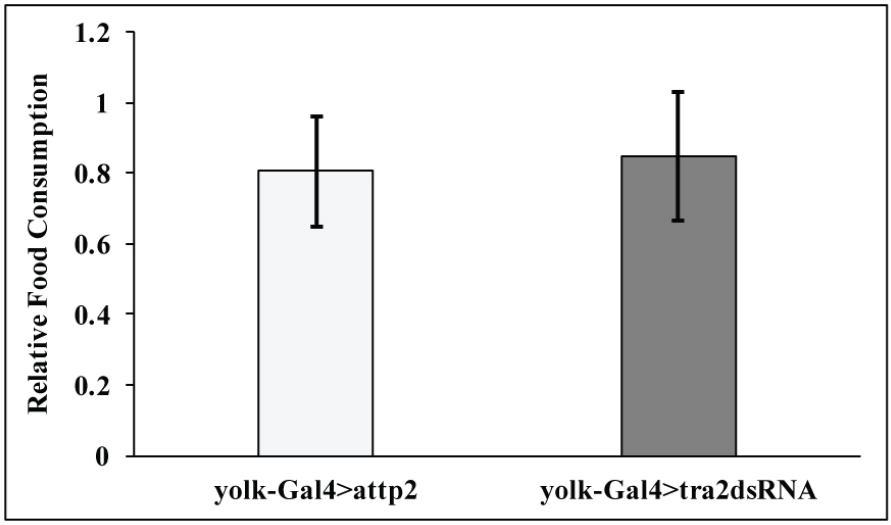


Figure 5. Feeding is not altered in flies with decreased *tra2* gene expression.

Food consumption was monitored over a 24-hour period using the CAFÉ assay in adult female yolk-Gal4>tra2^{dsRNA} flies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error.

It is also possible that the increased triglyceride storage phenotype observed in flies with decreased *tra2* levels can result from an increased number of fat cells within the fat body, an increased amount of fat stored per cell, or both. As the number of cells in an organ increases, the total DNA content of that organ also increases, allowing DNA content to be used as a surrogate measurement for cell number (DiAngelo and Birnbaum, 2009). We hypothesize that the number of fat cells present in tra2-RNAi flies would be increased, accounting for the augmented triglyceride storage phenotype. Surprisingly, we observed that the amount of DNA is actually decreased in tra2-RNAi flies, suggesting that the increased triglyceride phenotype in flies with decreased tra2 expression is not due to an increase in fat cell number (Fig 6A). To test whether

the amount of fat stored per cell was affected by decreasing *tra2*, triglyceride/DNA ratios were also measured (Fig. 6B). An increase in the triglyceride/DNA ratio was observed in tra2-RNAi flies, indicating that *tra2* regulates the amount of fat stored per fat body cell.

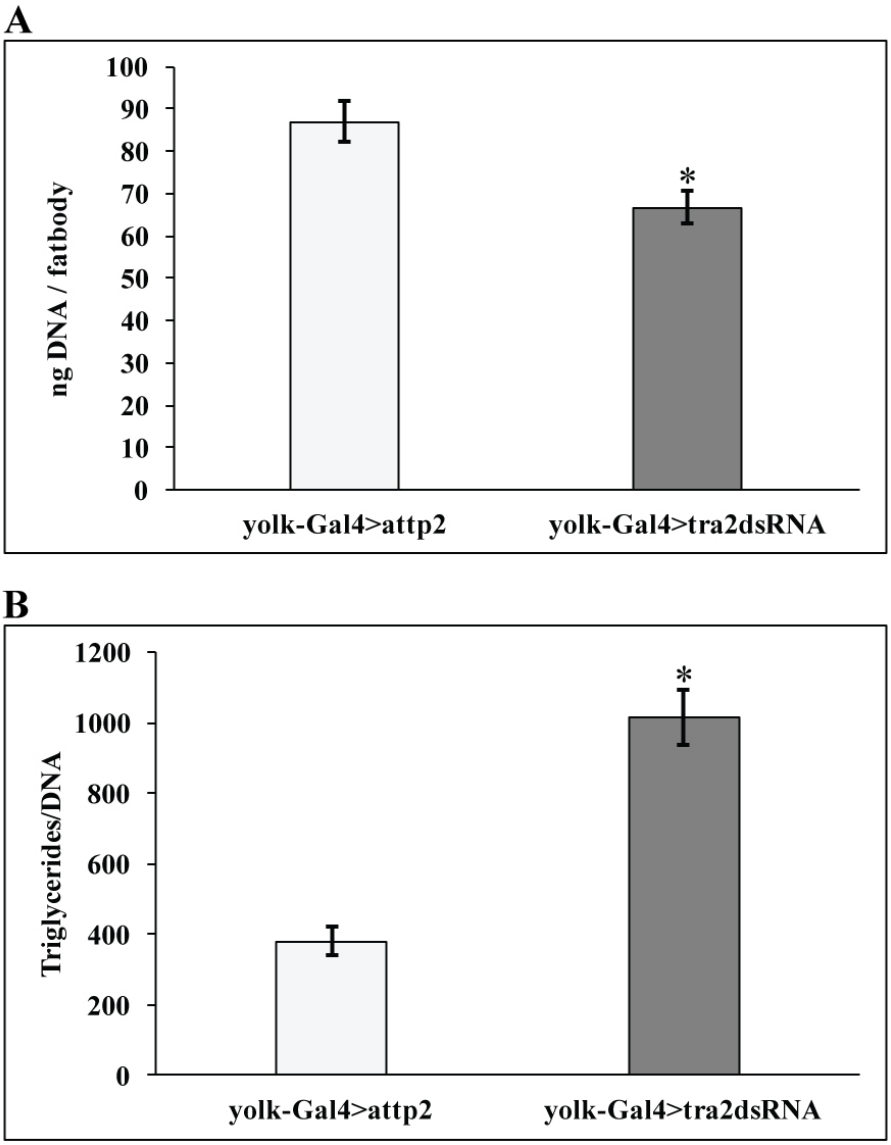
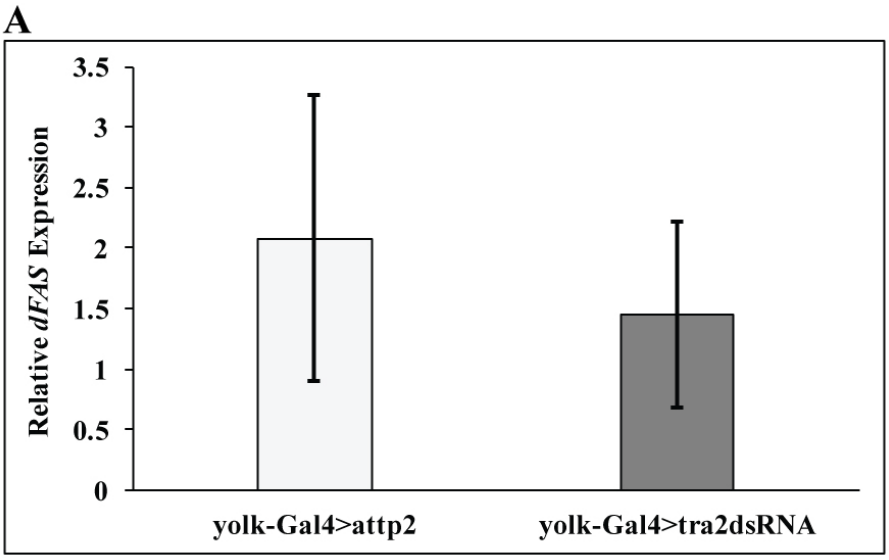


Figure 6. Decreasing fat body *tra2* results in fewer fat body cells and more fat per cell.

(A) DNA and (B) triglyceride levels were measured in yolk-Gal4>tra2^{dsRNA} fat bodies and compared to yolk-Gal4>attp2 controls. In panel B, total triglycerides were normalized by DNA content to represent the amount of fat per cell. Bars represent mean and error bars represent standard error. *p<0.05 using Student's t-test.

Since tra2-RNAi flies have increased triglycerides stored in each cell, we hypothesized that tra2 may control the expression of genes important for lipid synthesis, breakdown or both. To address this question, we started by measuring the expression of two genes in particular, *dFAS* and *dACC*, known for their rate-limiting role and high regulation in fatty acid synthesis. We performed qPCR to measure the expression of these two enzymes in flies with decreased *tra2*, and observed that the mRNA levels of these enzymes were not altered compared to control animals (Fig. 7).



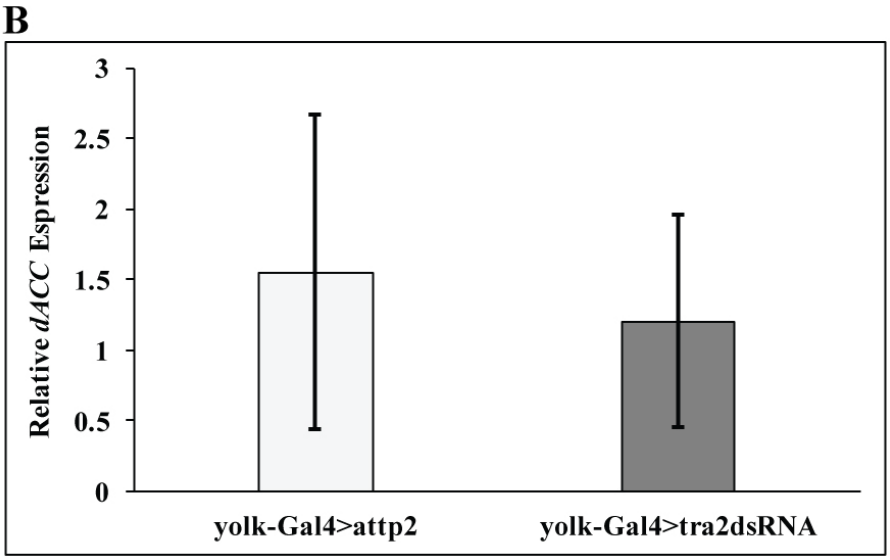


Figure 7. Decreasing *tra2* in the fat body does not affect the expression of enzymes involved in lipid synthesis.

The expression of *dFAS* (A) and *dACC* (B), enzymes important for lipid synthesis, was measured by qPCR in yolk-Gal4>tra2^{dsRNA} fat bodies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error.

In addition, we also measured the expression of *bmm*, the *Drosophila* homolog of adipose triglyceride lipase (ATGL), an enzyme important for lipolysis (Gronke et al., 2005), and the levels of this gene were also unaffected in tra2-RNAi flies when compared to yolk-Gal4>attp2 control flies (Fig. 8). Together, these results suggest that the increased triglyceride storage phenotype in yolk-Gal4>tra^{dsRNA} flies is not due to increased expression of lipid synthesis genes or decreased expression of genes important for lipid breakdown.

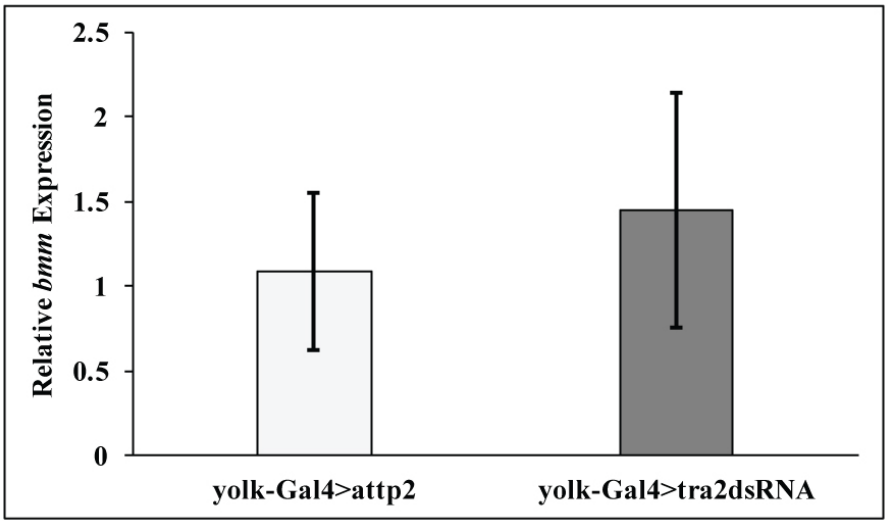
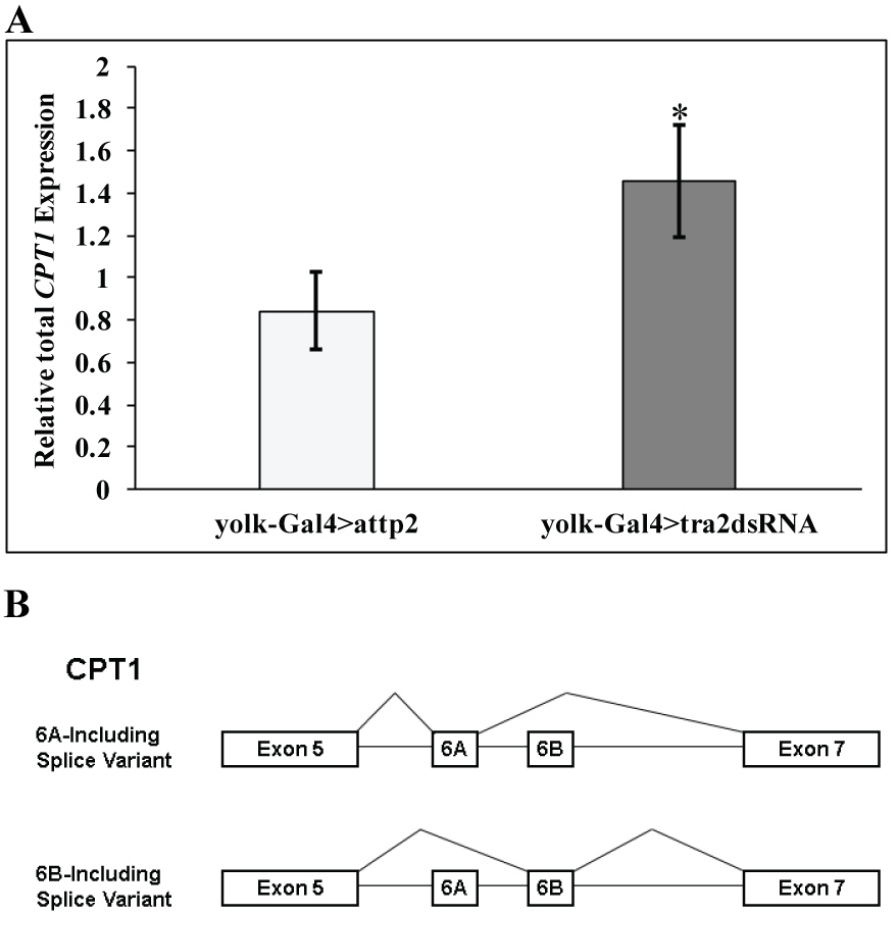


Figure 8. Decreasing *tra2* in the fat body has no effect on the expression of an enzyme involved in lipid breakdown.

The expression of *brummer* (*bmm*), an enzyme important for lipid breakdown, was measured by qPCR in yolk-Gal4>tra2^{dsRNA} fat bodies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error.

It is also possible that the processing of lipid metabolic genes could be altered in flies with decreased *tra2*. Our lab decided to investigate CPT1, the rate-liming step of β-oxidation. We hypothesized that total CPT1 expression would be lowered similar to the decreased triglyceride storage phenotype seen in tra2-RNAi flies. Surprisingly, total *CPT1* expression increased when compared to yolk-Gal4>attp2 flies (Fig. 9A). However, even though *CPT1* RNA levels increased, the activity of the CPT1 enzyme did not necessarily increase along with it. *Drosophila* contain two forms of *CPT1* resulting from the inclusion of an alternate sixth exon (6A or 6B) (Fig. 9B). CPT1 enzymes containing exon 6A have higher activity than those including 6B (Price et al., 2010). Therefore, it is possible that flies with decreased *tra2* may have altered *CPT1* splicing causing triglycerides to accumulate despite increased total *CPT1* expression. In order to investigate whether increased triglyceride levels in tra2-RNAi flies are

caused by alterative splicing of *CPT1* resulting in accumulation of the less active form of CPT1, we performed qPCR for the presence of these two alternative sixth exons of *CPT1* in flies with decreased *tra2*. Flies with less *tra2* in their fat bodies produced more *CPT1* transcripts including the 6B exon than the control flies (Fig. 9C), resulting in less CPT1 enzyme activity (Price et al., 2010). These data suggest that tra2 functions to control the splicing of *CPT1*, regulating the overall storage of triglycerides in the fly fat body.



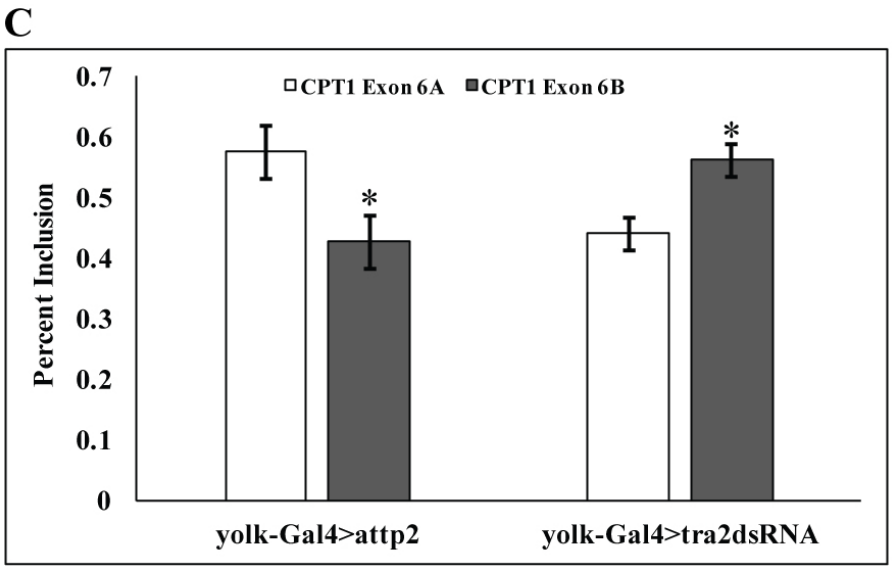


Figure 9. CPT1 splicing is altered in fat bodies with decreased *tra2*.

(A) The expression of total *CPT1*, a rate-limiting step of β-oxidation, was measured by qPCR in yolk-Gal4>tra2^{dsRNA} fat bodies and compared to yolk-Gal4>attp2 controls. Bars represent mean and error bars represent standard error. *p<0.05 using Student's t-test. (B) Diagram of 6A and 6B-including splice variants of *Drosophila CPT1* (adapted from Price et al., 2010). (C) qPCR was performed to amplify *CPT1* isoforms containing exon 6A and 6B. The percent of transcripts including exons 6A and 6B of *CPT1* in yolk-Gal4>attp2 and yolk-Gal4>tra2^{dsRNA} flies is shown above. Bars represent mean and error bars represent standard error. *p<0.05 using Student's t-test comparing percent inclusion of splice variant 6A and 6B in each genotype.

Chapter 4

Discussion

In this study, we set out to determine whether the splicing factor tra2 functions in the *Drosophila* fat body to regulate lipid metabolism. We have shown that fat body-specific knockdown of *tra2* resulted in increased triglyceride storage and starvation resistance. The augmented storage of triglycerides seemed to be due to an increase in the amount of fat stored per cell and not to increased food consumption or increased expression of genes coding for lipid metabolic enzymes. Interestingly, we found that the splicing of CPT1 was altered in flies with decreased *tra2*, resulting in a higher amount of the less active enzyme, potentially explaining the lipid storage phenotype.

Previous genome-wide RNAi screens performed by other researchers identified a group of genes whose knockdown resulted in smaller, more dispersed droplets (Guo et al., 2008; Beller et al., 2008). This group included genes such as *Rpr6*, *SmB*, *SmD* and *prp8*, which are components of various snRNPs in the spliceosome used for the removal of introns (Matera and Wang, 2014). The effects of altered expression of snRNP proteins on lipid storage in cultured cells is consistent with previous studies performed by the DiAngelo lab showing that fat body knockdown of components of the U1 and U2 snRNPs also results in decreased triglyceride levels (Guo et al., 2008; Beller et al., 2008; Gingras et al., 2014). Interestingly, altering the expression of SR proteins has different metabolic effects than snRNP gene knockdown. Knocking down SR proteins in the adult fat body has shown either no effect on triglycerides (with knockdown of B52 and SC35) or an increase in triglyceride storage (with knockdown of 9G8) (Gingras et al., 2014). It has also previously been shown that 9G8 binds to tra2 (which is also an SR protein) and

another RNA-binding protein called transformer (tra) to correctly process the *doublesex* (*dsx*) gene, which is important for controlling whether a fly develops into a male or female (Sciabica and Hertel, 2006). Since 9G8 and tra2 act together to regulate sex determination, it is possible that they play similar roles in controlling lipid storage and the results described here support that hypothesis.

Beyond its function in the splicing of *dsx* to control sex determination, we have uncovered a new role for *tra2* in the splicing of the gene coding for the metabolic enzyme CPT1. We show here that decreasing *tra2* results in the alternative splicing of the gene for CPT1, an enzyme important for lipid breakdown, consequently increasing the concentration of a less active CPT1 isoform. Interestingly, overall total CPT1 levels were also seen to increase in *tra2* knockdown flies. While this is not what we expected, we hypothesize that while more of this lipid breakdown enzyme is present, there are more CPT1 enzymes with lower activity, resulting in less lipid breakdown overall and explaining the increased lipid storage phenotype. It would be useful to determine whether having higher CPT1 mRNA levels enriched with the less active CPT1 isoform correlates to decreased overall CPT1 enzyme activity.

In order to further explain the alternative splicing of CPT1 in 9G8 knockdown flies and the resulting increased triglyceride levels, our collaborator, Alexis Nagengast, has generated an algorithm that predicts the location of 9G8 binding sites in the nucleotide sequence of a gene. Consistent with the altered splicing of CPT1 in 9G8-RNAi flies, one 9G8 binding site has been identified at the end of exon 5 in the *CPT1* gene (unpublished observations) (Fig. 10A). This 9G8 binding site could be responsible for the alternative splicing of exon 6 of *CPT1* resulting in

the inclusion of exon 6A and the loss of this splice site or the SR protein that binds to it may result in inclusion of exon 6B of *CPT1* instead, therefore affecting overall lipid breakdown (Fig. 10B). If our hypothesis is correct that tra2 and 9G8 work together to control the splicing of *CPT1*, we would expect to find a *tra2* binding sequence in close proximity to the 9G8 binding site described above (Fig. 10C).

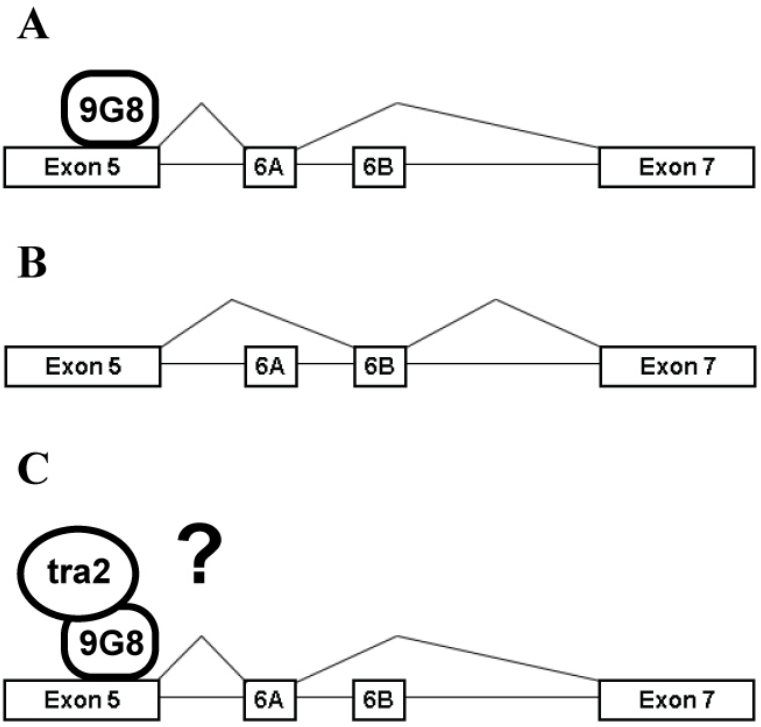


Figure 10. Model for the alternative splicing of *CPT1* by 9G8 and tra2.

(A) The binding of 9G8 to exon 5 in the *CPT1* gene results in the inclusion of exon 6A, and increased lipid breakdown. (B) In 9G8-RNAi flies, the lack of 9G8 binding to exon 5 results in the inclusion of exon 6B, and decreased lipid breakdown. (C) tra2 may be involved in the regulation of *CPT1* splicing via interactions with 9G8.

It is also possible that instead of binding directly to the CPT1 transcript and regulating its alternative splicing, tra2 could form a heterodimer with 9G8 and the entire complex binds to the *CPT1* transcript to control its processing. To test this hypothesis, immunoprecipitations for tra2 and 9G8 could be performed after crosslinking proteins to mRNA and the presence of the *CPT1*

mRNA could be determined by qPCR. In addition, it is possible that 9G8 and tra2 regulate the splicing of additional lipid metabolic genes such as fatty acid synthase (dFAS), acetyl-CoA carboxylase (dACC) or brummer (bmm), and experiments designed to measure the splicing of these genes will help provide insight into additional metabolic functions of 9G8 and tra2.

The results from this study have potential implications on human metabolism. The expression of the human homolog of *Drosophila tra2*, *SFRS10*, has been observed to be decreased in obese patients and, consistent with these results, knockdown of *SFRS10* in cells and mice leads to increased triglyceride storage (Pihlajamäki et al., 2011), similar to what we have shown here for *tra2*. However, one difference between tra2 and SFRS10 is that SFRS10 does not control the splicing of *CPT1* in humans (in fact, *CPT1* is not alternatively spliced in humans at all (Price et al., 2010)). SFRS10 does affect the splicing of another gene important for lipogenesis, *Lpin1*. Interestingly, the splicing pattern of *Lpin1* is similar to what has been seen in *CPT1* splicing. The Lpin1 enzyme catalyzes the removal of a phosphate from phosphatidic acid and can exist in more and less active forms, controlled by the presence of exon 6 in the *Lpin1* gene (Csaki and Reue, 2010). The knockdown of *SFRS10* leads to increased levels of the *Lpin1ß* isoform, leading to augmented expression of lipogenic genes (Csaki and Reue, 2010; Peterfly et al., 2005). Interestingly, altered splicing of *Lpin1* is responsible for the obese phenotype of animals and cells with less *SFRS10* as simultaneous knockdown of both *SFRS10* and *Lpin1ß* results in normal triglyceride levels and lipogenesis (Pihlajamäki et al., 2011). It would be interesting to investigate whether a similar phenotype can be seen when both *tra2* and *CPT1* are decreased in flies. While the *CPT1* gene has been identified as a target of the splicing factor tra2, it would also be interesting to determine whether decreasing *tra2* affects the splicing of the

Drosophila lipin homolog in a similar fashion to SFRS10.

Together, the findings in this thesis show that the knockdown of *tra2* in the *Drosophila* fat body results in an increased triglyceride phenotype, which could be explained by altered splicing of the gene coding for the lipid breakdown enzyme, CPT1. These findings are consistent with previous studies analyzing the SR protein, 9G8, providing support of the hypothesis that these two RNA-binding proteins may be functioning together to control the splicing of lipid metabolic genes and, therefore, overall lipid homeostasis. The increased triglyceride storage seen with the knockdown of the human *tra2* homolog, *SFRS10*, has also been consistent with our results suggesting that tra2 and other RNA binding proteins play a highly conserved role in regulating the expression and processing of important metabolic enzymes, many of which have yet to be identified. Together, the results from this thesis suggest a link between mRNA splicing, sex determination and lipid metabolism and may provide insight into the mechanisms underlying tissue-specific splicing and nutrient storage and the development of obesity.

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