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Examination of Interaction between the SR Proteins 9G8 and Repressor Splicing Factor 1 in the Alternative Splicing of Carnitine Palmitoyltransferase I in lipid Metabolism

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Abstract

The method of gene regulation underlying lipid metabolism related to obesity is poorly understood, yet alternative splicing (AS) appears to be an important mechanism for proper lipid storage. CPT1 (carnitine palmitoyltransferase I) is a beta-oxidation enzyme required for the breakdown of fatty acids. The gene coding for CPT1 is alternatively spliced to produce two different products that vary in their activity. When the splicing SR protein 9G8 is knocked down in the fat body of Drosophila, the CPT1 splicing pattern is altered. A linear search algorithm was developed that parsed through FASTA files of the CPT1 gene region and sought out sequences that matched known binding sequences of 9G8. We expected a result in exon 6A that would signal its inclusion but unexpectedly found a match in exon 5 of the CPT1 transcript. We theorized that 9G8 and the SR protein competitor RSF1, interact with one another for binding sites on the CPT1 gene to result in different isoforms. Additionally, TG data indicated that 9G8 and RSF1 KD flies displayed statistically significant higher TG levels than their control suggesting a potential cooperative relationship. We next performed a starvation resistance experiment and found that 9G8 and RSF1 KD flies phenocopied starvation resistance suggesting that both proteins interact to result in the same CPT1 product. We are using qPCR to determine if there is a difference in the AS of CPT1 among flies with decreased expression of 9G8, the SR protein antagonist RSF1 and the SR protein shuttle TRN-SR.

Keywords: Biochemistry, Alternative Splicing, Lipid Metabolism, CPT1, 9G8, RSF1

Introduction

The past 30 years have revealed a staggering trend in obesity data. According to the National Institute of Health, over 37% of all Americans are considered obese (BMI > 30) as of 2017. A recent study that came from Robert Wood Johnson Foundation Health and Columbia University found that one out of every five deaths is now attributed to obesity [1]. There are three major factors that influence obesity: nutrition, activity level, and gene regulation. As the prior two factors seem to affect gene regulation, it makes sense to study obesity from a molecular level instead of a behavioral. Consequently, two large genome wide RNAi screens were done in *Drosophila* S2 cells that examined lipid phenotypes [2]. Specifically, one of the phenotypes that was exhibited was a normal size and dispersion but reduced amount of lipid droplets when splicing factors were knocked down.

This thesis focuses on the role of alternative splicing related to the cause of obesity and lipid over storage. Splicing is the process of connecting exons, or protein coding segments of genes, into an mRNA transcript (Figure 1) [2]. This transcript then gets translated by the ribosomes in the cytoplasm to produce a protein with a function specific to the exons incorporated by the mRNA transcript. Splicing is a critical process in physiology because it allows there to be control or modification of the pre-mRNA strand before the mature mRNA transcript gets translated to produce a protein.

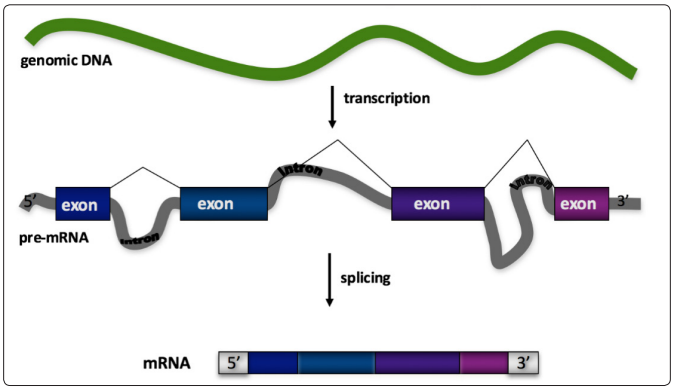

Figure 1: Overview of Splicing

Genomic DNA is transcribed into various exons and introns in a pre-mRNA transcript. Splicing is then the process of excising the introns away and ligating the different exons together, forming a mature contiguous sequence.

The process of splicing is comprised of a series of highly dynamic steps and interactions [3]. As such, the process of splicing can be summarized in three main stages: recruitment of proteins, removal of introns, and ligation of exons. In recruitment stage, a complex multi-subunit structure called the spliceosome is recruited to mRNA sequences that require splicing and intron and exon borders are defined. In the removal stage, the introns are removed by the spliceosome. The RNA within the spliceosome is catalytic and facilitates the reactions that need to occur during splicing and has a very complicated structure [4]. In the ligation phase, the exons are all ligated together to consolidate the transcript to only include protein-coding segments of the gene.

Under the broad category of general splicing, there is a significant subsection called alternative splicing. Alternative splicing is similar to general splicing but is responsible for the astounding extent of the biodiversity that is present in life [5]. General or constitutive splicing is removing the introns and ligating the exons to produce a contiguous mature mRNA transcript which will be translated by a ribosome and eventually yield a protein product, as can be seen in Figure 2. Alternative splicing maximizes the output of the genome by selectively incorporating various exons or introns into the mRNA transcript (Figure 2). Alternative splicing can also incorporate introns in addition to various exons for inclusion into the mRNA transcript. Since the function of the protein produced after translation is specific to the identity of the exons, alternative splicing is responsible for generating tremendous biodiversity using proteins. This is a very conserved process as 94% of human genes and 40% of *Drosophila* genes have been shown to be alternatively spliced [6, 7].

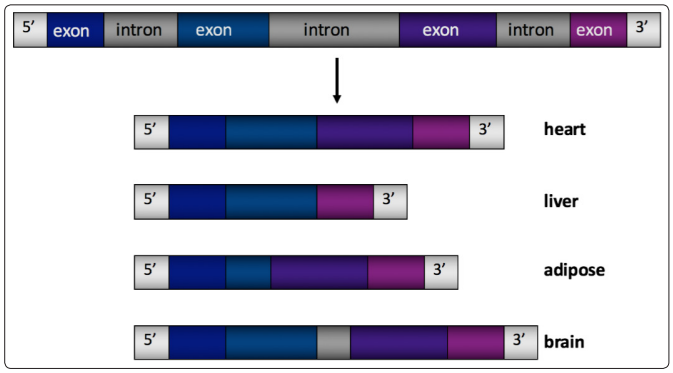

Figure 2: Alternative-Splicing Leads to Biodiversity

Figure 2 above shows the wide array of specific proteins that could be generated using alternative splicing. The same pre-mRNA strand can give rise to different protein types such as brain or adipose specific proteins by including a different series of exons or introns within the mature mRNA transcript.

There are five basic modes or types of common alternative splicing mechanisms that occur (illustrated in Figure 3) [8]. The most common type of alternative splicing is exon skipping. In exon skipping, exons can either be spliced out or retained in the mature mRNA transcript. This type of alternative splicing is also called cassette exon because the transcript is put together much like a cassette where there are selective parts included or excluded. Another mode of alternative splicing is mutually exclusive exons. In this pattern, one of two exons is incorporated within the mature mRNA transcript but not both. The rarest mode of alternative splicing that occurs is called intron retention. This is where the introns are retained in the final mRNA transcript alongside exons. This is another point of difference between splicing and alternative splicing. In general or constitutive splicing, introns are spliced out and exons are contiguously ligated together; whereas, in alternative splicing, both exons and introns are selectively included or excluded from the mature mRNA transcript. It is this difference which makes alternative splicing responsible for such diversity among the protein code. The other two modes are alternative 5' donor sites and alternative 3' acceptor sites.

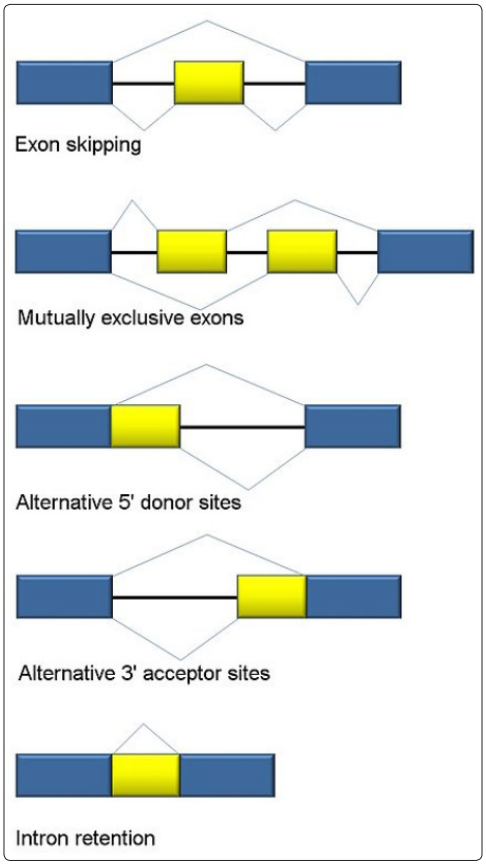

Figure 3: There are Five Different Modes of Canonical Alternative Splicing

The most common and well understood modes of alternative splicing are illustrated. The most common mode of canonical alternative splicing is exon skipping and the least common is intron retention [9].

These splicing modes can be seen across a wide array of different organismal species such as humans, flies, chickens. This suggests that the process of alternatively splicing one's genes is an evolutionarily conserved mechanism [10]. Figure 4 below depicts this concept with a visual representation of the prevalence that each mode has on two different organisms: *Drosophila* and humans. The most common form of splicing that is seen in humans is the exon skipping method, whereas, in *Drosophila*, all five modes are relatively equal to one another.

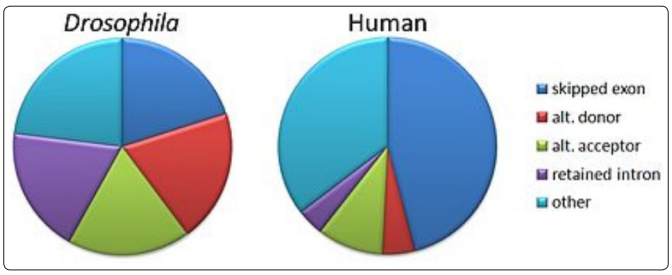

Figure 4: Comparison of the Various Alternative Splicing Modes between Drosophila and Humans

In *Drosophila*, all five modes are relatively evenly used. This observation is starkly contrasted with the modes of splicing in humans. In humans, the vast majority are other modes not currently fully understood and the skipped exon process, which accounts for nearly 50% of all alternatively spliced genes [9].

It is very easy to see how alternative splicing can produce a wide variety of protein structures from a total of 64 codons. Alternative splicing provides benefits such as selectively including various components of the mRNA strand and also giving rise to diversity. Although there are great benefits of alternatively splicing genes, there are some drawbacks that can be associated with this process. Because alternative splicing gives rise to a completely different type of protein structure, mutations that occur during this process are usually very deleterious to the organism and could be lethal (Figure 5).

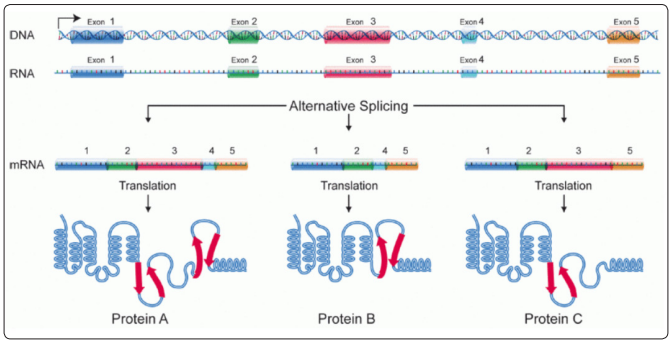

Figure 5: Mutations in Alternative Splicing Could Lead to Drastically Different Protein Structures

Because the identity of the protein is highly specific to the exons/introns included, if a mutation occurred that altered the composition of the mature mRNA transcript, then a vital protein type could be altered such as changing an intracellular to extracellular component of a transmembrane protein. This could be deleterious to the organism and produce potentially lethal consequences (Bioinformatics: Alternative Splcing. https://www.genome.gov/25020001/online-education-kit-bioinformatics-finding-genes/. DOA: 5/17).

Due to alternative splicing's strong effect on protein structure and phenotype, it has been linked to many diseases as well as cancers. mRNA transcripts which are abnormally spliced are prominently found in cancer diseases [11]. Previous studies have shown that varying expression of splicing proteins is an important aspect of cancer pathways [12]. Thus, alternative splicing is a very relevant process that has wide implications in the medical field and plays crucial importance in this experiment as well.

The actual splicing reaction is composed of two trans-esterification reactions (Figure 6) [13]. The first trans-esterification reaction is where cleavage occurs on the 5' end of the intron and is joined to a branchpoint adenosine by a phosphodiester bond. The second trans-esterification reaction is where cleavage occurs on the 3' end of the intron and the two exons are connected by another phosphodiester linkage. The excluded introns are released as a lariat and degraded.

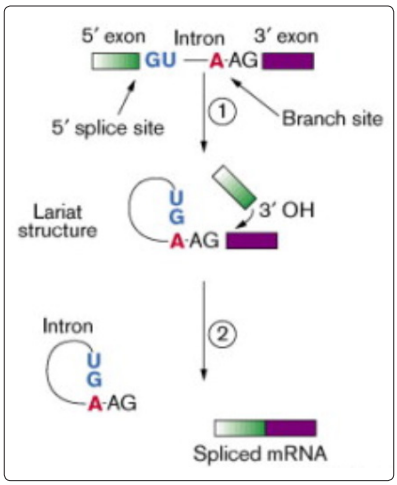

Figure 6: Overview of Trans-esterification Reaction of Splicing

There are two trans-esterification reactions that are catalyzed by the spliceosome. After catalysis, there is a lariat structure formed that releases the excluded mRNA intron so that it will get degraded [14].

The trans-esterification reaction is highly complex and is dependent on minimal sequences for the generation of a contiguous sequence. However, the identification of intron and exon borders are just as important for proper splicing to occur. These borders display conserved canonical nucleotide motifs and are identified by the presence of a GU nucleotide at the 5' splice site and an AG nucleotide at the 3' splice site. In alternative splicing (AS), the selectivity of various splice sites is regulated by special proteins with serine arginine (SR) dipeptide repeats known as SR proteins.

AS of pre-mRNA is regulated by SR proteins that, with varying concentration, can modulate the selection of various splice sites. SR proteins are critical for AS and make up an entire class of proteins in humans. The human SR protein family is comprised of nine canonical SR proteins that code for unique proteins with an N-terminal RNA binding domain and a C-terminal RS domain (Figure 7) [15]. In *Drosophila*, there are eight canonical SR proteins (B52, SC35, SF2, SRp54, XL6/9G8, Rbp1, Rbp1-like, and RSF1) [16]. SR proteins have a protein domain called the RNA recognition motif (RRM) that plays a crucial role in regulating pre-mRNA splicing by binding to the pre-mRNA transcript. The RS domain (arginine and serine dipeptide repeats) is involved in protein-protein interactions with other splicing factors. SR proteins are primarily found within a subdomain in the nucleus that functions to store or provide assembly sites for common transcription and splicing components. The RS domain can also function either as a localization or targeting signal to the splicing subdomains and can even assist in nucleo-cytoplasmic shuttling.

Before the SR protein can get shuttled into the nucleus for its splicing function, it first has to be activated in the cytoplasm. The protein is

activated when serines in the RS domain get phosphorylated, signaling shuttle transport. In *Drosophila*, RS domain phosphorylation is critical for nuclear import of SR proteins [17]. Transportin-SR (TRN-SR) shuttle protein interacts specifically with the phosphorylated RS domain and also modulates the import of these phosphorylated SR proteins into the nucleus for splicing to occur. Thus, shuttling seems to act as a mediating mechanism for AS regulation of common SR proteins.

Figure 7: Canonical SR Protein Family in Humans

There are nine major SR proteins in humans that encode for a variety of unique proteins. Each canonical SR protein is composed of a N-terminal RNA recognition motif (RRM), and a C-terminal arginine and serine (RS) domain. Additional SR proteins also contain an RNA recognition motif homolog (RRMH) [15]. 9G8 is unique in that it also contains a Zn finger domain.

SR proteins help to regulate either the inclusion or exclusion of specific exons based on their promoting or inhibiting function. 9G8 functions as an activating SR protein, as it helps to promote the use of weaker splice sites [18]. Conversely, other SR proteins behave in an inhibitory mode that blocks exons from inclusion in the transcript. Prior RNAi results in our lab revealed that 9G8 and another *Drosophila* canonical SR protein, repressor splicing factor 1 (RSF1), showed opposite triglyceride (TG) phenotypes when knocked down by RNAi in the fat body of larvae. A BLASTP alignment between the RRM domains of 9G8 and RSF1 revealed that they were 56% similar and 44% identical. This is possible due to the similarity of the RNA Recognition Motif (RRM) between 9G8 and RSF1 that binds to specific sequences or secondary structure in pre-mRNA. Their binding sequence similarity may allow the inhibitory SR protein, RSF1, to bind at the same sequence as the promoting SR protein, 9G8, and exclude an exon as a way of mediating exon selectivity through a competitive mechanism. Additionally, the RS domain of both 9G8 and RSF1 are critical for their ability to be phosphorylated and enter the nucleus for their splicing.

9G8 has been implicated in carnitine palmitoyltransferase 1 (CPT1) alternative splicing and lipid over storage in adult females [19]. CPT1 regulates the transport of long chain fatty acids into the mitochondria for β-oxidation to occur (Figure 8). The enzyme is associated with breaking down fat reserves for quick energy usage. CPT1 mRNA expression is elevated in a starvation environment to facilitate the breakdown of fatty acids. CPT1 has two main product isoforms – one that is more active and includes exon 6A, and one that is less active and includes exon 6B (Table1 + Figure 9) [17]. We theorized that the alternative splicing pattern of CPT1 depends on an SR protein binding to pre-mRNA sequences to promote the use of an alternative, weaker splice site. A 9G8 binding site has been found computationally near exon 6A and may promote the inclusion of the proximal exon 6A over 6B to result in the more active isoform of CPT1.

Prior experiments demonstrated that when 9G8 was knocked down by RNAi in the fat body of adult females, the less active isoform of CPT1 with exon 6B was produced and TG levels were high. Therefore in wild type scenarios when 9G8 is present, the proximal exon 6A is selected for in the CPT1 transcript, the resulting CPT1 enzyme activity is high, and normal TG levels are maintained by β-oxidation, giving a normal phenotype fly (Table 1). When 9G8 protein concentration is decreased due to RNAi knock down, the distal exon 6B is included, the resulting CPT1 enzyme activity is lower, β-oxidation decreases and TG levels increase (Table 1 and Table 2),

Table 1: CPT1 Splicing Patterns

6A	CPT1 ↑	β-oxidation ↑	TG =
6B	CPT1 ↓	β-oxidation ↓	TG ↑

Isoform 6A is associated with an increased CPT1 enzyme activity, which results in normal TG levels because of the increased rate of β-oxidation. Conversely, isoform 6B is associated with lower CPT1 enzyme activity producing a lipid overstorage phenotype fly with increased TG levels, due to a decrease in β-oxidation.

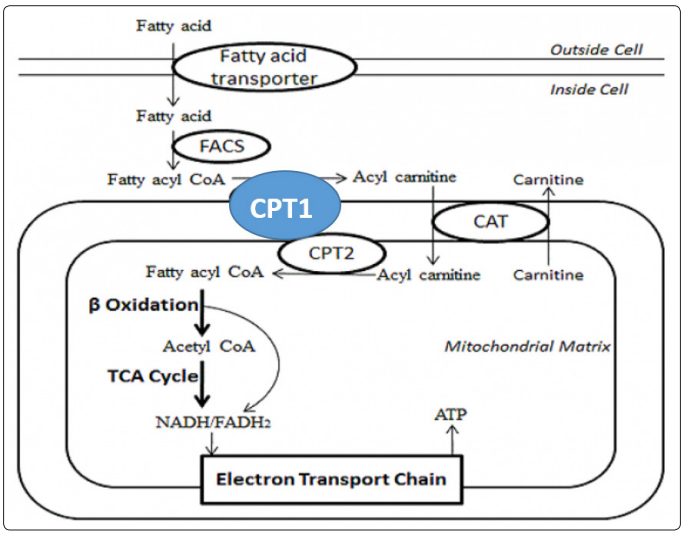

Figure 8: CPT1 role in β-oxidation

CPT1 transports long chain fatty acids into the mitochondria for breakdown into two carbon acetyl units, which is known as β-oxidation. CPT1 is the rate limiting step that regulates the levels of β-oxidation whose function is the catalysis of transferring long chain acyl groups of acetyl-coA to carnitine allowing the shuttling of fats into the mitochondria. (Fatty Acid Oxidation and Synthesis. https://www.diapedia.org/metabolism-insulin-and-other-hormones/5105592814/fatty-acid-oxidation-and-synthesis. DOA: 5/17)

It is currently unknown whether the SR proteins 9G8 and RSF1 both compete for the binding sites on the CPT1 gene once in the nucleus. Therefore, this thesis will investigate whether RSF1 plays a role in TG levels in adult whole animal samples and if RSF1 competes or cooperates with 9G8 in lipid metabolism.

If 9G8 and RSF1 are competing antagonistically, then CPT1 splicing would be opposite to 9G8 knock down if RSF1 were knocked down (Figure 10). Following the 9G8 RNA$_i$ data, it is expected that in a RSF1 RNAi environment, the more active isoform exon 6A would be produced and TG levels would be normal. Therefore, monitoring the levels of triglyceride in an RSF1 RNAi environment may indicate changes in the splicing patterns of the CPT1 gene [18].

However, if 9G8 and RSF1 are working together in a cooperative manner, then CPT1 splicing when RSF1 is knocked down should match splicing when 9G8 is knocked down (Figure 10). If a cooperative interaction is observed, then the isoform ratios would switch from wild type (60% 6A:40% 6B) and be 40% 6A:60% 6B, which is associated with decreased CPT1 activity, decreased β-oxidation, and increased TG levels producing a lipid overstorage fly phenotype [19].

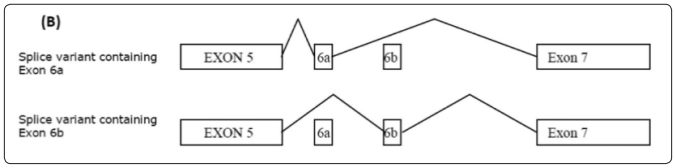

Figure 9: CPT1 Slicing Patterns and Isoforms

CPT1 has two splice variants that can include either exon 6A or exon 6B within the sequence [19]. The 6A isoform codes for a more active form of the enzyme and the 6B isoform codes for a less active CPT1 enzyme. More active CPT1 means more active carnitylation of activated fatty acids so that they can get localized into the mitochondrial matrix for β-oxidation to occur. In a 9G8 KD background, inclusion of exon 6B is increased and higher triglyceride levels are reported, presumably due to decreased activity of CPT1 and decreased fatty acid breakdown.

Phosphorylation signals may also be activated under different nutrient conditions to allow the correct isoform of CPT1 to be produced. For example, under low nutrient conditions, 9G8 may be activated by glucagon (AKH) signaling and imported into the nucleus to splice the exon 6A, more active CPT1 product for increased β-oxidation. Likewise when 9G8 is artificially knocked down by RNAi, more of the exon 6B, less active isoform is produced and there is less -oxidation and increased TG. In the competitive model, under high nutrient conditions, RSF1 may be phosphorylated by insulin signaling and activated to be imported into the nucleus to block the use of exon 6A, resulting in more of the exon 6B, less active form of CPT1 and decreased β-oxidation. This would suggest that knock down of RSF1 would produce the more active form of CPT1 and less TG, opposite of the 9G8 knock down phenotype.

This thesis aims to improve knowledge of the SR protein model of CPT1 alternative splicing related to CPT1 enzyme activity and subsequent triglyceride phenotype in adult females. The current understanding of the model is that the selectivity of different splice sites on CPT1 pre-mRNA is highly influenced by which SR protein binds to promote or block exon 6A or 6B inclusion. The two isoforms of CPT1 are 6A and 6B (Figure 9) and vary in their activity related to beta-oxidation and triglyceride levels. The more active isoform contains exon 6A and due to higher CPT1 activity, the phenotype that is produced is a normal fly because of homeostatic levels of beta-oxidation resulting in standard TG levels. The lesser active isoform contains exon 6B and exhibits low CPT1 activity and produces a phenotype of a lipid over storage fly because of the decreased beta-oxidation and fatty acid breakdown, resulting in increased TG levels [20] (Table 2).

Table 2: Preferred Exon and CPT1 Activity

Condition	Major Exon	CPT1 Activity	TG Levels
Wild type	6A	High	Normal
9G8 KD	6B	Low	High

The wild type (wt) splicing pattern of CPT1 is a 60% 6A: 40% 6B isoform ratio, preferring the 6A exon and resulting in normal TG levels due to high CPT1 activity. When 9G8 is knocked down, exon 6B is preferred, possibly because RSF1 can bind at exon 6A and prevent exon 6A inclusion [19].

The SR protein 9G8 primarily acts as an activator for splice sites whereas, RSF1 acts as a repressor as shown by RNA-seq analysis of AS events in *Drosophila* S2 cells [16]. This interaction between 9G8 and RSF1 suggests a competitive model. However in the RNA-seq analysis, 9G8 is approximately equal in preferring co-regulation with another SR protein or unique splicing done solely by 9G8 [18]. RSF1 prefers co-regulation meaning it predominantly acts with another SR protein for splicing [16]. These results do not clearly distinguish between a cooperative or competitive model and further investigation needs to be done to better understand the role of splicing in regulating lipid storage levels.

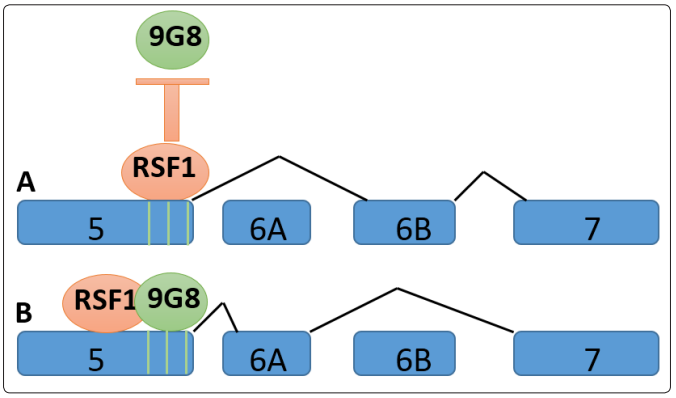

Figure 10: 9G8 + RSF1 Competitive and Cooperative Model

9G8 and RSF1 can interact either competitively or cooperatively with one another for binding sites in exon 5. Panel A shows a competitive relationship as RSF1 binding to exon 5 will prevent 9G8 binding and will promote the use of the distal 6B splice site. Conversely, panel B illustrates a cooperative model in which both 9G8 and RSF1 work together to promote the use of the proximal 6A splice site.

Materials and Methods

Fly Stocks

Table 3: Fly Lines of All Fly Samples used for the Gal4-UAS system of RNAi knock down and the parental sex

Parental Sex	Stock No	Lines	Chromosome with ransgene Insertion
M	145	w^{1118}; attB: UAS-IR: 9G8 VDRC	II
M	130	w^{1118}; UAS-IR: RSF1 VDRC	III
M	178	W^{1118} VDRC	n/a
M	223	yv; attP2: UAS-IR-TRN-SR	III
M	177	yv; attP2	III
F	44	yolk-Gal4/FM7	I

The female flies (stock 44) were all crossed with the above males using the takeout-gal4 UAS driver and their progeny collected, dissected, and analyzed. IR means inverted repeat which induces RNAi. RNAi is when the RNAi induced silencing complex (RISC) cleaves miRNA into one stranded siRNA which complimentary base pairs to the mRNA of a gene of interest and the mRNA message is degraded and expression inhibited.

The flies were raised in vials using a standard molasses food source sprinkled with yeast. The crosses were maintained at 25 °C in an incubator. Progeny were transferred to fresh food within 3 days of eclosion and aged approximately one week before use in triglyceride assays or starvation resistance analysis.

Bioinformatics

In order to determine how 9G8 and RSF1 interact on the CPT1 gene, a bioinformatics search was performed using a program that Abhay Arahay developed to search for the binding motifs of both SR proteins to see if there would be a hit on the CPT1 exon coding regions. Prior literature shows the individual binding motifs of 9G8 and RSF1 to be CUUCGA, CUACAA, CUUCAA and ACUUCU, respectively [16]. These binding sites were discovered by MEME-CHIP. The search parameters were run by using FASTA files for all exon regions of CPT1 and iterated through them, index by index, to find hits for the target sequence of the SR proteins 9G8 and RSF1.

Quantify TG levels under RNAi Knock down in Female FB

SR proteins 9G8 and RSF1 gain access into the nucleus for splicing of CPT1 which is implicated with lipid storage related to measurable TG levels. Using the gal4-yolk driver, 9G8, RSF1 and TRN-SR were knocked down by RNAi in the adult female fat body with the background transgene strain w1118 used as a control. Progeny from the crosses were moved to a fresh vial of food within three days of eclosion and analyzed by StanBio TG Liquicolor assay (catalog # 2200225) within one week of transfer because adult fat body takes one week to form. Triglyceride levels were normalized to protein levels using a Pierce BCA Protein Standard Kit (catalog #23225). At least 12 different samples from each knock down were analyzed in triplicate by TG assays. The UAS-RSF1 stock is not homozygous and the transgene is heterozygous to the TM3 Sb (Stubble) balancer chromosome. Therefore, to ensure adequate sample numbers, two vials of crosses was set up for RSF1 RNAi. Knock down was performed in adults because there is also no visual marker for Stubble in larvae so there would be no way to identify which larvae have the RSF1 knock down as opposed to TM3, whereas Stubble is easily visible in adults. The TG assay was done using the lab standard sample prep using a 200 μL volume of extract and TG assay protocol. The TG level results were normalized to protein to account for any variability among the flies using the lab standard protein assay protocol. Mothers of the genotype w-; to-gal4 were crossed to males with UAS transgenes to knock down 9G8, RSF1, and TRN-SR shuttle (complete genotypes listed in Table 3).

Starvation Survival

Triplicates of 20-25 flies per each genotype were followed over the course of several days the number of fly deaths per each 6-hour time interval was recorded. The flies were starved of food on 2% agar and kept in a dry place in room temperature. The data log was compiled in Microsoft Excel and then uploaded to the Online Application for the Survival Analysis of Lifespan Assays (OASIS) for figure generation and statistical analyses [20].

Dissections + qPCR

Because previously published results used dissected fat body, it was necessary to dissect out the fat bodies of the flies to concentrate the signal enough for quantitation and also because different tissues, such as muscle, have different isoform 6A to isoform 6B ratios. Two dissections were done to see which would provide the best detection values – a crude and a fine dissection. The crude dissection separated the abdomen from the thorax; whereas, the fine dissection carefully extracted the fat body from the carcass. Quantitating the RNA concentration by Nanodrop revealed that there was no significant difference between the crude and fine dissections. However, there was not enough time to generate cDNA and perform qPCR to detect the splicing pattern of CPT1 in these RNA samples.

Table 4: Primer Sequences Used

	Primer	Primer Sequence (5' to 3')
Total CPT1	CPT1-4-1	GCAAGTGCAAAT TGAGGAAA
	CPT1-5-5	AAGTGCTCCTCACCTTCCAC
Exon 6A containing CPT1	CPT1-5-2	CCGCTGGTTTGAC AAGTG
	CPT1-6A-4	TCATCGACGATCAGGTTCTC
Exon 6B containing CPT1	CPT1-6B-1	AATGGTCGCGTT GGCTTC
	CPT1-6B-2	TCCCAAAACCGGTGCATC
Rp49	Rp49-1	CCGCCACCAGTCGGATC
	Rp49-2	TTGGGCTTGCGCCATT

The individual forward and backward primer sequences that were used for CPT1 exon 6A and 6B and the house-keeping gene, rp49.

RNA was isolated from whole adult males and cDNA was generated using an oligo DT primer with 5 g of RNA for the cDNA reactions. The qPCR parameters that were used were incubating for 2 min at 50 °C, followed by 10 min at 95 °C. This was followed by 45 cycles consisting of 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C. All of the samples were subjected to a melting curve analysis consisting of 30 s at 95 °C, 60 s at 60 °C, and 30 s at 72 °C.

Results

Bioinformatics suggests 9G8 + RSF1 can bind to similar sequences in CPT1 to affects its splicing

Prior experimentation show that 9G8 plays a role in lipid metabolism correlated to a change in CPT1 splicing; this suggests that 9G8 is able to bind to the transcript of CPT1 to affect splicing [19]. Prior studies in *Drosophila* S2 cells by *Bradley et al* identified individual SR proteins binding motifs by using MEME-ChIP [16]. The consensus binding regions of 9G8 and RSF1 are listed below in Table 5.

Table 5: 9G8 and RSF1 Binding Motifs Share Degeneracy

SR Protein	Binding Motifs
9G8	CUUCGA, CUACAA, CUUCAA
RSF1	ACUUCU

There is a degree of overlap between the binding domains as CUUC is shared among the binding regions of both SR proteins.

Since the binding motifs of both SR proteins share a strong sense of overlap, we used bioinformatics to search for the binding sequences on the CPT1 transcript. The location will suggest if they can potentially interact with one another. FASTA files for all exon regions of CPT1 were examined and a script iterated through them to find hits for the target sequences of both 9G8 and RSF1.

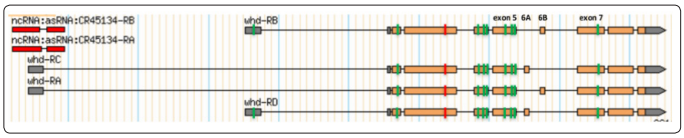

Figure 11: Bioinformatics Reveals Multiple 9G8 and Only One RSF1 Binding Site on CPT1 Transcript

Multiple 9G8 binding hits (green) and only one RSF1 binding sequence (red) are detected.

The bioinformatics search revealed multiple 9G8 binding sites but only one RSF1 binding sequence on the transcript of CPT1 (Figure 11). The location of 9G8's binding sites, particularly on exon 5 and 7, suggest that 9G8 plays a role in intron definition and helps to promote the use of the proximal splice site on exon 6A. The only match for RSF1 that yielded a 100% target hit was found to be on exon 3, however, it is likely that RSF1 can also bind to one of 9G8's consensus sequences, particularly on exon 5, due to the strong sequence overlap of their binding motifs. Under the competitive model, RSF1 could block 9G8 from binding to exon 5 and result in inclusion of the distal exon 6B. Under the cooperative model, RSF1 could bind to sequences in exon 5 to strengthen 9G8's interaction and promote the use of the proximal exon 6A. The bioinformatics search also corroborated our past result that showed in a 9G8 KD scenario, more of the distal isoform 6B splice site is used, which is associated with decreased CPT1 activity and decreased β-oxidation, resulting in higher TG levels. The bioinformatics does not narrow down the specific type of interaction, as 9G8 and RSF1 can still behave in a competitive or cooperative manner. Prior larval data suggested competition but larval data may be flawed due to the TM3 balancer chromosome in the RSF1 line so we tested in adults to better understand a potential 9G8-RSF1 interaction.

RNAi of 9G8 + RSF1 in adult males does not significantly alter TG levels

Previous results showed that 9G8 knock down in the adult female fat body resulted in increased TG levels which was associated with a change in CPT1 splicing patterns. 9G8 knock down in the fat body of larvae showed decreased TG levels and RSF1 knock down showed an increase in TG levels. However, it is unknown what phenotype would be found in adults. Thus, it is predicted that RNA_i of 9G8 and its SR protein competitor, RSF1, in adult male fat body would produce different splicing products of CPT1 detectable by qPCR and altered triglyceride levels due to CPT1 activity. As such, 9G8 or RSF1 was knocked down in the adult male fat body using a to (takeout)-gal4 driver and TG levels were measured in whole animals (Figure 12). The SR protein shuttle TRN-SR also was tested as an overall indicator of SR protein activity because it allows passageway of activated SR proteins from the cytoplasm to the nucleus for splicing. If multiple SR proteins are involved in splicing of lipid associated genes, the knock down of TRN-SR may have higher TG levels. As seen in Figure 12, the TG levels among the knock down experiments were not significantly different from the controls; however, the TRN-SR shuttle's TG levels approached significance ($p = 0.09$), which is a promising result. A possible reason as to why the TRN-SR TG levels did not reach significant values might have to do with insufficient sample numbers or poor RNAi efficiency for the line.

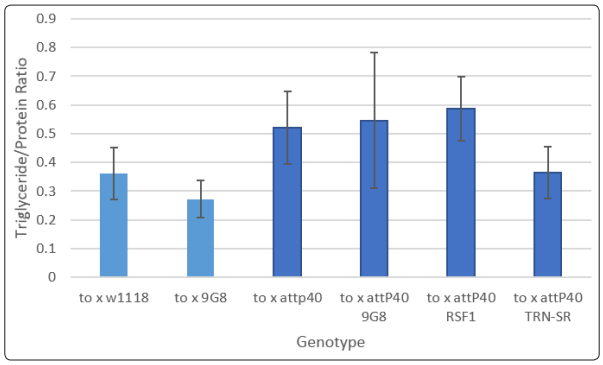

Figure 12: Triglyceride Assays for Different RNAi conditions in male fat body

TG assays were normalized to protein for the different genotypes. All samples were done in triplicates with N=6 total flies per bar.

The TG assays were inconclusive and were not able to distinguish between a competitive or cooperative model because knock down of 9G8 itself did not have a phenotype. Furthermore, we looked at the TRN-SR shuttle to see if overall SR proteins may show an effect and the data came back inconclusive, as RNAi of the shuttle also was not significant in males. Adult male flies do not have as much TG when compared to female flies and males may not be as sensitive to changes in splicing that affect TG levels. Therefore, we decided to test female adults where the original CPT1 splicing phenotype was observed.

RNAi of 9G8 + RSF1 in adult females suggests cooperative model

RNAi knock down of 9G8 in the larval FB resulted in significantly decreased TG levels, whereas, RSF1 KD flies displayed significantly higher TG levels. To further investigate if RSF1 might contribute to CPT1 splicing, TG assays were performed on female adult flies undergoing RNA_i of 9G8 and RSF1 for comparison to their w1118

control. We performed TG assays in adult female flies because larval flies do not visually display any markers for stubble and there would be no way to distinguish flies with RFS1 KD as opposed to the balancer chromosome, TM3. If 9G8 and RSF1 behave in an antagonistic manner, then we would expect to see 9G8 promoting the proximal splice site isoform 6A, and would produce homeostatic normal TG levels; whereas, RSF1 would prevent 9G8 from using that splice site by binding to the same sequence and would promote the use of the distal isoform 6B splice site which would result in a lipid overstorage phenotype. If the two SR proteins are cooperating together, then 9G8 and RSF1 KD will show a similar phenotype and will both promote the use of exon 6A.

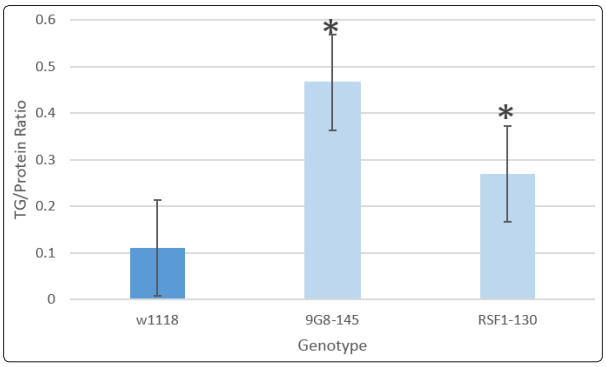

Figure 13: Both 9G8 and RSF1 Display Statistically Significant High TG Levels when Knocked Down in Female Fat Body

Knock down of 9G8 and RSF1 both result in a much higher TG phenotype than their w1118 control. Asterisk indicates $p < 0.05$.

Not only do both 9G8 and RSF1 exhibit statistically significant higher TG levels than their w1118 control, but also they do so in the same magnitude (Figure 11). Both 9G8 and RSF1 exhibit high TG levels when decreased by RNAi. The fact that both SR proteins' TG values are significant in the same direction suggest that 9G8 and RSF1 might be working in a cooperative manner in lipid metabolism.

We then did another TG assay to include the TRN-SR shuttle in a different genetic background because of SR proteins need for nuclear localization. SR proteins are in the cytoplasm of the cell but splicing takes place in the nucleus. Therefore, SR proteins need a mechanism to get into the nucleus to perform splicing. The TRN-SR shuttle has been known to provide a mechanism for SR proteins to gain access into the nucleus and both 9G8 and RSF1 use this shuttle for nuclear localization [21].

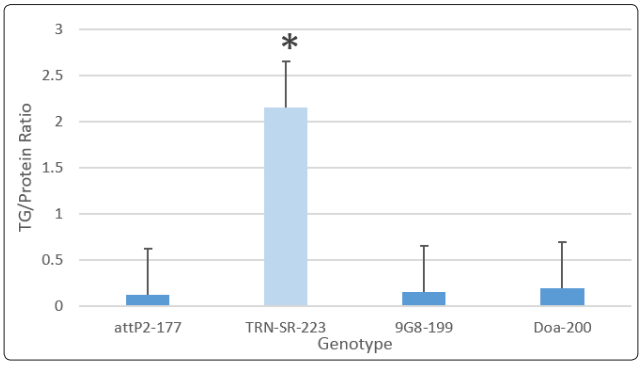

Figure 14: RNAi of TRN-SR Shuttle Displays Statistically Significant High TG Levels in Female Fat Body

In this TG set, the only genotype that was statistically significant was the TRN-SR shuttle when compared to the attP2 control. Asterisks indicates $p < 0.05$.

Figure 14 above shows that the only genotype that was significant with knock down in the female fat body was the TRN-SR shuttle when compared to the attP2 control. This corroborates the claim that the TRN-SR shuttle transports SR proteins for splicing related to lipid metabolism. Moreover, the scale of the TRN-SR TG data is different from the scale of the 9G8 and RSF1 TG data (Figure 13). The shuttle exhibited significantly higher TG levels than the individual properties of the 9G8 and RSF1 knock down. This suggests that there are other SR proteins that play a role in lipid metabolism and the regulation of TG storage.

RNAi of 9G8 in the attP2 genetic background did not alter TG levels (Figure 14). This may be because this particular RNAi line is not as efficient at decreasing 9G8 levels. In support of this, knock down of 9G8 in larval fat body using the 9G8-199 line does not result in male lethality like knock down of 9G8 using the 9G8-145 line. It is known that 9G8 is phosphorylated by Darkener of apricot (DOA) for its role in sex determination splicing [22]. Doa is an SR protein kinase that phosphorylates and activates cytoplasmic SR and SR-like proteins such as TRA, TRA2 and RBP1, marking them for nuclear localization for their role in splicing of sex determination genes. The TG data of DOA knock down was also not significant (Figure 14) and suggests that the observed 9G8 TG phenotype is not related to its sex determination function.

The TG results also confirm 9G8, RSF1, and TRN-SR all play a role in lipid metabolism and specifically, result in statistically significant higher TG levels when compared to their respective controls. The TG data between 9G8 and RSF1 showed that not only are both SR proteins significant, but also significant in the same direction, as they both produced a fat fly phenotype with higher than normal TG levels. This supports 9G8 and RSF1 behaving cooperatively with one another because an antagonistic interaction would have shown one genotype having increased TG levels and the other decreased, or vice versa. Because both genotypes display increased TG levels, this suggests a cooperative interaction. The TRN-SR nuclear transport shuttle was also examined in its corresponding genetic background and that was the only genotype that exhibited statistically significant high TG levels when compared to its control.

Starvation Experiment Shows 9G8 + RSF1 Phenocopy Starvation Resistance

Having higher TG levels with RNAi knock down does not indicate the organism's ability to mobilize or use those TG reserves, as other genes related to TG mobilization could be affected too. To investigate the ability of 9G8, RSF1 and TRN-SR knock down flies to access the increased TG stores, we performed a starvation resistance experiment and monitored approximately 25 flies of each genotype maintained on 2% agar without food for several days and documented at what time intervals flies died. If knock down flies were unable to access TG stores, they would be expected to die at a time point earlier than the control.

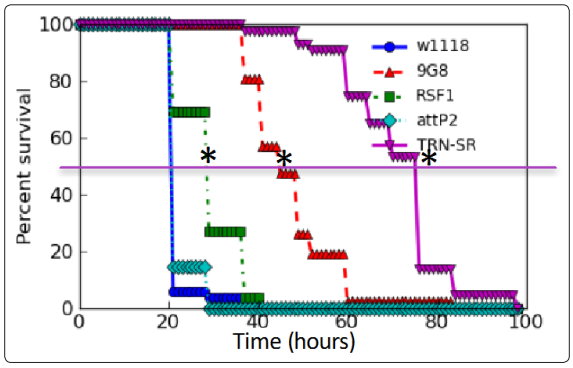

Figure 15: RNAi of 9G8 and RSF1 Phenocopy Starvation Resistance Phenotype

Both SR proteins 9G8 and RSF1 phenocopy each other and mirror a starvation resistant phenotype when compared to their w1118 control. The TRN-SR shuttle outlived all the other genotypes when compared to its attP2 control. This figure was created with OASIS [20]. Asterisks indicate $p < 0.05$.

Figure 15 above shows the starvation resistance graph and two conclusions can be drawn. The first is that all genotypes were statistically significant in resisting starvation when compared to their respective control. 9G8 (red) outlived its RSF1 (green) counterpart, which is consistent with 9G8 KD having increased TG levels when compared to RSF1 KD, as can be seen in Figure 13. The fact that RSF1 phenocopies 9G8 in exhibiting increased starvation resistance further suggests a possible cooperative interaction between the two proteins, as they both significantly outlived the w1118 control. The starvation survival experiment and the TG data suggest that neither 9G8 nor RSF1 KD flies have trouble accessing their lipid stores. The nuclear transport shuttle was also included in the starvation survival experiment to see if TRN-SR KD flies could mobilize their fat stores. The TRN-SR KD flies lived the longest, also consistent with the increased TG levels seen in Figure 13.

Genotype	No. Subjects	Hours	Std. Error	50% Lethality (hours)
w1118	51	21.86	0.51	22
9G8	42	47.31	1.47	45
RSF1	26	28.85	1.24	29
attP2	41	22.17	0.44	22
TRN-SR	43	70.72	1.81	76

Table 6: 9G8, RSF1, and TRN-SR Show Statistically Significant Increased Lifespans

All experimental genotypes displayed statistical significance when compared to their respective controls. The blue color of the table illustrates the 9G8 and RSF1 experimental genotypes being compared to their w1118 control. The purple color of the table is examining the TRN-SR shuttle to its attP2 control.

To further confirm the 9G8-RSF1 cooperative model, the CPT1 AS patterns will be monitored to see how they are affected with the knock down of RSF1 and the TRN-SR shuttle. We theorize that knock down of RSF1 would result in the same CPT1 splicing patterns as 9G8 knock down (Table 2). This would result in the inversion of wild type isoform ratios and would produce a fly with a fat phenotype because of the 40% 6A to 60% 6B isoform distribution. Having a TRN-SR KD scenario would be expected to enhance the fat phenotype because of the prevention of SR protein nuclear localization. Thus, we theorize that if TRN-SR is knocked down, then the isoform ratio would be heavily skewed towards inclusion of isoform 6B and would result in an 80-100% inclusion of the less active CPT1 activity isoform.

We have three independent pieces of evidence that support 9G8 and RSF1 interacting with one another in lipid metabolism. The bioinformatics search revealed multiple 9G8 binding sites, but only one binding site for RSF1 on the transcript of CPT1. Furthermore, the degeneracy of 9G8 and RSF1's binding motifs suggest that one SR protein can potentially bind to the binding sequence of the other to cooperatively promote the proximal exon 6A inclusion. The TG data showed that 9G8 and RSF1 knock down in the female fat body exhibited statistically significant higher TG levels when compared to their w1118 control, as can be seen in Figure 13. Not only were TG levels significant, but they both were significant in the same direction suggesting a potential cooperative interaction to ensure exon 6A inclusion. The starvation survival experiment showed that 9G8 and RSF1 KD flies phenocopied each other and displayed statistically significant starvation resistance in the same direction. Taken together, these three independent experiments all suggest that 9G8 and RSF1 might be working cooperatively with one another to promote proximal exon 6A inclusion.

Discussion

Many metabolic genes are alternatively spliced and SR proteins regulate the splicing patterns of such genes by binding to exons and marking the boundaries for spliceosome recruitment. Our lab has focused on the canonical SR protein 9G8 because it is known to play a role in lipid metabolism. Prior experimentation has shown that not only 9G8, but also the SR protein RSF1 generates a TG phenotype in the fat body of larvae when it is knocked down. When 9G8 is knocked down TG levels decreased but when RSF1 was knocked down, TG levels increased. Thus, we decided to investigate what role RSF1 plays in whole adult flies in a known target of 9G8 called CPT1.

CPT1 is responsible for -oxidation and demonstrates a mutually exclusive splicing pattern. In wild type conditions, the major isoform produced is isoform 6A with 60% exon 6A and 40% 6B produced [19]. This is associated with increased CPT1 activity, -oxidation, and results in a fly with normal TG levels. However, when 9G8 is knocked down, the isoform ratios are inverted and yield a higher TG phenotype because of the 40% exon 6A and 60% exon 6B pattern. These changes in splicing patterns and phenotypes suggests that 9G8 binds to CPT1 to influence its splicing.

To investigate where on CPT1's transcript 9G8 and RSF1 can bind, we ran a bioinformatics search to identify those locations. Prior literature discovered the binding motifs of both 9G8 and RSF1 and by using the FASTA files of the exon regions of CPT1, we were able to locate hits for our target sequences, as can be seen in Figure 11. The bioinformatics revealed multiple 9G8 binding sites and only one RSF1 site on the transcript of CPT1. The degeneracy of 9G8 and RSF1 binding sequences (CUUC) suggest that the SR proteins

can potentially bind to the other's site. Furthermore, the presence of multiple 9G8 binding motifs at the end of exon 5 suggests that 9G8 promotes the use of the proximal splice site isoform 6A over the distal 6B. This corroborates our prior experimentation that showed when 9G8 is knocked down, CPT1 splicing is skewed towards the inclusion of isoform 6B (40% 6A/60% 6B) and produces a lipid overstorage phenotype fly.

Although the bioinformatics search was useful in identifying where the SR proteins 9G8 and RSF1 bind, it does not distinguish between a competitive or cooperative interaction. To distinguish between the two types of interactions, we ran TG assays on male flies undergoing RNAi in the fat body, as can be seen in Figure 12. A competitive interaction would result in high TG levels when 9G8 is knocked down and low when RSF1 is knocked down, or vice versa. A cooperative interaction would show both 9G8 and RSF1 would phenocopy and both display either low or high TG levels. The male TG data came back inconclusive because none of the genotypes tested had significantly different TG levels. We then repeated the assay in females because females have a yolk-gal4 driver that is more robust than the male's takeout-gal4 driver and also because splicing in females has shown that more 6B is produced, yielding fatter flies in 9G8 KD.

The female TG data showed that both 9G8 and RSF1 had statistically significant TG levels when compared to their w1118 control, as can be seen in Figure 13. Not only were they significant, but they were significant in the same direction, as both exhibited fat phenotypes. Because 9G8 and RSF1 displayed the same fat phenotype, this suggests that they might be involved in a cooperative interaction. Bradley et al has shown that the canonical SR protein 9G8 interacts with other SR proteins in a predominantly cooperative way. Because splicing is very complex and multi-factorial, we next wondered if these are the only two SR proteins that are involved in TG storage. To investigate whether 9G8 and RSF1 are the only SR proteins important for TG storage, we performed RNAi KD of the TRN-SR shuttle. This shuttle is responsible for the nuclear localization of phosphorylated SR proteins to allow splicing to commence. The TRN-SR KD TG data showed significantly increased TG levels when compared to its respective control, attP2, as can be seen in figure 14. The scaling between the 9G8 and RSF1 TG data and the TRN-SR TG data is different and shows just how much more TG the TRN-SR has when compared to the 9G8 and RSF1 TG data. The fact that the shuttle KD has more TG levels than the additive TG levels of both 9G8 and RSF1 suggests that there are other SR proteins that play a role in TG storage and is not limited to just 9G8 and RSF1 activity. Other SR proteins that may be involved in generating a lipid phenotype can be SRp54, SF2, and B52 because these SR proteins are involved in most AS events [16].

The TG data revealed that 9G8, RSF1, and TRN-SR KD mutants have increased TG levels but does not comment to the accessibility of those reserves as required for CPT1 activity and -oxidation. An organism can have increased TG levels but might not be able to mobilize those reserves. To investigate whether the 9G8, RSF1, and TRN-SR KD mutants were able to access the increased TG stores, we performed a starvation resistance experiment. The mutants were starved and monitored over several days, documenting at what time intervals they died. Following the completion of the experiment, a survival curve was generated using OASIS and it revealed that all knocked down mutants lived significantly longer than their respective controls, as can be seen in Figure 15. Moreover, 9G8 KD out lived the RSF1 KD, which is consistent with our TG results showing that 9G8 has more TG levels than the RSF1 KD mutant. The TRN-SR KD mutant out lived both the 9G8 and RSF1 mutant, which is also consistent with our TG data showing that the shuttle had more than double the additive TG levels of 9G8 and RSF1, further corroborating our prior data. This also suggests that there are other SR proteins that play a role in the AS of genes that influence lipid metabolism such as B52, SRp54, and SF2.

The bioinformatics revealed the location of multiple 9G8 binding sites, particularly the cluster of sites at the end of exon 5 suggest 9G8's role in intron definition and promoting the use of the proximal isoform 6A splice site. However, the bioinformatics does not distinguish between a competitive or cooperative interaction. The female TG data shows that not only do 9G8 and RSF1 exhibit statistically significant TG levels, but also they show the same phenotype suggesting a potentially cooperative interaction. The shuttle TG data suggests that there are other SR proteins, possibly SRp54, SF2, and B52, involved in addition to 9G8 and RSF1 because the shuttle's TG levels were more than the additive TG levels of both 9G8 and RSF1. Furthermore, the starvation resistance experiment showed that all mutants lived statistically longer than their controls and that 9G8 and RSF1 phenocopy the starvation resistant condition once again suggesting a potentially cooperative interaction.

Future Directions

Because the shuttle TG data suggests that there are other SR proteins involved in TG storage, a future direction would be to look for other SR proteins binding sites in CPT1 exons and introns. Even though we have identified bioinformatically the presence of 9G8 and RSF1 binding sites on CPT1, we need to biochemically determine if they physically bind to CPT1 by developing an antibody for 9G8. We also cannot definitively say the exact nature of 9G8 and RSF1 interaction. The only way to specifically prove such an interaction would be through biochemical experiments like performing a double KD of 9G8 and RSF1. However, another way to answer the question of 9G8 and RSF1 interaction would be to look at CPT1 splicing. A future direction would be to look at CPT1 splicing when both RSF1 and TRN-SR is knocked down. If CPT1 splicing when RSF1 is knocked down matches 9G8 KD, then we would be able to better determine if 9G8 and RSF1 are working cooperatively together.

Because qPCR was already done on males, the same procedure can be utilized on females. To verify the published effect of 9G8 knock down on CPT1 splicing patterns, qPCR was then done in duplicates on samples from adult males undergoing knock down of 9G8 or the w1118 control in the fat body using the qPCR cycling parameters adapted from Price et al. CPT1 primers were optimized for qPCR using these conditions and efficiency was calculated for the control and 9G8 RNAi to be 110% and 126%, respectively. The house keeping gene that was used for these reactions was rp49 and the primer sequence for both the house keeping gene and the experimental CPT1 is found in Table 3. However, no message was detected for rp49 by qPCR and it was not possible to calculate the ratio of exon 6A to 6B in the w1118 or 9G8 RNAi samples.

The dissection of fat body from adult males was tedious and time consuming because males are smaller and do not contain as much fat as females. Additionally, the knock down of TRN-SR in adult male fat body did not result in a significant TG phenotype (Figure 12).

Thus, the knock down experiments will be repeated in female flies using the yolk-gal4 driver because 9G8 KD females have been shown to have increased exon 6B inclusion and increased TG levels [19].

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