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The regulation of carnitine palmitoyltransferase 1 (*CPT1*) mRNA splicing by nutrient availability in *Drosophila* fat tissue

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ABSTRACT

After a meal, excess nutrients are stored within adipose tissue as triglycerides in lipid droplets. Previous genome-wide RNAi screens in *Drosophila* cells have identified mRNA splicing factors as being important for lipid droplet formation. Our lab has previously shown that a class of mRNA splicing factors called serine/arginine-rich (SR) proteins, which help to identify intron/exon borders, are important for triglyceride storage in *Drosophila* fat tissue, partially by regulating the splicing of the gene for carnitine palmitoyltransferase 1 (CPT1), an enzyme important for mitochondrial β-oxidation of fatty acids. The *CPT1* gene in *Drosophila* generates two major isoforms, with transcripts that include exon 6A producing more active enzymes than ones made from transcripts containing exon 6B; however, whether nutrient availability regulates *CPT1* splicing in fly fat tissue is not known. During ad libitum feeding, control flies produce more *CPT1* transcripts containing exon 6B while fasting for 24 h results in a shift in *CPT1* splicing to generate more transcripts containing exon 6A. The SR protein 9G8 is necessary for regulating nutrient responsive *CPT1* splicing as decreasing 9G8 levels in fly fat tissue blocks the accumulation of *CPT1* transcripts including exon 6A during starvation. Protein kinase A (PKA), a mediator of starvation-induced lipid breakdown, also regulates *CPT1* splicing during starvation as transcripts including exon 6A did not accumulate when PKA was inhibited during starvation. Together, these results indicate that *CPT1* splicing in adipose tissue responds to changes in nutrient availability contributing to the overall control of lipid homeostasis.

1. Introduction

Obesity and related issues of excess energy storage have been health concerns and the focus of decades of medical research. Defined as an increase in body weight as a result of excess fat storage, obesity remains an epidemic plaguing more than 40% of individuals across the globe, compromising their health and quality of life [1]. Obesity has been linked to increased rates of diseases such as hypertension, coronary artery disease, and type II diabetes [2]. While genome-wide association studies and transcriptomic analyses have increased our understanding of the genetic underpinnings of obesity and other related metabolic diseases, there are limitations to these approaches to identify and study novel genes associated with these diseases. The use of a genetic model organism can help expand our knowledge of the genes that regulate lipid metabolism.

In addition to mammalian model systems, *Drosophila melanogaster* has been used extensively to study human diseases. 75% of human disease genes are found in the *Drosophila* genome and flies have a fast life cycle and are economically advantageous to study in a laboratory [3]. Moreover, *Drosophila* adipose tissue functions similarly to mammals employing conserved lipogenic and lipolytic signaling pathways to use and metabolize fats [4]. Therefore, *Drosophila* genetics provides a powerful system to study obesity and related human metabolic diseases related to lipid metabolism.

Previous genome-wide RNA interference (RNAi) screens performed on cultured *Drosophila* cells identified genes important for triglyceride storage and lipid droplet formation and genes encoding members of the mRNA-processing spliceosome complex were found to regulate lipid droplet size and number from these screens [5,6]. Previous studies using RNAi in fly fat tissue from our lab identified many regulatory mRNA splicing factors called SR (serine-arginine rich) proteins that are necessary for regulating lipid metabolism [7–9]. SR proteins regulate the

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splicing of an important lipid breakdown protein, carnitine palmitoylacyltransferase 1 (CPT1). Specifically, CPT1 catalyzes the transfer of long chain fatty acyl CoAs across and into the mitochondrial matrix, where they are further broken down via beta oxidation [10]. In *Drosophila* adipose tissue, the *CPT1* gene is alternatively spliced resulting in two major isoforms, differing by a single exon: 6A and 6B. Transcripts containing exon 6A produce a more active CPT1 enzyme, suggesting a higher rate of fatty acid metabolism than exon 6B-containing transcripts ([11]; Fig. 1A). Previous work from our lab found that the SR proteins 9G8, tra2 and SF2 play a role in regulating *CPT1* splicing to limit triglyceride storage [7–9]. For example, *9G8-RNAi* flies have more exon 6B-containing isoforms of *CPT1* than exon 6A-containing isoforms in their fat bodies [8]. In addition, we have also shown that the nuclear SR protein import protein Tnpo-SR, which shuttles splicing factors such as 9G8 in and out of the nucleus, alters *CPT1* splicing to limit lipid accumulation; *Tnpo-SR-RNAi* flies have more exon 6B-containing *CPT1* isoforms than exon 6A-containing isoforms in fat tissue [12]. Together, the data suggest that splicing factors play a key metabolic role in regulating lipid storage by modulating the splicing of the *CPT1* gene. However, the metabolic signals that control *CPT1* splicing are not fully understood.

In this study, we set out to identify and characterize the metabolic signals that regulate the splicing of *CPT1* in *Drosophila* fat tissue. While previous studies have summarized the effects of nutrient availability on lipid storage [13], there is still limited information on whether the presence or absence of food affects *CPT1* splicing. More importantly, the role of splicing factors in regulating *CPT1* splicing under different nutrient conditions requires further understanding. Our results show that a 24-h starvation period promotes preferential inclusion of exon 6A in the *CPT1* gene in *Drosophila* fat tissue and that the SR protein 9G8 and the SR protein transporter Tnpo-SR are necessary for this to occur. In addition, PKA, a kinase known to be activated during fasting, is shown to mediate *CPT1* splicing under starvation to preferentially produce exon 6A-containing *CPT1* transcripts. Together, these data suggest that starvation promotes increased lipid breakdown by regulating *CPT1* splicing, thus increasing our understanding of how alternative splicing affects overall lipid homeostasis.

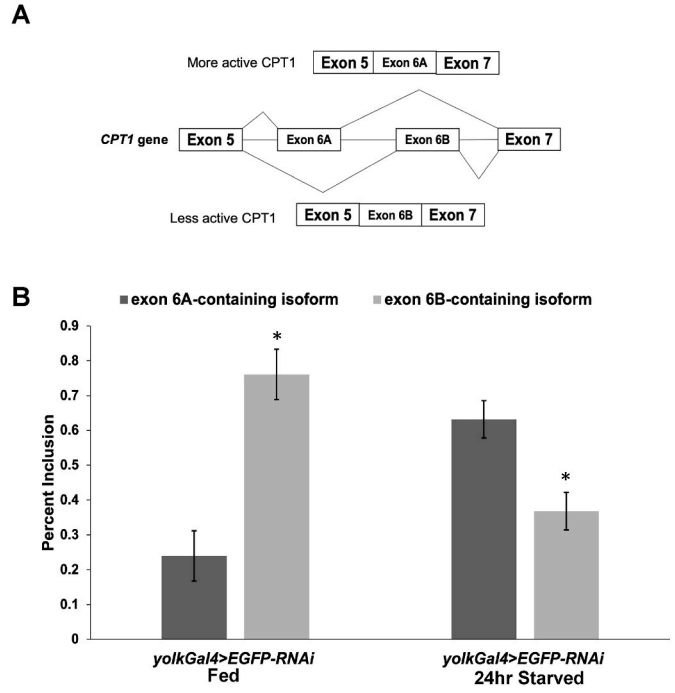

Fig. 1. Starvation regulates *CPT1* splicing. (A) Schematic diagram of *CPT1* splicing that displays two isoforms that include different versions of exon 6. Enzymes produced from transcripts including exon 6A are more active than enzymes produced from transcripts including exon 6B (Adapted from Ref. [11]). (B) RNA was isolated from fat bodies dissected from 1 week old *yolkGal4 > EGFP-RNAi* females either fed or starved for 24 h (n = 5–10). qPCR was performed for *CPT1* isoforms and quantities were then normalized by *rp49,* and fractions of each transcript including either exon 6A or exon 6B that were expressed were calculated. Values are mean ± standard error. *, $P < 0.05$ by Student's *t*-test comparing the percent inclusion of *CPT1* isoforms containing exon 6A to those containing exon 6B in each condition.

2. Materials and methods

Fly Genetics – The following flies were used in this study: *yolkGal4* [14]; *y* [1] *sc[*] v* [1]; *P{y[+ t7.7] v[+ t1.8] = VALIUM20-EGFP}attP2* (BL#35782, referred to as *UAS-EGFP-RNAi); w[1118]; attB: UAS-9G8RNAi* (VDRC #100226); *y* [1] *v* [1]; *P{y[+ t7.7] v[+ t1.8] = TRiP.JF02010}attP2* (BL#25988, referred to as *UAS-Tnpo-SR-RNAi*); *w [*]; P{w[+ mC] = UAS-GFP.S65T}Myo31DF[T2]* (BL#1521, referred to as *UAS-GFP*); and *w[*]; P{w[+ mC] = UAS-PKA-R1.BDK}35* (BL#35550, referred to as *UAS-PKA-R1-BDK*). Flies were grown on standard cornmeal-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) at 25 °C in a 12 h:12 h light:dark cycle.

Triglyceride, Glycogen, Glucose and Protein Assays – Two one-week old female flies were homogenized in lysis buffer (140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton-X) and 1X Complete Protease Inhibitor (Roche). Protein concentrations were determined using the Pierce BCA Assay kit (ThermoFisher Scientific), triglycerides were determined using Infinity Triglyceride Reagent (ThermoFisher Scientific), and free glucose was determined using the Pointe Scientific Glucose Oxidase Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Total glucose levels were determined by treating homogenized samples with 8 mg/mL amyloglucosidase diluted in 0.2 M citrate buffer, pH. 5.0 and incubated for 2 h at 37 °C. Glycogen levels were then calculated by subtracting the free glucose from the total glucose levels. Triglyceride, free glucose, and glycogen levels were then normalized by total protein content.

Starvation Assay and RNA Isolation – For starvation experiments, one-week old female flies were starved on 1% agar for 24 h before fat body dissection and compared to flies that had been given regular access to food. To maximize the yield of fly fat body tissue, cuticles with fat bodies attached were dissected from 15 one-week old adult female flies and homogenized in Ribozol RNA Extraction Reagent (AMRESCO) according to manufacturer's instructions.

DNase treatment, cDNA synthesis and qPCR – 5 μg of isolated total RNA was DNase treated using the DNA-Free Turbo Kit according to the manufacturer's instructions (Ambion). 0.25 μg of DNase treated RNA was then reverse transcribed using qScript-XLT cDNA Supermix (Quanta Biosciences) according to the manufacturer's protocol. 1 μL of the generated cDNA was amplified with 1X Perfecta SYBR Green Supermix (Quanta Biosciences). qPCR analysis was performed using a Step-One Plus qPCR machine and the primers used to amplify all *CPT1* isoforms and the qPCR cycling conditions used were previously described [11]. Relative expression of *CPT1* isoforms was normalized by the *rp49* expression levels.

Statistical Analysis – Standard error was calculated for all sample groups and a *t*-test was conducted to determine whether there were any differences between experimental and control groups. A P value ≤ 0.05 was determined to be statistically significant.

3. Results

3.1. Regulation of CPT1 splicing by starvation

To understand the signals that control fat storage, the regulation of *CPT1* splicing was first examined in *Drosophila* fat bodies under starvation conditions. Since starvation induces lipolysis in adipocytes [15], we

hypothesized that starvation is a potential signal that alters the splicing of *CPT1* in *Drosophila* fat tissue. To test this hypothesis, *CPT1* isoform production was detected by qPCR on cDNA made from RNA isolated from the fat bodies of *EGFP-RNAi* control flies fed ad libitum or starved for 24 h. The results confirm that a 24-h starvation period increases the relative amount of exon 6A-containing isoforms of *CPT1* compared to fed *EGFP-RNAi* flies (Fig. 1B). These data suggest that starvation serves as a signal that regulates *CPT1* splicing promoting the inclusion of exon 6A, thus producing a more active enzyme [11].

3.2. The role of SR proteins in starvation-induced CPT1 splicing

Since it was shown that starvation alters *CPT1* splicing, the next step was to investigate whether splicing factors are regulated by starvation to control the splicing of *CPT1*. Previous studies in *Drosophila* have shown that the SR protein nuclear transporter Tnpo-SR regulates the splicing of *CPT1* transcripts to promote the inclusion of exon 6A [12]. Since starvation also seems to promote the inclusion of exon 6A, we hypothesized that Tnpo-SR is necessary for starvation-induced splicing of *CPT1*. To test this hypothesis, *Tnpo-SR* levels were decreased in the fat body by RNAi and *CPT1* splicing was measured in the fat body of starved or fed flies as described above. If Tnpo-SR is necessary for promoting the inclusion of exon 6A in *CPT1* during starvation, then it is expected that starved *Tnpo-SR-RNAi* flies would accumulate *CPT1* isoforms with exon 6B. Consistent with this hypothesis, when *Tnpo-SR-RNAi* flies were starved, the amount of exon 6B-containing transcripts remained higher than exon 6A-containing transcripts similar to fed flies (Fig. 2A) suggesting that Tnpo-SR modulates starvation-induced *CPT1* splicing.

Among the SR proteins that are shuttled by Tnpo-SR, 9G8 (also known as SFRS7 or x16) was identified to be an important splicing factor that promotes the usage of exon 6A in *CPT1* [8,16]. Since 9G8 increases the levels of *CPT1* transcripts with exon 6A, we investigated whether 9G8 functions to promote exon 6A usage during starvation. To address this question, *CPT1* splicing was measured in fat bodies from starved *9G8-RNAi* flies. If 9G8 is important for exon 6A inclusion in *CPT1* during starvation, then we would expect that starving *9G8-RNAi* flies would still accumulate *CPT1* transcripts with exon 6B. Consistent with this hypothesis, when *9G8-RNAi* flies were starved, the amount of exon-6B containing isoforms remained higher than exon-6A containing isoforms similar to fed flies (Fig. 2B). Together, these results suggest that 9G8 and Tnpo-SR are necessary proteins that regulate *CPT1* splicing under starvation to increase exon 6A-containing isoforms.

3.3. PKA regulates CPT1 splicing

So far, we have shown that starvation affects *CPT1* splicing via SR proteins; however, the signaling molecules and pathways linking starvation to splicing factors are not known. In *Drosophila*, lipid breakdown is promoted during starvation by activating cyclic-AMP (cAMP)-dependent and Ca^{2+}-dependent signaling pathways [17,18]. Since *CPT1* is the rate-limiting step in the oxidation of fatty acids, a process activated during fasting [10], we hypothesized that PKA (one of the potential kinases activated to promote lipolysis) also plays a role in regulating fatty acid oxidation by controlling *CPT1* splicing.

Since PKA promotes lipid and glycogen breakdown [13], then inhibiting PKA is expected to result in an accumulation of TAG and/or glycogen. Previous studies have decreased PKA activity by engineering a mutated form of the PKA regulatory subunit 1 (known as R1-BDK) so that it cannot bind to cAMP; this mutation prevents the release of the catalytic subunits and decreases PKA signaling [19]. Overexpression of *PKA-R1-BDK* specifically in the adult fat body results in a significant increase of TAG and glycogen (Fig. 3). These findings suggest that overexpression of *PKA-R1-BDK* effectively inhibits PKA activity in *Drosophila* fat tissues.

To determine whether PKA activity affects *CPT1* splicing in starved flies, PKA activity was inhibited by expressing *PKA-R1-BDK* and *CPT1*

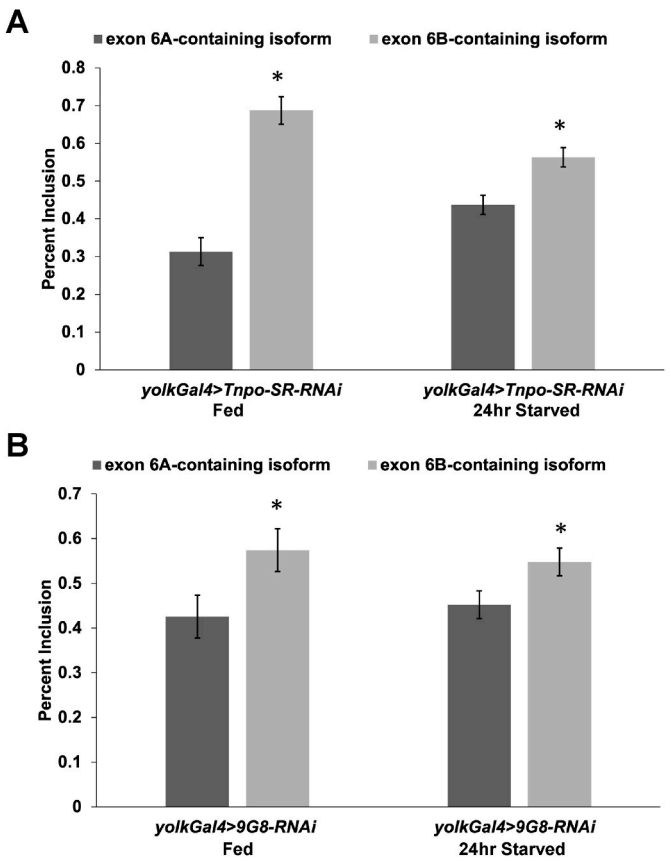

Fig. 2. Tnpo-SR and 9G8 are necessary to regulate *CPT1* splicing during starvation. RNA was isolated from fat bodies dissected from 1-week old **(A)** *yolkGal4 > Tnpo-SR-RNAi* (n = 8) and **(B)** *yolkGal4 > 9G8-RNAi* (n = 9–14) females starved for 24 h and compared to fed controls of the same genotypes. qPCR was then performed for *CPT1* isoforms including either exon 6A or 6B and quantities were normalized by *rp49,* and fractions of each transcript including either exon 6A or exon 6B that were expressed were calculated. Values are mean ± standard error. *, $P < 0.05$ by Student's *t*-test comparing the percent inclusion of *CPT1* isoforms containing exon 6A to those containing exon 6B in each condition.

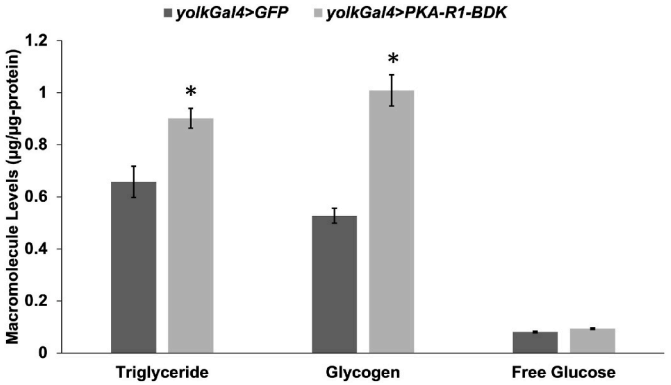

Fig. 3. Inhibiting PKA in fat tissue results in triglyceride and glycogen accumulation. Triglyceride, glycogen, and glucose were measured from 1 week old female *yolkGal4 > PKA-R1-BDK* flies compared to *yolkGal4 > GFP* controls (n = 29–30). Triglyceride, glycogen, and free glucose were then normalized by protein levels. Values are mean ± standard error. **, $P < 0.05$ by Student's *t*-test comparing macromolecule levels in control flies to flies with PKA genetically modified.

splicing was measured in both fed and starved flies and compared to *GFP*-expressing controls. Under fed conditions, both *GFP*-expressing control and *PKA-R1-BDK* flies contain equal amounts of exon 6A-containing isoforms and exon 6B-containing isoforms of *CPT1* (Fig. 4A), suggesting that inhibiting PKA under fed conditions does not change *CPT1* splicing. Since starvation induces *CPT1* splicing to include exon 6A, and PKA activity is high during starvation, we hypothesized that inhibiting PKA activity during starvation would prevent the accumulation of *CPT1* isoforms that include exon 6A. Like the data in Fig. 1B, starving *GFP*-expressing control flies increased the amount of exon 6A-containing isoforms (Fig. 4B). Consistent with the hypothesis, starving *PKA-R1-BDK* flies does not result in the accumulation of exon 6A-containing *CPT1* isoforms; in fact, the amount of exon 6B-containing isoforms increases in these flies (Fig. 4B). Together, these findings suggest that starvation activates PKA to regulate starvation-induced changes in *CPT1* splicing to produce isoforms that include exon 6A.

4. Discussion

In this study, the metabolic signals that regulate the splicing of *CPT1* in *Drosophila* fat tissue were investigated. We focused on starvation as previous work in *Drosophila* has shown that lipid levels decrease during starvation ultimately leading to death of the flies starting from 48 to 72 h after the onset of starvation, depending on the genetic background and lipid storage [18,20]. Consistent with the decrease in lipids during starvation, the expression of the lipid breakdown gene *Lipase 3* increases during starvation [21]; however, whether other lipid breakdown genes like *CPT1* were regulated by starvation was not fully understood. We found that a 24-h starvation period induces the preferential inclusion of exon 6A in *CPT1* transcripts. While we chose a 24-h starvation period for this study, we did not test whether differing lengths of the starvation period would alter the effect on *CPT1* splicing. It is very possible that longer starvation times would result in a stronger *CPT1* splicing phenotype; however, additional experimentation is necessary to address this question. Consistent with previous studies on *CPT1* splicing in *Drosophila* adipose tissue, our results support that starvation is a contributing signal to the production of a more active CPT1 enzyme to increase the rate of fatty acid oxidation [11]. The starvation-induced splicing of *CPT1* was found to be dependent on the nuclear protein transporter Tnpo-SR and the SR protein 9G8, suggesting that 9G8 and Tnpo-SR may promote beta oxidation in *Drosophila* fat tissue when starved.

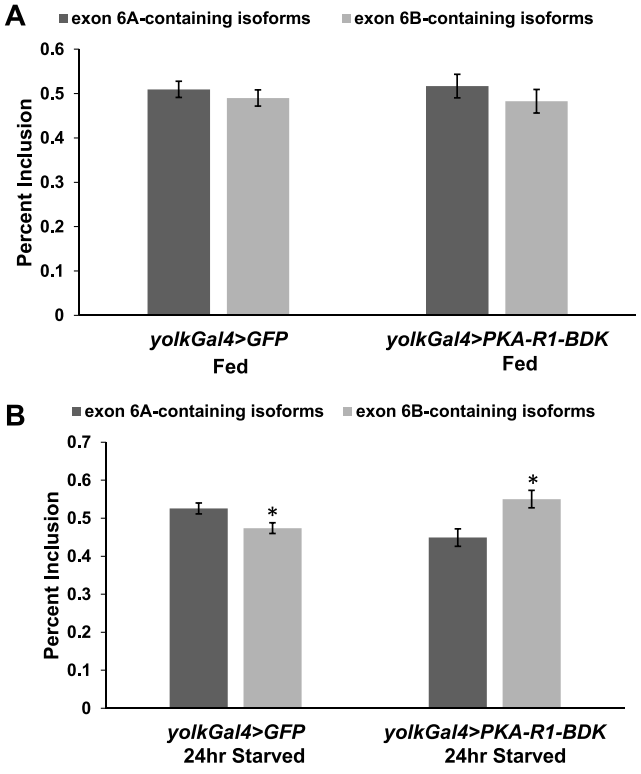

Fig. 4. PKA is necessary for the starvation-induced splicing of *CPT1*. RNA was isolated fat bodies dissected from 1 week old female **(A)** fed and **(B)** 24 h starved *yolkGal4 > GFP* and *yolkGal4 > PKA-R1-BDK* flies (n = 8). qPCR was performed for *CPT1* isoforms including either exon 6A or 6B and quantities were then normalized by *rp49,* and fractions of each transcript including either exon 6A or exon 6B that were expressed were calculated. Values are mean ± standard error. *, P < 0.05 by Student's *t*-test comparing the percent inclusion of *CPT1* isoforms containing exon 6A to those containing exon 6B in each condition.

We have also identified the starvation activated kinase, PKA, as playing a role in *CPT1* splicing in *Drosophila* fat tissue. In *Drosophila*, starvation induced lipid breakdown is regulated by the hormone adipokinetic hormone (AKH) [22], which signals through the AKH receptor leading to cAMP and Ca^{2+}-dependent signaling pathways to become activated [17,18]. We showed that overexpressing the *R1-BDK* mutant of PKA specifically in fat tissue results in more triglyceride and glycogen storage suggesting less PKA activity consistent with previous larval growth and development studies [19,23]. Inhibiting PKA during starvation also blocks the inclusion of exon 6A in *CPT1* transcripts, suggesting that PKA relays a starvation signal to promote *CPT1* splicing to produce more active CPT1 enzymes. These findings also indicate that when presented with limited nutrients, PKA is activated to stimulate beta oxidation by promoting the preferential splicing of *CPT1* to include exon 6A, thus producing a more active beta oxidation enzyme. However, since Ca^{2+}-dependent signaling pathways are also activated during starvation to regulate lipid breakdown in *Drosophila* [17], it is possible that additional enzymes such as phospholipase C and protein kinase C could also be important for regulating starvation-induced *CPT1* splicing and future experiments would need to be performed to test this hypothesis.

We have shown in this study that the SR protein 9G8 controls *CPT1* splicing under starvation conditions, but 9G8 regulation by other protein kinases to regulate lipid storage is poorly understood in *Drosophila* fat tissue. Previous studies have shown that the *Drosophila* SR protein kinase homologs SRPK and SRPK79D both promote exon 6B inclusion because knockdown of either *SRPK* or *SRPK79D* in fat tissue led to more *CPT1* transcripts containing exon 6A [24]. 9G8, on the other hand, promotes exon 6A inclusion because knocking down *9G8* results in more *CPT1* transcripts containing exon 6B [8]. These two results suggest that phosphorylation of 9G8 by SRPKs may prevent 9G8 from promoting the inclusion of exon 6A in *CPT1* transcripts. Moreover, PKA promotes lipid breakdown [10,13] and we have shown that it alters *CPT1* splicing during starvation (Fig. 4B), but whether PKA regulates SR proteins directly to control lipid homeostasis is unknown. Previous studies have shown that PKA phosphorylates 9G8 in human neuronal cells to alter the splicing of *tau*, which encodes a microtubule-associated protein [25], but whether PKA phosphorylates 9G8 to control *CPT1* splicing and overall lipid storage in *Drosophila* requires further studies.

While it is unknown how *CPT1* splicing is regulated in humans, previous studies performed in mammalian systems have found other lipid metabolic enzymes whose splicing are regulated by nutrients. For example, the splicing of *glucose-6-phosphate dehydrogenase* (*G6PD*), which encodes an enzyme of the pentose phosphate pathway that produces nicotinamide adenine dinucleotide phosphate (NADPH) necessary for fatty acid synthesis, is regulated by starvation in mammals [26]. Interestingly, another study identified *LPIN1*, which encodes the protein lipin, to be alternatively spliced in cultured mammalian liver cells [27]. In humans, *LPIN1* splicing is mediated by the splicing factor SFRS10 [27], but whether nutrient availability affects *LPIN1* splicing is currently not known. And despite our understanding of the *G6PD* and *LPIN1* genes in mammals, there is limited research on the alternative splicing of the

Drosophila G6PD homolog, *Zwischenferment* (*Zw*), and the *Drosophila LPIN1* homolog, *Lpin*. Whether starvation plays a role in altering the splicing of the *Zw* and *Lpin* genes in *Drosophila* requires further investigation.

In summary, we have shown that starvation regulates *CPT1* splicing to produce *CPT1* transcripts containing exon 6A to promote lipid breakdown. The SR protein 9G8 and the SR protein transporter Tnpo-SR are necessary to carry out starvation-induced *CPT1* splicing, and PKA plays a role in modulating *CPT1* splicing during starvation. While only two major *CPT1* isoforms exist in *Drosophila* [11], three *CPT1* isoforms (*CPT1A, CPT1B, CPT1C*) have been identified in mammals [28]; however, whether starvation regulates the splicing of these isoforms remains unknown. Furthermore, the *CPT1* splicing data described here increases our knowledge of how starvation regulates splicing of genes encoding for lipid metabolic enzymes such as G6PD and LPIN1 to regulate lipid storage in humans [26,27]. Understanding the metabolic signals that regulate lipid storage allows us to better understand human metabolic diseases such as obesity, perhaps better equipping us to generate new approaches to achieve metabolic homeostasis during these disease states.

CRediT authorship contribution statement

Huy G. Truong: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Alexis A. Nagengast:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Justin R. DiAngelo:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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