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## The control of lipid metabolism by mRNA splicing in *Drosophila*

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### Abstract

The storage of lipids is an evolutionarily conserved process that is important for the survival of organisms during shifts in nutrient availability. Triglycerides are stored in lipid droplets, but the mechanisms of how lipids are stored in these structures are poorly understood. Previous *in vitro* RNAi screens have implicated several components of the spliceosome in controlling lipid droplet formation and storage, but the *in vivo* relevance of these phenotypes is unclear. In this study, we identify specific members of the splicing machinery that are necessary for normal triglyceride storage in the *Drosophila* fat body. Decreasing the expression of the splicing factors U1-70K, U2AF38, U2AF50 in the fat body resulted in decreased triglyceride levels. Interestingly, while decreasing the SR protein 9G8 in the larval fat body yielded a similar triglyceride phenotype, its knockdown in the adult fat body resulted in a substantial increase in lipid stores. This increase in fat storage is due in part to altered splicing of the gene for the  $\beta$ -oxidation enzyme CPT1, producing an isoform with less enzymatic activity. Together, these data indicate a role for mRNA splicing in regulating lipid storage in *Drosophila* and provide a link between the regulation of gene expression and lipid homeostasis.

### Keywords

*Drosophila*; Fat storage; mRNA splicing; 9G8; CPT1

## 1. Introduction

Throughout evolution, animals have developed such that excess fats and sugars not immediately required for energy are stored. This biological function, invaluable for survival during times of scarce food and famine, has become less practical in the developed world, where sustenance abounds. The excess triglyceride storage resulting from the modern influx of nutrient-rich foods has become the foundation for metabolic diseases such as obesity. A

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better understanding of the mechanisms by which fat storage is regulated may prove to be medically indispensable for such diseases. In mammals, triglycerides are stored within structures known as lipid droplets [1]; however, much is still unknown about how lipid droplet formation and morphology is regulated. In an attempt to identify genes involved in this process, genome-wide RNAi screens have been performed in *Drosophila* cell culture since lipid droplet structure is well conserved from flies to humans [2,3]. These screens identified several classes of genes that when knocked down generated visible and quantifiable lipid droplet morphology phenotypes. One subset of these genes included various components of the spliceosome.

Alternative splicing is a critical part of regulating gene expression; over 90% of the human transcriptome is alternatively spliced [4]. Identifying the role of alternative splicing in the function of multiple biological and disease processes has driven many research initiatives in recent years [5]. Specifically, links have been made between mRNA processing and the control of carbohydrate and lipid metabolism. For example, alternative splicing of glucose 6-phosphate dehydrogenase, the rate-limiting enzyme of the pentose phosphate pathway, is regulated in response to altered dietary input [6]. Additionally, down-regulation of the splicing factor SFRS10 in humans has been linked to enhanced lipogenesis and lipid accumulation in obese patients [7]. However, the mechanisms whereby the splicing machinery controls triglyceride storage and lipid droplet morphology are not fully understood.

In *Drosophila*, around 61% of multi-exon genes are alternatively spliced [8]. The regulation of nutrient storage and metabolism is also highly conserved in *Drosophila* [9,10]. Therefore, the fly can serve as an excellent system for understanding the role of the splicing machinery in the control of lipid metabolism. In this study, we used the *Drosophila* fat body (the mammalian liver and adipose equivalent) to assess the role of the proteins involved in the earliest stages of the splicing reaction (including parts of the U1 snRNP, U2AF, and SR proteins) in the regulation of lipid storage. The expression of these representative splicing factors involved in the splicing commitment complex were decreased using RNAi in larval and adult fat bodies and assayed for total triglyceride content. Not surprisingly, we found that decreasing the expression of a number of general splicing factors produced animals with decreased triglyceride levels. Interestingly, knockdown of the SR protein 9G8 results in distinctive triglyceride storage defects, depending on the stage of development. These changes in triglyceride levels were not, however, accompanied by a similar change in glycogen storage, suggesting that triglyceride metabolism is being specifically affected by 9G8-induced splicing of target genes. Decreasing 9G8 also affects the splicing of the  $\beta$ -oxidation enzyme CPT1, potentially contributing to the observed lipid storage phenotype. This study provides *in vivo* evidence for a complex connection between mRNA splicing and fat metabolism.

## 2. Materials and methods

### 2.1. Fly genetics

The following lines were used in this study: cg-Gal4 [11], yolk-Gal4 [12], y [1] v [1]; P{y[+t7.7]=CaryP}attP2 (BL#36303, referred to as attP2), UAS-U1-70K-IR (BL#33396),

UAS-U2AF38-IR (BL#29304), UAS-U2AF50-IR (BL#27542), UAS-SF1-IR (BL#28036), UAS-pUF68-IR (BL#34785), w<sup>1118</sup> (VDRC background stock), UAS-9G8-IR (VDRC#100226), UAS-B52-IR (VDRC#101740), UAS-SC35-IR (VDRC#104978). Flies were grown on standard cornmeal–sugar medium and all experiments were performed on flies grown at 25 °C.

## 2.2. Triglyceride, glycogen, protein and DNA measurements

Triglyceride, protein, and DNA measurements were made as described previously [13]. Briefly, single wandering third instar larvae or single approximately 1-week old adult flies were homogenized in lysis buffer (140 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% Triton-X and 1X complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)). Triglycerides were measured using the Stanbio LiquiColor Triglycerides kit (Stanbio Laboratory, Boerne, TX) and protein was measured using the BCA protein assay kit (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions. To measure glycogen levels, homogenized samples were treated with 8 mg/ml amyloglucosidase (Sigma–Aldrich, St. Louis, MO) for 2 h at 37 °C and then total glucose was measured using Glucose Oxidase Reagent Set (Pointe Scientific, Inc., Canton, MI). Glucose concentrations were also measured in samples that were not treated with amyloglucosidase to determine free glucose and free glucose was subtracted from total glucose to determine glycogen levels. DNA was measured using the Quant-iT High Sensitivity DNA Assay kit (Invitrogen, Carlsbad, CA) from three fat bodies dissected from adult females.

## 2.3. Feeding assay

Food consumption over a 24-h period was measured by using a modified version of the Capillary Feeder (CAFE) Assay as described previously [14]. Three female adult flies were placed in a vial with 1% agar as the only water source and 5% sucrose in a 5- $\mu$ l glass micropipette (Fisher Scientific) as the sole food source. After 24 h had elapsed, the amount of liquid consumed by the flies was measured and was corrected for any evaporation that occurred during the experiment.

## 2.4. RNA isolation and quantitative PCR

Total RNA was isolated from dissected abdomens from approximately 1-week old female flies using Ribozol reagent (Amresco) according to manufacturer's instructions. Remnant genomic DNA was removed using the DNA free kit (Ambion) and cDNA was generated from 1  $\mu$ g of total RNA using oligo (dT) as a primer using the RETROscript kit (Ambion) according to manufacturer's instructions. Quantitative PCR for CPT1 isoforms was carried out as previously described [15].

## 3. Results

### 3.1. Decreasing splicing factor expression in the fat body affects triglyceride storage

In order to assess the role of mRNA splicing on the control of lipid metabolism and storage in *Drosophila*, we first set out to confirm whether decreasing splicing factor expression affects fat storage *in vivo* and, specifically, which splicing factors contribute to a lipid

storage phenotype. In order to accomplish this task, we induced fat-body specific RNAi knockdown of several splicing factors via the Gal4/UAS system. The driver *cg-Gal4*, which expresses at high levels in the larval fat body [11], was used to induce RNAi of 8 different splicing factors. We specifically focused on splicing factors involved in the earliest events in the splicing mechanism (i.e., members of the U1 snRNP and U2 auxiliary factor) as well as SR proteins, which are important for the identification of intron/exon boundaries [16].

Decreased expression of the U1 protein U1-70K and the large and small subunits of the U2 auxiliary factor, U2AF38 and U2AF50, in the fat body results in a decrease in larval triglyceride storage compared to the control (Fig. 1A). This result is consistent with the genome-wide RNAi screens in cultured cells where RNAi to splicing factor components led to fewer lipid droplets [2,3]. These phenotypes are specific to these genes and not all splicing factors as RNAi towards SF1, a protein that binds to U2 auxiliary factors to help identify the 3' splice site [17,18], and pUf68, an hnRNP, have no effect on lipid levels (Fig. 1A). We also measured triglyceride storage in larvae with fat body-specific knockdown of the arginine-serine (SR) domain-containing proteins 9G8, B52, and SC35. Loss of 9G8 in the fat body resulted in decreased lipid storage, while decreasing B52 and SC35 had no effect (Fig. 1C). The role of these splicing factors in controlling nutrient storage is specific for triglycerides as glycogen levels were normal in fat body-specific RNAi of these splicing factors (Fig. 1B and D). Additionally, fat body expression of U1-70K, U2AF38, U2AF50, and 9G8 is important for fly development as animals with decreased levels of these genes were developmentally delayed by 24–36 h and arrested during the pupal stage of development (data not shown). Together, these data indicate a role for specific members of the splicing machinery in the fat body in controlling fly development and lipid storage and confirm the *in vitro* genome-wide RNAi screen data in an *in vivo* system.

Since the knockdown of several splicing factors resulted in a developmental delay, it is possible that the decreased triglyceride phenotypes could be secondary to the developmental phenotype. To address this possibility, *yolk-Gal4*, which expresses specifically in the adult female fat body [12], was used to drive expression of the select splicing factor RNAi lines that yielded triglyceride phenotypes in larvae. Triglyceride levels were significantly decreased when U1-70K and U2AF38 levels were decreased (Fig. 2A), which is consistent with the larval lipid data and suggests that these splicing factors control fat storage independently of fly development. However, inducing RNAi to U2AF50 in the adult fat body resulted in no significant change in fat storage (Fig. 2A), suggesting that U2AF50 may play different roles in *Drosophila* larvae and adults. Interestingly, when 9G8 was knocked down in the adult fat body, triglyceride levels increased more than 10-fold (Fig. 2B), indicating that 9G8 also functions differently in the larval and adult fat bodies. Together, these data confirm that members of the splicing machinery regulate triglyceride storage independently of any effects on the developmental timing of the animal.

### **3.2. 9G8 regulates triglyceride storage by controlling both fat cell number and the amount of fat per cell**

In order to change total triglyceride storage, the amount of fat stored per cell may be altered, the number of cells within the fat body may be altered, or both [19]. Determining the

mechanism by which triglyceride storage is affected when these splicing factors are decreased may shed light on which genes are being alternatively spliced to cause a lipid storage phenotype. To further characterize how splicing factors regulate triglyceride levels, an analysis of the DNA content of the fat body, a measurement previously shown to accurately reflect cell number [13], was carried out to determine whether or not there was a significant change in the number of fat storing cells in the fat body. Triglyceride/DNA and triglyceride/protein ratios were also assessed to determine whether the amount of fat stored per cell was affected by splicing factor knockdown. We focused on 9G8 since it resulted in such a drastic lipid phenotype. Decreasing 9G8 in the adult fat body exhibited a striking 30-fold increase in triglyceride/DNA and triglyceride/protein ratios as well as a small, but statistically significant increase in fat body DNA content (Fig. 3A and B). These data indicate that 9G8 controls lipid storage in the adult fat body by regulating both the amount of fat per cell as well as the number of fat cells.

### 3.3. 9G8 regulates the splicing of *Drosophila* CPT1

One potential cause of the increased triglycerides in fat body cells with decreased 9G8 is that these animals eat more than the controls. To test this hypothesis, food consumption of adult flies was monitored over a 24-h period using a capillary feeding (CAFE) assay [14]. Surprisingly, average food consumption per fly was found to be less when 9G8 was decreased in the fat body as compared to the controls (Fig. 4A). This indicates that increased feeding is not the cause of the elevated fat levels in the fat body specific 9G8-IR flies.

Another explanation for the increased triglyceride phenotype observed in the 9G8-IR flies is that loss of 9G8 could lead to a decrease in lipid synthesis or an increase in lipid breakdown, presumably by altering the splicing of a lipid metabolic gene leading to a more or less active form of the protein. The *Drosophila* genome contains one homolog of *CPT1*, the rate-limiting step in  $\beta$ -oxidation. This gene has two distinct isoforms resulting from an alternate sixth exon (exon 6A or 6B; [15]). Enzymes resulting from *CPT1* isoforms that include exon 6A have higher activity than those including exon 6B [15]. To determine whether 9G8 affects triglyceride storage by regulating the splicing of *CPT1*, we performed quantitative PCR for the presence of these two alternative sixth exons of *CPT1* in 9G8 knockdown fat bodies. In control flies, the *CPT1* isoforms including exon 6A, the splice variant leading to the more active enzyme, were present at levels twice that of the isoforms containing exon 6B (Fig. 4B). Interestingly, flies with decreased fat body 9G8 expressed the *CPT1* isoforms including exon 6B (the splice variant leading to the less active enzyme) at levels twice that of those including exon 6A (Fig. 4B), suggesting that flies with less 9G8 may undergo less  $\beta$ -oxidation contributing to the increased fat phenotype. Together, these data indicate that 9G8 regulates the splicing of *CPT1* to control fat body triglyceride storage.

## 4. Discussion

In this study, we have shown that fat body-specific knockdown of the key splicing factors U1-70K and U2AF lead to decreased triglyceride storage phenotypes in larvae. Additionally, there was no accompanying defect in glycogen storage observed in these animals. It is interesting that decreasing the expression of these constitutive splicing factors in the fat body would only lead to an alteration in the splicing of genes involved in triglyceride

metabolism and not glycogen as well. This suggests that the activity of lipid metabolic genes are more regulated by changes in the splice variants produced than enzymes involved in glycogen metabolism. Decreased fat body-specific expression of U1-70K and U2AF38 in adult females also yielded a decreased triglyceride level similar to the phenotype observed in larvae. The previous genome-wide RNAi screens have indicated that *in vitro* knockdown of the constitutive splicing factors U1-snRNA, SmB, SmD, and U2AF50 all result in lipid storage phenotypes [2,3]. These findings are consistent with our data.

We have also shown that knockdown of the SR protein 9G8 in the larval fat body also leads to a decreased triglyceride storage phenotype. Surprisingly, the knockdown of 9G8 in the adult female fat body resulted in a large increase in triglyceride levels and this is mainly due to an increase in the amount of lipid stored in each fat body cell. SR proteins affect splicing of target genes in a concentration-dependent manner, and bind to exonic splice enhancers to promote the use of alternative splice sites [20]. 9G8 expression has been reported to increase in the fat body as the fly transitions through larval and pupal development and has higher expression in the adult female than male (modENCODE data on Flybase), which may provide an explanation for the marked difference between larval and adult triglyceride phenotypes observed here.

The elevated triglyceride phenotype observed in flies lacking 9G8 in their fat bodies could occur due to a role for 9G8 in regulating food consumption, lipid synthesis or breakdown, or a combination of these. We have shown that food intake is actually decreased in 9G8-IR flies, which may be a compensatory response to the higher triglycerides. Regardless, changes in feeding do not seem to account for the lipid storage phenotype observed when 9G8 is decreased. Conversely, we have provided evidence that 9G8 affects lipid breakdown by regulating the splicing of *CPT1*, the rate-determining enzyme in  $\beta$ -oxidation. 9G8-IR flies express twice the amount of *CPT1* mRNA including exon 6B than *CPT1* mRNA including exon 6A leading to twice as much of the less active CPT1 enzyme (Fig. 4; [15]). Therefore, it is likely that flies with decreased 9G8 in their fat body break down triglycerides through  $\beta$ -oxidation more slowly resulting in the accumulation of triglyceride shortly after eclosion. While we believe that altered splicing of *CPT1* contributes to the lipid storage phenotype observed in the 9G8-IR flies, it is probably not the only metabolic gene that is improperly spliced in these flies. A number of important lipid metabolic genes such as dFAS and the adipose triglyceride lipase homolog brummer are annotated to have multiple isoforms (Flybase) and it is possible that 9G8 is involved in controlling the splicing of these genes as well. In fact, the 9G8 splicing enhancer sequence has been identified [21,22] making it possible to identify metabolic genes in *Drosophila* that may rely on 9G8 to regulate their alternative splicing.

In summary, this study has provided evidence for a role of mRNA splicing in controlling triglyceride storage. We have specifically identified the SR protein 9G8 as a regulator of lipid metabolism by affecting the splicing of the gene encoding for the rate-limiting step in  $\beta$ -oxidation, CPT1, leading to the production of an enzyme with altered kinetics. This study also provides support for using the *Drosophila* fat body as a system with which to further study the mechanisms of mRNA splicing in an *in vivo* context.

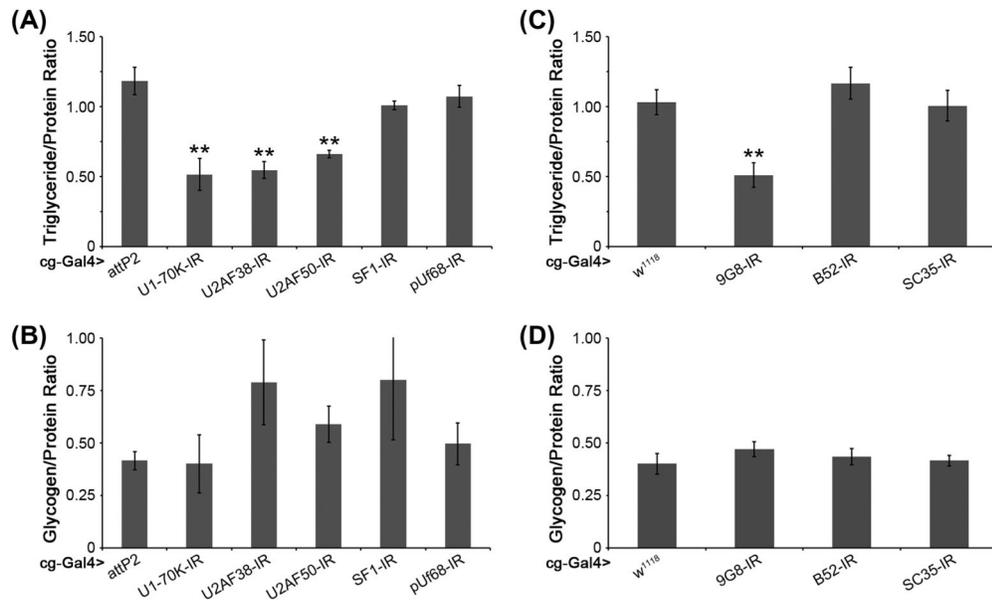
## Acknowledgments

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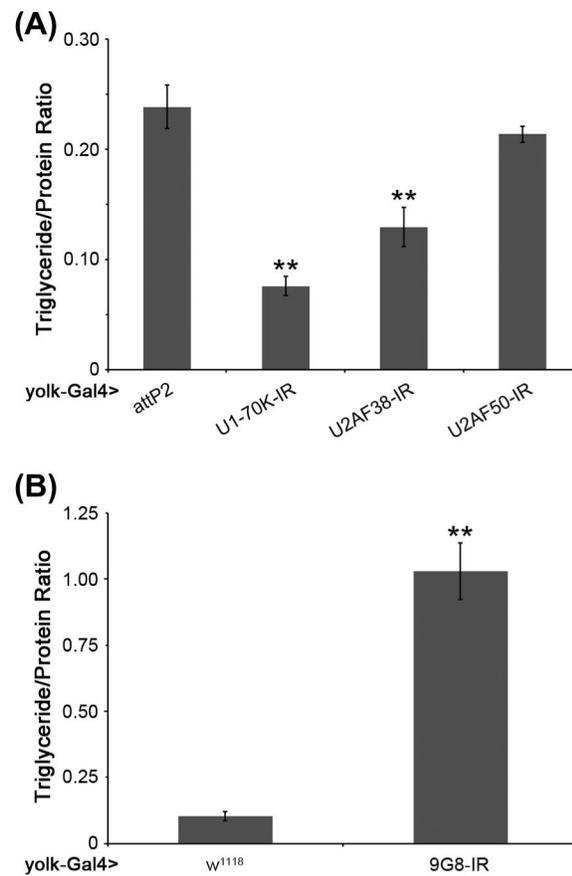
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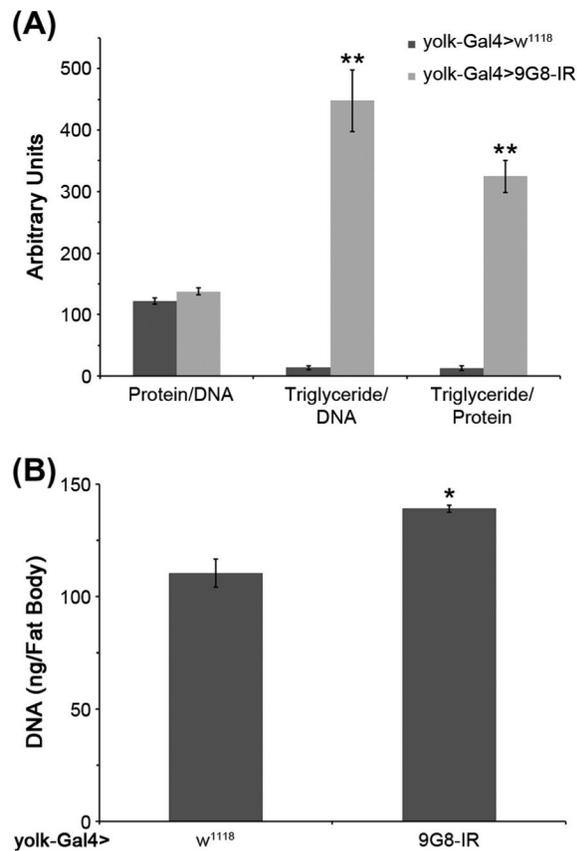
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**Fig. 1.**

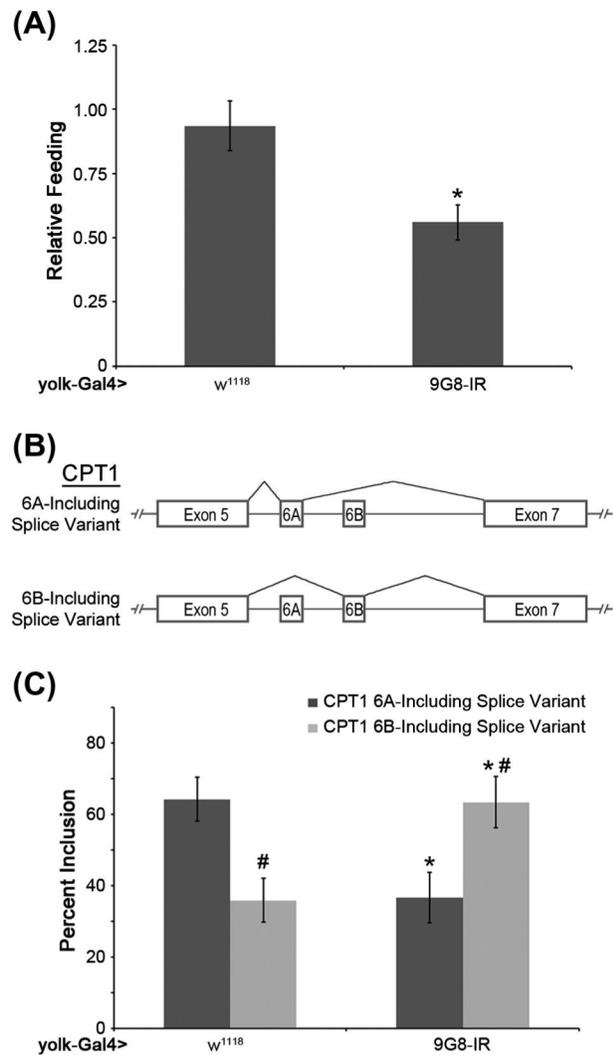
Several splicing factors are necessary for the normal storage of triglycerides. (A) Triglyceride/protein and (B) glycogen/protein ratios of whole larvae with fat body expression of U1-70K-IR, U2AF38-IR, U2AF50-IR, SF1-IR, pUf68-IR, and a genetic background control (cg-Gal4/attP2). (C) Triglyceride/protein and (D) glycogen/protein ratios of whole larvae with fat body expression of 9G8-IR, B52-IR, SC35-IR, and a genetic background control (cg-Gal4/w<sup>1118</sup>). Each experiment was performed at least three times and values represent mean  $\pm$  SEM. \*\* $P < 0.01$  by unpaired Student's *t*-test compared to the appropriate background control.



**Fig. 2.** Splicing factor knockdown in the adult fat body affects triglyceride storage. (A) Triglyceride/protein ratios of adult females with fat body-specific expression of U1-70K-IR, U2AF38-IR, U2AF50-IR, or a genetic background control (yolk-Gal4/ attP2). (B) Triglyceride/protein ratios of adult females with fat body-specific expression of 9G8-IR or a genetic background control (yolk-Gal4/w<sup>1118</sup>). Each experiment was performed at least three times and values represent mean  $\pm$  SEM. \*\* $P < 0.01$  by unpaired Student's *t*-test compared to the appropriate background control.



**Fig. 3.** 9G8 regulates fat body triglyceride storage by controlling the amount of fat stored per cell as well as the number of fat cells. (A) Protein/DNA, triglyceride/DNA, and triglyceride/protein ratios of dissected female fat bodies from *yolk-Gal4 > 9G8-IR* or *yolk-Gal4/w<sup>1118</sup>* genetic background control animals. (B) Total DNA content of dissected fat bodies from *yolk-Gal4 > 9G8-IR* or *yolk-Gal4/w<sup>1118</sup>* genetic background control animals. Each experiment was performed at least three times and values represent mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$  by unpaired Student's *t*-test compared to controls.



**Fig. 4.** 9G8 regulates the splicing of *CPT1* in *Drosophila* fat body. (A) Food consumption of adult female *yolk-Gal4 > 9G8-IR* or *yolk-Gal4/w<sup>1118</sup>* genetic background control flies was measured using the CAFE assay over a 24-h period. \* $P < 0.05$  by unpaired Student's *t*-test compared to controls. (B) Schematic splice site diagram of the 6A and 6B-including splice variants of *Drosophila CPT1* (Adapted from [15]). (C) Percent inclusion of exons 6A and 6B of *CPT1* in *yolk-Gal4 > 9G8-IR* or *yolk-Gal4/w<sup>1118</sup>* genetic background controls. Each experiment was performed at least three times and values represent mean  $\pm$  SEM. \* $P < 0.05$  by unpaired Student's *t*-test compared to inclusion of that splice variant in control flies. # $P < 0.05$  by unpaired Student's *t*-test compared to inclusion of exon 6A-including splice variant.