SYNDECAN KNOCKDOWN IN THE INSULIN PRODUCING CELLS OF *DROSOPHILA MELANOGASTER* AFFECTS ENERGY METABOLISM AND LIFE SPAN

by

JONATHAN LUND WARREN JR.

MARIA DE LUCA, COMMITTEE CHAIR
BARBARA GOWER
DOUGLAS MOELLERING

A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2014

SYNDECAN KNOCKDOWN IN THE INSULIN PRODUCING CELLS OF *DROSOPHILA MELANOGASTER* AFFECTS ENERGY METABOLISM AND LIFE SPAN

JONATHAN LUND WARREN JR.

DEPARTMENT OF NUTRITION SCIENCES

ABSTRACT

Drosophila melanogaster is a powerful model organism for studying human metabolic disease due to the conservation of various signaling processes and pathways. The insulin producing cells (IPCs) in the brain of adult flies sense circulating nutrients and respond by producing three insulin-like peptides (dILP2, dILP3, and dILP5). Several studies have shown that dILPs play a major role in Drosophila reproduction, metabolism, growth, and longevity. Previous data showed that flies homozygous for a hypomorphic mutation in the Drosophila *syndecan* (*dSdc*) gene had defects in energy metabolism and lower expression of brain *dilp2-3* and *dilp5* genes. Syndecan is a transmembrane proteoglycan that serves as a co-receptor in the binding of a variety of ligands, such as growth factors and cytokines. The objective of this study was to investigate the role of *dSdc* in the IPCs and dILP regulation. An experimental fly model with reduced expression of *dSdc* specifically in the IPCs was generated. IPCs-specific *dilp2-GAL4* driver flies were crossed over *UAS-dSdc RNAi* flies or w^{1118} control flies. Gene expression was measured by quantitative PCR and protein quantity by Western blot in the offspring of each cross. Total glycogen and triacylglycerol storage were measured using standard assay kits. Total protein content was assessed by Lowry assay. Food intake was tested by capillary feeding method and metabolic rate by flow-through respirometry. An

oral glucose tolerance test was performed by subjecting flies to an overnight fast, re-feeding in a glucose medium, and measuring whole-body glucose over the subsequent four hours. Adult lifespan and starvation resistance were also assessed. Flies with reduced *dSdc* in the IPCs showed increased *dilp2* and *dilp3* expression, but decreased circulating dILP2 peptide compared with controls. They had lower glucose tolerance, higher glycogen and triacylglycerol storage, and lower protein levels. Furthermore, the IPC-specific *dSdc* knockdown flies displayed increased life span and starvation resistance. Taken together, these findings suggest that *dSdc* plays a cell autonomous role in mediating the proper functioning of the IPCs and the release of dILP2 in *D. melanogaster* and provide a framework for approaching the study of the role of mammalian *syndecan* in the function of the pancreatic beta-cell.

Keywords: *Drosophila melanogaster*, carbohydrate metabolism, syndecan

ACKNOWLEDGMENTS

The author would like to express sincere appreciation for the following individuals and facilities for assistance provided throughout the course of this work:

Maria De Luca, PhD
Barbara Gower, PhD
Douglas Moellering, PhD
Susan Miller, MS, RD, LD
José Fernández, PhD
Su Bu, PhD
Qiuli Liang, PhD
Matthew Eveland
Lauren Nelson
Hayley Leuch
Kelley Johnston
Dipti Halade
Dr. Jeff Leips
Chia-Hue Lue
Dr. Pierre Leopold
Dr. Eric Rulifson

DRC Bioanalytical Redox Biology Core

Funding provided by P30 DK079626/DK/NIDDK NIH

TABLE OF CONTENTS

LIST OF TABLES

Table *Page*

LIST OF FIGURES

LIST OF ABBREVIATIONS

AKH	adipokinetic hormone
AKT	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
CAFÉ	Capillary Feeding Assay
dEGFR	Drosophila epidermal growth factor receptor
dFOXO	Drosophila forkhead box protein O
dilp2>Sdc-IR	flies with reduced expression of *dSdc* in the IPCs
dILP	Drosophila insulin-like-peptide
dIR	Drosophila insulin receptor
drp49	Drosophila ribosomal protein 49
dSdc	Drosophila syndecan
DTKR	Drosophila tachykinin-related peptide receptor
GABA	gamma-aminobutyric acid
IGF-1	insulin-like-growth factor 1
IIS	insulin/insulin-like-growth factor signaling
IPC	insulin-like-peptide producing cells
IRS	insulin-receptor-substrate
JAK/STAT	Janus kinase/signal transducers and activators of transcription
p70s6k	p70s6 kinase

PI3K	phosphatidylinositol-3 kinases
T2DM	Type 2 diabetes mellitus
TAG	triacylglycerol
Upd2	Unpaired2
UAS	Upstream activating sequence

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a disease that continues to affect the population of the United States and the world at an alarming rate. The implications of T2DM are far reaching, affecting not only the patients and their families, but also the national economy by increasing medical costs to the individual and insurance providers. Nearly 30 million people in the US alone (almost 10% of the total population) are living with the disease and almost $250 billion is being spent on total costs associated with T2DM [1, 2]. Worldwide, the number of patients with T2DM is nearly 350 million, with the vast majority of new cases diagnosed in low- and middle-income countries [3, 4]. Clearly, there is a great interest among the medical and research communities to decipher the causes of T2DM, and bring an end to a disease that has become a global burden by utilizing the wide variety of innovative tools at our disposal.

T2DM is characterized by resistance of the peripheral cells to insulin action and impaired insulin secretion by the beta-cells of the pancreas [5]. Insulin is an anabolic hormone produced in the beta cells of the pancreas that acts as a signal to the various tissues in the body to indicate that glucose is available for uptake. Specifically, binding of insulin to the insulin receptor triggers a signaling cascade that directs glucose transporters from the cytosol of the cell to the membrane for the import of glucose. In a normal patient, blood insulin levels rise in a biphasic pattern as a response to a postprandial increase in blood glucose concentration. There is an initial surge followed by a period of

low, sustained release [6]. As the cells respond to insulin, glucose is cleared from the blood and entry is facilitated into the muscle and adipose tissues. Subsequently, insulin levels fall with the decrease in blood glucose back to homeostatic levels. In addition to insulin secretion after food intake, there appears to be a circadian pattern with levels rising in early morning, and then peaking at early afternoon [7]. The cells of a patient with T2DM are resistant to insulin signaling and/or do not produce enough insulin to clear sufficient glucose from the blood to return to homeostatic levels. This can lead to chronic hyperglycemia, which can create harmful glycation products and oxidative damage. If undiagnosed or unmanaged, hyperglycemia can result in the possibilities of blindness, periodontitis, peripheral neuropathy, and kidney complications [2]. Additionally, T2DM can promote the risk of cardiovascular complications associated with chronic, low-level oxidative stress due to insulin resistance [8].

The fruit fly *Drosophila melanogaster* has emerged in recent years as a powerful model for studying human metabolic disease due to a wide range of conserved metabolic regulatory systems and pathways [9]. Flies have 14 insulin-like-peptide producing cells (IPCs) localized to the brain. The IPCs of adult *D. melanogaster* sense circulating nutrients and respond by producing three insulin-like peptides (dILP2, dILP3, and dILP5), with well-studied homology to mammalian insulin [10]. The IPCs display several commonalities with the human beta-cells of the pancreas with respect to insulin release, relying on membrane depolarization and influx of calcium in response to carbohydrate for release of dILPs [11]. Among the dILPs, *dilp2* is the most highly expressed in the IPCs and the most abundant dILP in circulation [10]. Genetic partial ablation of the IPCs in the fly produces phenotypes similar to the human diabetic model including increased

circulating carbohydrate and impaired ability to clear carbohydrate from circulation following food intake [12]. Additionally, IPC-ablated flies have retarded growth, increased levels of glycogen and triacylglycerol (TAG), increased resistance to oxidative stress, increased starvation resistance, and a longer life span compared to control flies [12].

The IPCs project their axons into the corpora cardiaca that produces the adipokinetic hormone (AKH), which is a putative glucagon homolog, and controls energy homeostasis in insects by inducing mobilization of glycogen and fat reserves from the fat body [13]. The latter is a diffuse organ that has characteristics of both mammalian liver and mammalian white adipose tissue and has been shown to produce a functional homolog of human leptin, called Unpaired2 (Upd2) [14]. Upd2, which is produced in the fed state by the fat body, can activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling in neurons that produce gamma-aminobutyric acid (GABA) and innervate the IPCs [14]. The activation of the JAK/STAT signaling by Upd2 relieves the inhibitory effect of GABAergic neurons on the production of dILPs [15]. Secreted dILPs bind a single Drosophila insulin receptor (dIR), a receptor tyrosine kinase, that once activated undergoes autophosphorylation like in mammals [16].

The Drosophila insulin/insulin-like growth factor signaling (IIS) pathway triggered by the engagement of the dIR with the dILPs displays several conserved commonalities with the mammalian reaction cascade for insulin and insulin-like-growth factor 1 (IGF-1) pathways (Figure 1) [17]. The activated dIR recruits a homolog of the mammalian insulin-receptor-substrate (IRS) called chico in the fly that promotes the recruitment and activation of the phosphatidylinositol-3 kinases (PI3K)/AKT pathway

[18]. As in mammals, the PI3K/AKT pathway regulates phosphorylation of proteins that are key for metabolic control, such as shaggy (a glycogen synthase kinase homolog) [19]. Phosphorylation of the transcription factor Drosophila forkhead box protein O (dFOXO) also occurs via the PI3K/AKT pathway, inhibiting its transcriptional function and promoting cell growth [20]. Mutations in several Drosophila genes involved in the IIS pathway have been shown to exhibit increased fat storage and a longer life span than controls [21, 22]. Decreased IIS promotes the function of the transcription factor dFOXO in the adult fat body, resulting in increased Drosophila life span and suggesting that the targets of dFOXO control life span extension [20].

The IIS pathway triggered by circulating dILPs in the fly serves a dual role in the control of metabolism and promotion of growth and aging [23]. In mammals, insulin-like-growth factor 1 (IGF-1) produced in the liver drives similar growth and aging processes, but a shared binding capacity exists between insulin and IGF-1 and their respective receptors [24]. Mammalian insulin also drives pathways directing glucose transporters (GLUT) from the cytoplasmic space to the cell membrane for glucose uptake. To this point, the IPC-derived dILPs have not been shown to facilitate glucose uptake in this manner [25].

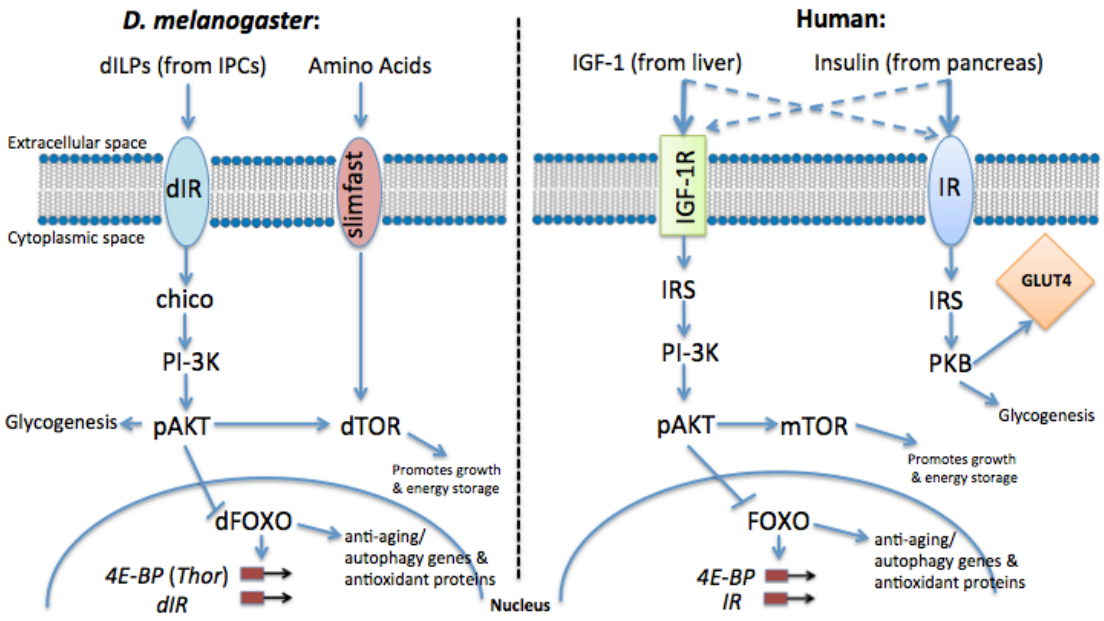

Figure 1: Comparison of dILP action to insulin and insulin-like-growth factor 1 in mammals.

In addition to the differences seen between the function of the dILPs and mammalian insulin, the fly and many other invertebrates, utilize trehalose, a disaccharide molecule made up of two glucose molecules, as the primary circulating carbohydrate source as opposed to glucose, the primary sugar in mammals [25]. In contrast to mammals, which seek to maintain circulating glucose levels to within discrete homeostatic concentrations, the concentrations of circulating trehalose in the fly can vary markedly. As a disaccharide, trehalose is non-reducing and stable across a wide range of temperatures and pH levels; thus is much less likely to form the harmful glycosylated metabolites with other circulating nutrients in high concentrations in the hemolymph. The utilization of trehalose appears to be an evolutionary conserved adaptation to allow the fly to respond and thrive in a number of stressful environments.

Previous work in the laboratory of Dr. Maria De Luca first identified a gene called *syndecan* (*dSdc*) as a factor controlling inter-individual variability in fat storage of

D. melanogaster [26]. In the same study, it was also reported that flies homozygous for a hypomorphic mutation in *dSdc* had decreased expression of *dilp2* in the brain as well as decreased energy metabolism and life span.

Syndecan is a member of a family of transmembrane proteoglycans, which are characterized by a core protein with extracellular attachment sites for heparan and chondroitin sulfate chains (Figure 2). Through these chains, they interact with various extracellular signaling molecules, including growth factors and cytokines, and their respective receptors to direct various cellular processes important for development [27]. A short intracellular domain contributes to the role of syndecan as a signaling molecule, allowing for direct communication between the dynamic extracellular matrix and the interior cytoskeleton. One process that has been shown to regulate syndecan function is the "shedding" of the extracellular domain by matrix metalloproteinase [28]. Once released from the cell surface, the extracellular domains can sequester soluble factors and compete for cell surface binding [27, 29].

Unlike invertebrates such as *D. melanogaster* that contain only one *dSdc* gene that is expressed in most tissues, mammals have four different genes with respective proteins (SDC1-4) [30]. Considerable conservation between mammalian syndecans and Drosophila syndecan exists in both structure and function [30]. Thus, insights gained from the studies in Drosophila are likely to apply to mammals as well.

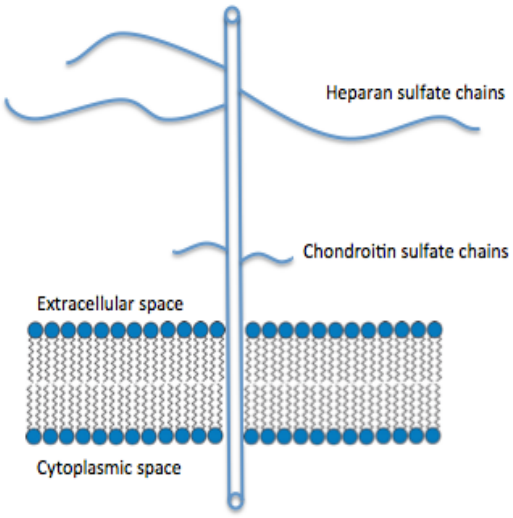

Figure 2: Syndecan protein structure.

Previous work has shown mammalian SDC4 to be present in abundance in basement membranes of pancreatic beta-cells *in vivo* and mouse-derived MIN6 pancreatic cell line, tissues important for preserving beta-cell function [31]. However, little is known about the role of syndecan in these membranes and on their role in beta-cell longevity and function. Based on these observations and the findings in Drosophila reported above, the objective of this study was to test the hypothesis that syndecan plays a cell autonomous role in the function of the Drosophila IPCs, thus regulating changes in dILP production and energy storage.

To address this hypothesis, an experimental fly model was generated exhibiting knockdown of *dSdc* expression specifically in the IPCs. These flies showed increased *dilp2* and *dilp3* expression, but decreased levels of the dILP2 protein in the periphery compared with controls. The experimental flies also had lower glucose tolerance, higher glycogen and triacylglycerol storage, and lower total protein levels. Additionally, they

displayed increased life span and increased starvation resistance. No changes in insulin signaling or energy balance were observed.

METHODS

Fly Stocks

All flies were reared in temperature-controlled incubators at 25°C in and 60-75% relative humidity on a standard cornmeal and yeast formula with 12 hr light-dark cycles. Newly eclosed flies were collected as virgins and aged for 3-6 days before performing various assays.

A GAL4/upstream activating sequence (UAS) approach was used to achieve tissue specific reduction of *dSdc* in the IPCs. *dilp2*-GAL4 driver flies containing a GAL4 coding sequence inserted by a P-element downstream of *dilp2* were generously provided by Dr. Eric Rulifson, University of California, San Francisco. Males of this line were crossed with female UAS-*dSdc* interference RNA (dSdc-IR) (Vienna Stock Center, #13322) or wild-type w^{1118} (Bloomington Stock Center, #5905) female flies to generate flies with reduced expression of *dSdc* in the IPCs (dilp2>Sdc-IR) and controls, respectively (Figure 3). A characteristic of the dSdc-IR transgenic flies is the presence of a mRNA anti-sequence to *dSdc* downstream of the UAS element. As such, the binding of GAL4 protein to the UAS element in the heterozygous offspring of the experimental cross resulted in knockdown of *dSdc* in the IPCs.

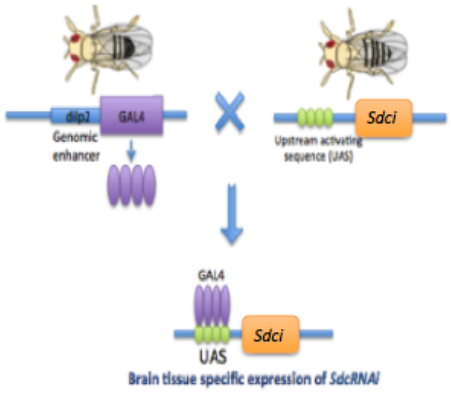

Figure 3: Experimental cross design producing offspring with *dSdc* specifically reduced in the IPCs.

Quantitative PCR (qPCR)

Four replicates of each genotype and sex containing either 20 heads or 10 bodies were collected in order to measure gene expression in the IPCs and periphery. These samples were frozen on liquid nitrogen between 9:00 am and 10:00 am on collection days and stored at -80°C. On the day of processing, mRNA was extracted from the heads or bodies using a Quiagen RNeasy Mini column filtration kit (Qiagen; Valencia, CA) following the manufacturer's protocols with lab optimization. Resulting mRNA was then immediately used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems by Life Technologies; Grand Island, NY). Stocks of cDNA were standardized to 500 ng/uL for storage at -20°C. A standard curve was created by pooling together an equal amount of cDNA from all diluted samples and preparing a subsequent serial dilution. Working dilutions of 200 ng/uL cDNA samples used for running of the RT-PCR protocol.

Each qPCR was performed on a StepOnePlus instrument using ABI SYBR-Green PCR master mix in a 15 µL reaction (Applied Biosystems by Life Technologies). The

ratio of the quantity of the gene of interest versus the Drosophila gene *ribosomal protein 49* (*drp49*), a common reference gene in Drosophila genetic analysis, was used to normalize target gene expression.

Primers (forward and reverse) used are as follows: dSdc-F, CAG CAT CAT CGG CAA ACC AC; dSdc-R, CAC ACC CAC ATA CGC AGA GT; dSdcRA-F, ACA TCG ACG ACT TCG ACA CCA AT; dSdcRA-R, GGA CTC CGT ATC CAG ATC CT; dSdcRB-F, CCC TGT CTC TCC AAC CAA CGC; dSdcRB-R, CTC CGA TGA GAA TTG CGA CTA; dilp2-F, TCC ACA GTG AAG TTG GCC C; dilp2-R, AGA TAA TCG CGT CGA CCA GG; dilp3F, AGA GAA CTT TGG ACC CCG TGA A; dilp3R, TGA ACC GAA CTA TCA CTC AAC AGT CT; dilp5-F, GCT CCG TGA TCC CAG TTC TCC; dilp5-R, GCC GAA TGC TCG ACA GTG AG; Thor-F, TCC TGG AGG CAC CAA ACT TAT; Thor-R, GGA CGCC ACG GAG ATT CTT; dIR-F, GGA CGC CCA AGT CTG AAG AA; dIR-R, AAC TAC CAG CCG AAC CAC TG; drp49-F, ATC CGT TAC GGA TCG AAC AA; drp49-R, GAC AAT CTC CTT GCG CTT CT.

Knockdown of the *dSdc* in the IPCs and *Thor* and *dIR* in the body of flies was tested only in males and statistically significant differences in *dSdc* isoforms were calculated by non-parametric Wilcoxon test. Statistical differences in the expression of *dilp*s were analyzed by analysis of variance (ANOVA) after testing for normality, using the following model: y = genotype + sex + genotype*sex + error. Analyses were performed using SAS 9.3 software (SAS Institute, Cary, NC).

Western Blotting

Heads of flies were dissected from the bodies, and both heads and bodies were snap-frozen in liquid nitrogen and stored at -80°C. Fifty heads per genotype were

homogenized in 200 µL of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, supplemented with protease inhibitor and phosphatase inhibitor. Twenty bodies per genotype were homogenized in 200 µL of the same lysis buffer. Homogenized samples were centrifuged and the supernatant were transferred to new tubes. Protein concentrations were determined by BCA protein assay. For dILP2 western blot, 50 µg of protein was loaded per lane onto 15% acrylamide bis-acrylamide gels. For p-AMPK, p-AKT and p-p70s6k western blots, 50 µg of protein was loaded per lane onto 12% acrylamide bis-acrylamide gels. The protein was separated and transferred to PVDF membrane, blocked in 5% BSA, and blotted with 1:1000 rat anti-dilp2 (a kind gift from the lab of Dr. Pierre Leopold, Institute of Biology Valrose), 1:1000 rabbit anti-p-AMPK (Cell Signaling; Danvers, MA), 1:1000 rabbit anti-p-AKT (Cell Signaling) and 1:1000 mouse anti-p-p70s6k antibodies (Thermo Fisher Scientific; Waltham, MA). The primary antibodies were incubated overnight at 4°C. Secondary antibody was 1:5000 HRP-conjugated Donkey anti-rat, goat anti-rabbit, or goat anti-rabbit for 1 hr at room temperature. The protein image was obtained using Image Lab 5.0 software. A statistical difference between genotypes in protein staining was assessed by two-tailed *t*-test.

Total Glycogen, TAG, and Protein Levels

Whole-body TAG, glycogen, and protein levels were measured using the same homogenate. Between 16 and 20 replicates per sex, per genotype were collected. TAG was measured spectrophotometrically using a commercially available kit following manufacturer's protocol (Sigma-Aldrich; St. Louis, MO). Total glycogen was assessed after incubating an aliquot of homogenate with amyloglucosidase at 37°C to cleave

glycogen into measureable glucose using a commercially available glucose oxidase kit (Sigma-Aldrich). Total protein was assessed by standard Lowry method [32]. Statistical differences in TAG and glycogen levels were analyzed by ANCOVA using the following model: y = genotype + sex + bodyweight + protein + genotype*sex + error. Protein levels were assessed by using the following model: y = genotype + sex + bodyweight + genotype*sex + error. Analyses were performed using SAS 9.3 software (SAS Institute; Cary, NC).

Glucose Tolerance Testing

Glucose tolerance testing was adapted from the laboratory of Dr. Aaron Haselton [12] and personal communications with members of the laboratory of Dr. Carl Thummel. Six replicates per time point, per sex, per genotype containing 5 flies each were fasted overnight on a 1% agar solution. After the fast and before re-feeding, a set of 6 replicates per sex, per genotype was frozen ("fasted"). The remaining replicates were re-fed in vials containing 10% glucose/1% agar medium for 2 hrs. A second set of six replicates per sex per genotype was collected immediately following the glucose re-feeding (post-prandial, 0 hrs, "PP0"), while the remaining flies were then transferred back to the fasting medium. Six replicates per sex, per genotype were collected 2 hrs and 4 hrs post-prandial (PP2, PP4). Flies were homogenized in 100 uL of PBS buffer and glucose was measured using Sigma Glucose Oxidase kit. Glucose measures were normalized to protein quantities obtained by Lowry method using the same homogenate. Statistical differences in males were analyzed by two-tailed *t*-tests between genotypes at each of the four time points.

Capillary Feeding Assay (CAFÉ)

The capillary feeding assay was adapted from Ja et al. [33]. Vials containing a 1% agar medium were used as the feeding chambers. A capillary tube filled with a liquid food media made up of 5% glucose and 5% autolyzed yeast was pierced through the foam top of the vial. Flies were allowed to acclimate to the capillary system overnight before measurements were taken. Five replicates per sex, per genotype were utilized containing five flies each. Measurements began with the initiation of the light cycle the next day (7:00 am). Food consumed was calculated as the difference in initial volume provided minus the volume remaining at the end of the light cycle and end of the dark cycle. Two-tailed *t*-tests were used to examine statistical differences between genotypes.

Metabolic Rate

Metabolic rate was measured as CO_2 production using flow through respirometry adapted from Van Voorhies et al. [34] as described previously in De Luca et al. [26]. Statistical differences in metabolic rate were analyzed by ANCOVA using the following model: y = genotype + sex + time + genotype*sex + sex*time + genotype*time + genotype*sex*time + body weight + error. Analyses were performed using SAS 9.3 software (SAS Institute).

Adult Life Span

Adult life span experiments were performed in the laboratory of Dr. Jeff Leips at the University of Maryland at Baltimore County using the protocol described in De Luca et al. [26]. Briefly, population cages were used in order to assess differences in adult life span between flies with reduced *dSdc* in the IPCs and controls. To standardize the parental density of each genotype, flies were allowed to lay eggs for five days. Offspring

were collected as virgins over a period of four days and reintroduced to the population cage at the end of the four days. Three population cages of each genotype and sex were monitored while deceased flies were counted and removed every other day. Cox regression analysis was used to compare rate of survival between genotypes, analyzing each sex separately and using replicate cage as a covariate.

Starvation Resistance

Survival under starvation conditions was assessed by placing 10 flies per sex and genotype on a 1.5% agarose medium. The number of flies alive was recorded at 8 hr intervals until all flies were deceased. Cox regression analysis was used to compare rate of survival between genotypes, analyzing each sex separately.

RESULTS

Genetic Analysis and Protein Quantification

To first assess knockdown of *dSdc* in the IPCs, overall expression of four isoforms of *dSdc* was measured using two primer sets by qPCR. While no difference was found in *dSdc-RA, dSdc-RD,* and *dSdc-RF* transcript levels between the dilp2>dSdc-IR flies and controls ($p = 0.289$), the levels of *dSdc-RB* mRNA were reduced by 39% in the head of the dilp2>dSdc-IR flies ($p = 0.034$) (Figure 4).

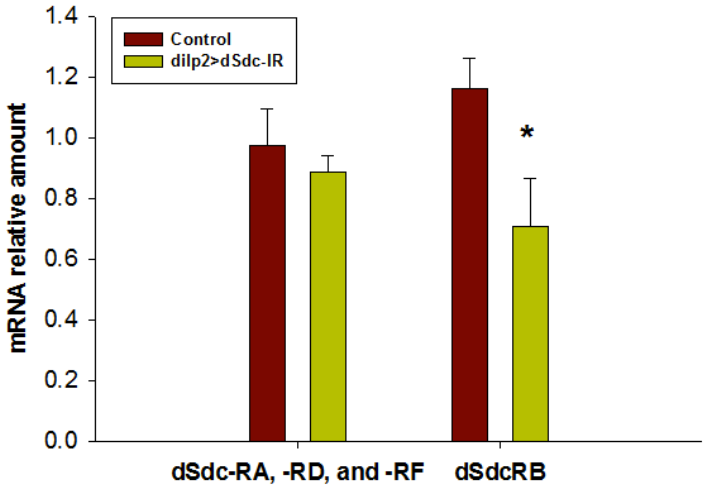

Figure 4: *dSdc* isoform transcript levels between genotypes in males.

Genetic expression of the dILPs produced in the IPCs was analyzed to observe any alterations in transcript due to *dSdc* reduction in these cells. While no significant changes were observed in *dilp5* mRNA levels between genotypes, the expression of *dilp2*

and *dilp3* was found to be increased by 27% and 13% respectively in the heads of dilp2>dSdc-IR compared to controls. (Table 1 and Figure 5). There was, however, a significant interaction between genotype and sex for both *dilp2* and *dilp3* transcript levels (Table 1), thus indicating that the increase in expression was driven by one of the sexes in both cases (*dilp2*: Female- dilp2>dSdc-IR = 1.024 $\pm$ 0.067, control = 1.020 $\pm$ 0.067, p = 1.000; Male- dilp2>dSdc-IR = 1.012 $\pm$ 0.067, control = 0.571 $\pm$ 0.077, p = 0.006; *dilp3*: Female- dilp2>dSdc-IR = 1.308 $\pm$ 0.057, control = 1.033 $\pm$ 0.049, p = 0.019; Male- dilp2>dSdc-IR = 1.328 $\pm$ 0.049, control = 1.297 $\pm$ 0.057, p = 0.974).

Despite the increase in expression, quantification of dILP2 protein in peripheral tissues conducted by Western blot revealed a significant 75% decrease in dilp2>dSdc-IR compared to controls (p = 0.046) (Figure 6).

Table 1. ANOVA results of *dilp*s mRNA levels for dilp2>dSdc-IR and control flies.

Gene	**Source**	**df**	**SS**	**F value**	***p* value**
dilp2	Genotype	1	0.18	10.2	0.009*
	Sex	1	0.20	10.9	0.007*
	Genotype*Sex	1	0.18	9.79	0.010*
dilp3	Genotype	1	0.08	8.23	0.017*
	Sex	1	0.07	7.08	0.024*
	Genotype*Sex	1	0.05	5.20	0.046*
dilp5	Genotype	1	0.01	0.09	0.769
	Sex	1	1.11	10.9	0.007*
	Genotype*Sex	1	0.01	0.09	0.769

*$p < 0.05$

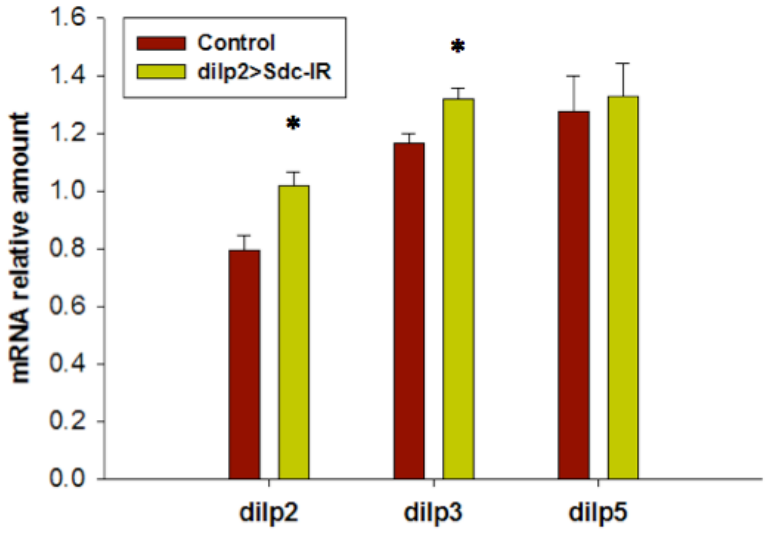

Figure 5. *dilp* transcript levels between genotypes.

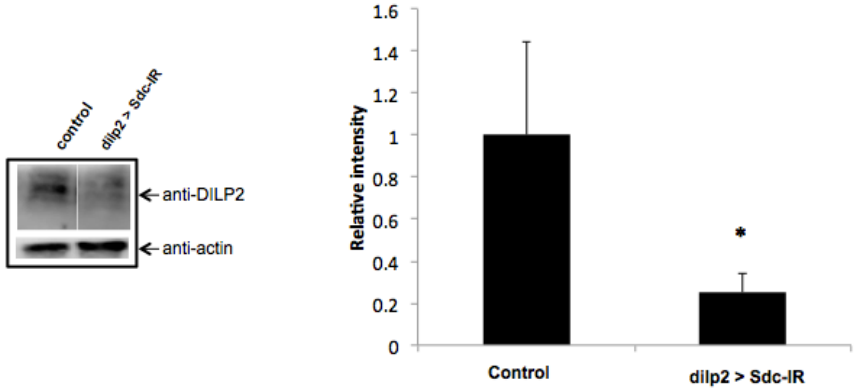

Figure 6. dILP2 protein measured in thoraces and abdomen samples.

Total Glycogen, TAG, and Protein Levels

Quantifying differences in energy reserves between the genotypes provides a context for potential mechanisms directed by alteration in dILP2 due to *dSdc* reduction in the IPCs. Total protein levels were significantly decreased by 9% in dilp2>dSdc-IR compared with controls (Table 2 and Figure 7).Total fat storage measured as TAGs in

whole body homogenate in dilp2>dSdc-IR was significantly increased by 10% compared with controls (Table 2 and Figure 8). Glycogen storage in dilp2>dSdc-IR flies was also significantly increased by 87% compared with control flies (Table 2 and Figure 9). There was, however, a significant interaction between genotype and sex (Table 2), indicating that the increase in glycogen was driven by the males (Female- dilp2>dSdc-IR = 1.024 $\pm$ 0.067, control = 1.020 $\pm$ 0.067, $p = 1.000$; Male- dilp2>dSdc-IR = 1.012 $\pm$ 0.067, control = 0.571 $\pm$ 0.077, $p = 0.006$).

Table 2. ANCOVA results of total protein, TAG, and Glycogen levels for dilp2>dSdc-IR and control flies.

Trait	Source	df	SS	F value	*p* value
Protein	Body weight	1	19.73	0.44	0.509
	Sex	1	855.61	19.07	<.0001*
	Genotype	1	439.48	9.80	0.003*
	Genotype*Sex	1	4.74	0.11	0.746
TAG	Body weight	1	0.00	0.00	0.978
	Protein	1	66.41	14.48	0.001*
	Sex	1	19.09	4.16	0.045*
	Genotype	1	20.23	4.41	0.039*
	Genotype*Sex	1	0.19	0.04	0.841
Glycogen	Body weight	1	80.83	0.38	0.540
	Protein	1	427.51	2.01	0.162
	Sex	1	30.66	0.14	0.706
	Genotype	1	1862.05	8.74	0.005*
	Genotype*Sex	1	5364.48	25.18	<.0001*

*$p < 0.05$

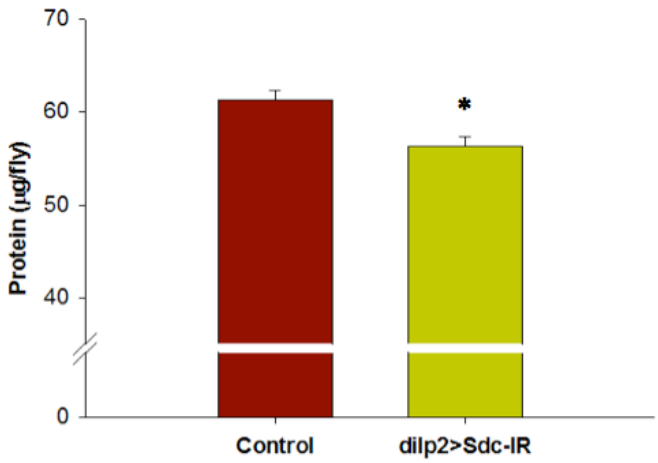

Figure 7. Total protein between genotypes.

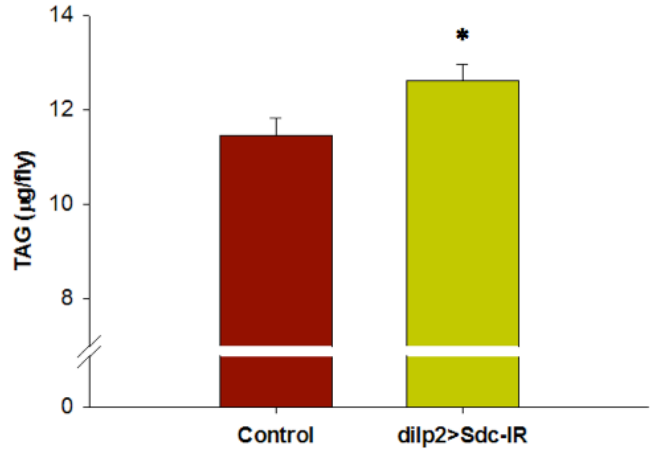

Figure 8. TAG storage between genotypes.

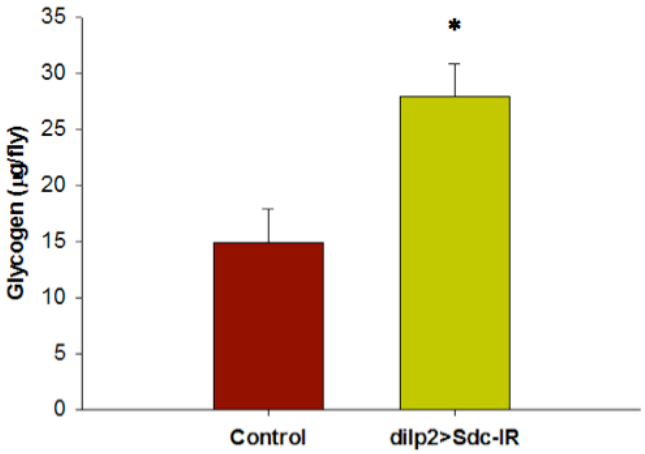

Figure 9. Glycogen levels between genotypes.

Glucose Tolerance Testing

To assess any differences in carbohydrate metabolism after feeding due to dILP deregulation, a glucose tolerance test was conducted in male flies (Figure 10). After an overnight fast, there was no difference in whole-body glucose levels between dilp2>dSdc-IR and control lines ($p = 0.426$). Immediately following 2 hrs of feeding on a glucose medium (PP0), the male dilp2>dSdc-IR flies displayed significantly elevated glucose ($p = 0.029$), as well as 2 hours after being placed back on the fasting medium (PP2) ($p = 0.002$). By 4 hrs post-prandial, differences in glucose between genotypes were no longer apparent ($p = 0.822$).

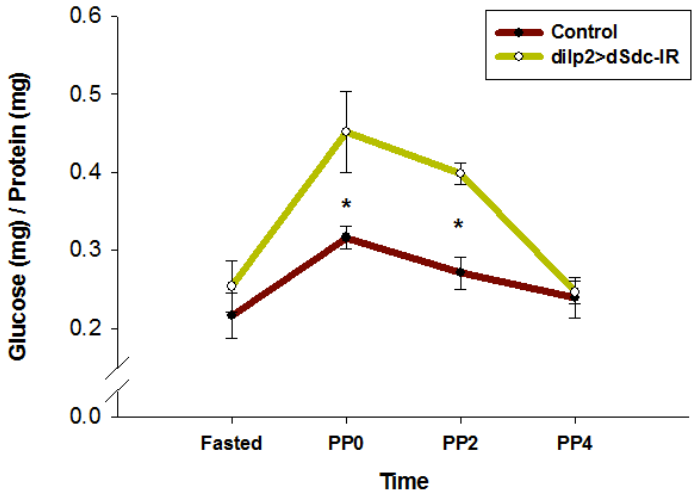

Figure 10. Glucose tolerance testing.

Energy Balance

To confirm the effects on energy storage were due to alterations driven by *dSdc* knockdown in the IPCs and not variations in energy balance, food intake and metabolic rate were measured by CAFÉ assay and flow through respirometry, respectively. No differences in food intake between lines were found during 12 hrs of light cycle (p = 0.120), 12 hrs of dark cycle (p = 0.323), or a calculated 24 hr average (p = 0.977) (Figure 11). Also, there were no differences in metabolic rate between genotypes (Table 3 and Figure 12).

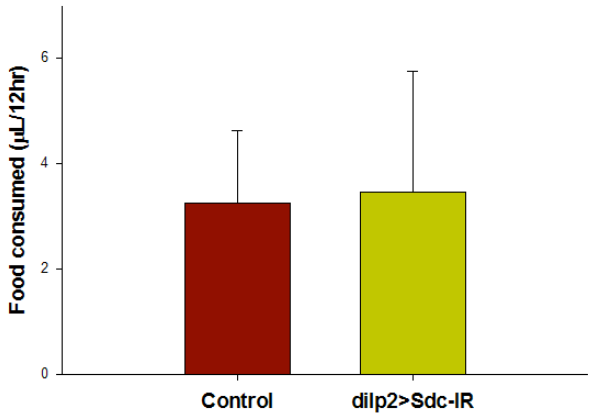

Figure 11. Food intake by CAFÉ method between genotypes.

Table 3. ANCOVA results of metabolic rate for dilp2>dSdc-IR and control flies.

Source	df	SS	F value	*p* value
Body weight	1	0.22	3.81	0.063
Time	2	0.36	3.05	0.067
Genotype	1	0.21	3.65	0.069
Sex	1	0.04	0.70	0.410
Time*Sex	2	0.16	1.41	0.265
Genotype*Sex	1	0.03	0.51	0.484
Genotype*Time	2	0.00	0.00	0.999
Genotype*Time*Sex	2	0.21	1.80	0.188

*$p < 0.05$

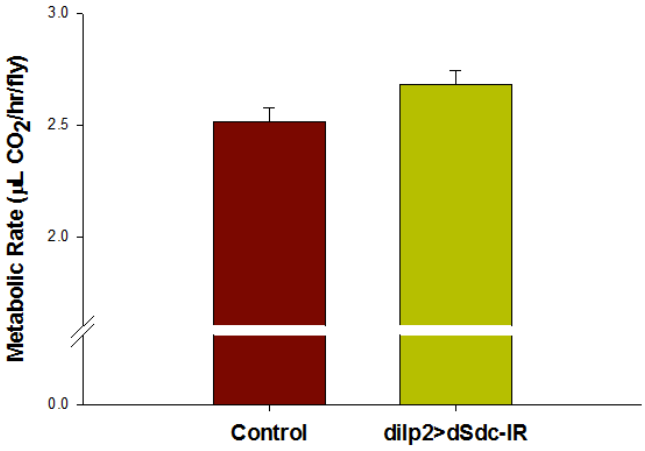

Figure 12. Metabolic rate as CO_2 production between genotypes.

Adult survival

Previous studies have reported that ablation of the IPCs in *Drosophila melanogaster* leads to increased longevity of adult flies due to a reduction in insulin signaling [12]. Under normal feeding conditions, significantly increased life span was observed in both male ($\chi^2 = 8.69$, $p = 0.003$) and female ($\chi^2 = 22.20$, $p < 0.0001$) dilp2>dSdc-IR flies compared with control flies (Figure 13).

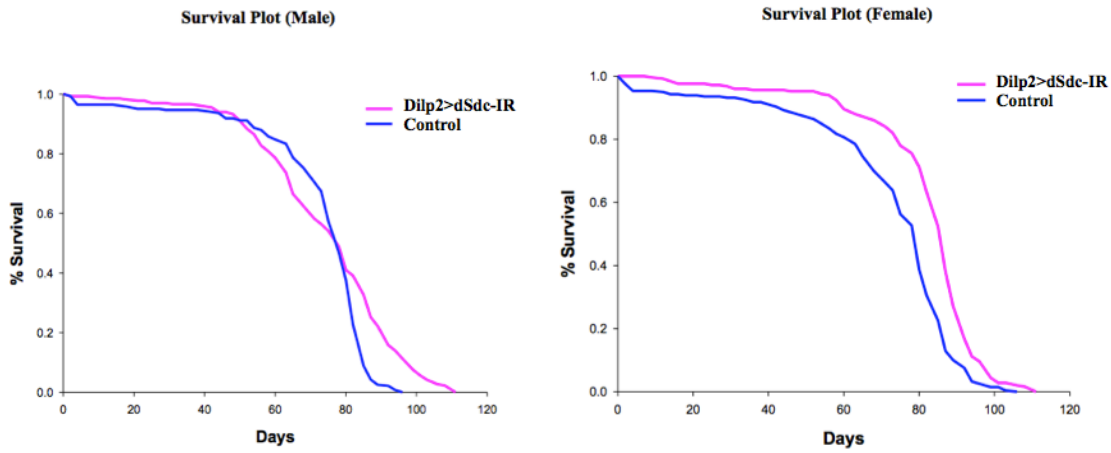

Figure 13. Adult lifespan.

Starvation resistance

Similarly to the increased longevity observed in flies with ablated IPCs, previous studies have shown a significant increase in starvation resistance likely due to increased stored energy reserves when IIS is decreased [12]. In the present study, significantly increased resistance to starvation conditions was observed in male dilp2>dSdc-IR flies compared with controls ($\chi^2 = 16.25$, $p < 0.0001$), but not in females ($\chi^2 = 0.003$, $p = 0.96$) (Figure 14).

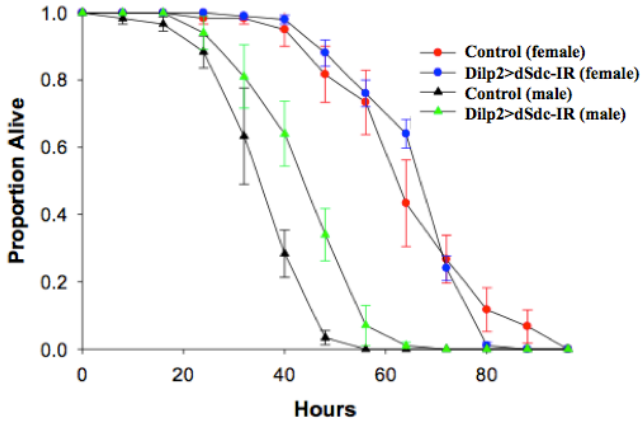

Figure 14. Starvation resistance.

Insulin Signaling

To determine whether the effects on energy storage, life span, and starvation resistance were due to a decrease in IIS, mRNA expression levels of *dIR* and *Thor* (downstream effectors of dFOXO) and protein quantification of phosphorylated protein kinase B (AKT), adenosine monophosphate-activated protein kinase (AMPK), and p70s6 kinase (p70s6k) were assessed. While there was no difference between genotypes found in *dIR* mRNA levels ($p = 0.995$), *Thor* expression was found to be significantly reduced by 30% in the dilp2>dSdc-IR line compared to control flies ($p = 0.043$) (Figure 15). No differences in phosphorylated AKT, AMPK, or p70s6k were observed between dilp2>dSdc-IR and control flies (Figure 15).

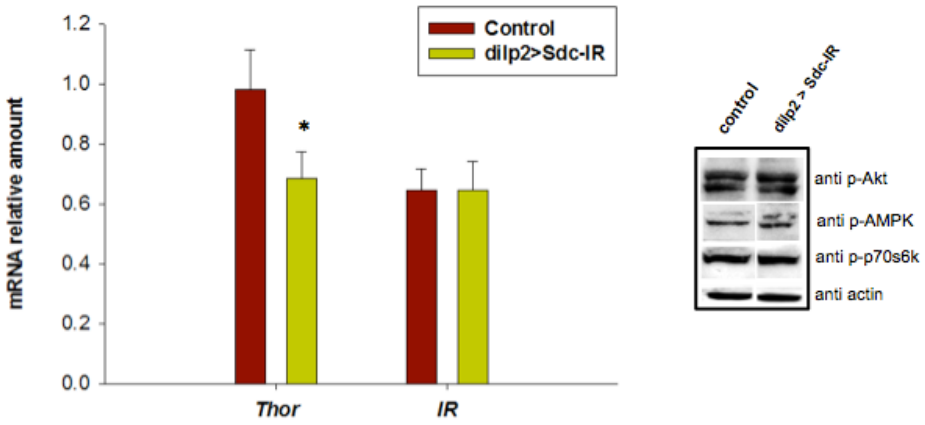

Figure 15. Insulin signaling analyses using qPCR for peripheral gene expression and Western blot for protein quantification.

DISCUSSION AND CONCLUSIONS

Previous work showed decreased levels of IPC-derived *dilp2-3 and 5* transcripts in flies with a hypomorphic mutation in the *dSdc* gene [26], suggesting that *dSdc* might play a cell autonomous role in the IPCs regulating *dilp* transcript and release. This idea was tested in the present study by generating an experimental line with *dSdc* knockdown specifically in the IPCs (dilp2>dSdc-IR).

Contrary to the results in the global *dSdc* mutant and the original hypothesis, this work showed that the expression of *dilp2* and *dilp3* was significantly increased in flies with *dSdc* reduced specifically in the IPCs compared to controls. However, the levels of dILP2 peptide in the periphery were found to be significantly decreased in thoraces and abdomen samples of dilp2>dSdc-IR. This pattern of increased *dilp* expression with decreased dILP secretion suggests that dILPs might accumulate in the IPCs as a result of alteration of a mechanism critical to the release of dILP. Indeed, this idea is further supported by the observation of decreased tolerance to a glucose meal under fasted conditions. This assay showed increased total glucose following a glucose meal that is consistent with decreased dILP2 secretion from the IPCs [12].

The mechanism by which syndecan is regulating *dilp* expression and dILP release cannot be determined from this study. However, there are a variety of nutrient and growth factor receptors located on the cell membranes of the IPCs that have been identified to independently regulate genetic expression and release of the IPC-derived dILPs. The role

of *dSdc* in the IPCs may be as a regulator of any of these receptors. For example, knockdown of *Drosophila* tachykinin-related peptide receptor (DTKR) in the IPCs has shown *dilp2* and *dilp3* transcript to be increased in the fed state, but not *dilp5* [35]. Similarly, another study reported that expression of a dominant negative form of the Drosophila epidermal growth factor receptor (dEGFR) led to significant reductions in *dilp2* and *dilp3* expression, but not *dilp5* [36]. To this end, syndecan could be a mediator of the binding capabilities of DTKR or dEGFR, thus influencing *dilp2* and *dilp3* transcript levels. Consistent with this hypothesis, preliminary data from the De Luca laboratory has shown expression of dEGFR to be increased two-fold in flies with *dSdc* reduced specifically in the fat body compared with corresponding control flies [personal communication].

Other potential signaling ligands through which syndecan could mediate dILP release are Upd2 and dILP6. As mentioned previously, knockdown of Upd2 has been shown to increase dILP2 accumulation in the IPCs [14]. Additionally, dILP6, synthesized in the fat body in response to nutrients, plays a role in regulating *dilp* expression and dILP peptide secretion in the IPCs [37].

Numerous studies across several species have shown an increase in life span when IIS is downregulated. In the present study, life span was significantly increased in experimental flies compared with controls, but no changes were observed in the IIS pathway. Though *Thor* expression was reduced, this is likely not related to alterations by insulin signaling since its transcriptional regulator dFOXO can be affected by a variety of other pathways in addition to IIS (e.g. environmental and oxidative stressors) [38]. Additionally, dILP2>dSdc-IR flies showed an increase in TAG and glycogen storage

despite no change in energy balance. These results closely mirror those seen in flies exhibiting ablation of the IPCs in adults [12, 39], strongly suggesting that the release of IPC-derived dILP peptides is indeed reduced. How the reduction of circulating dILP peptides affects life span and energy storage is still unknown and is an area of extensive investigation. Experimental male flies also displayed improved starvation resistance likely due to increased energy storage. The differences seen in life span and starvation resistance between the sexes may be driven in part by the reallocation of resources for reproductive processes [40].

Other considerations to be discussed are the role of the nutritional state of the fly and the age of flies during the experiments performed in this study. With the exception of glucose tolerance testing, all measures were taken from fed flies. Protein levels of dIR are reduced during fasting [41] and could have a considerable effect on the phenotypes observed in this study if altered. Differences in feeding behavior following a fast were also not tested here. Additionally, these phenotypes were measured during the early adult life stage of Drosophila. Profound effects of energy storage and IIS could become apparent between genotypes during life with a chronic reduction of dILP2 peptide.

Taken together, the results from the present study suggest that the role of syndecan in energy metabolism and life span is occurring directly at the level of the IPCs via decreased secretion of dILP2 (and presumably the other IPC-derived dILPs). The role of syndecan in the mammalian beta-cells remains largely unstudied. Staining for mammalian syndecan-4 and its extracellular heparan sulfate chains in pancreatic tissue from mouse, rat, and rabbit revealed syndecan-4 to be present preferentially on the basement membrane of the beta-cells compared with the alpha-cells [31]. Studies have

suggested that the basement membrane of the beta-cells is important for their optimal environment and plays a distinct role in its longevity and function [42]. The present study suggests a similar role for *dSdc* in the IPCs of Drosophila. Since preserving the function of the beta-cell is paramount for the prevention of T2DM, further studies investigating the mechanism behind the regulatory action of syndecan in *D. melanogaster* may assist in elucidating the role of mammalian syndecan in the longevity of the beta-cell.

REFERENCES

1. Centers for Disease Control and Prevention, *National Diabetes Statistics Report, 2014*. 2014: Atlanta, GA.

2. American Diabetes Association, *Economic costs of diabetes in the U.S. in 2012.* Diabetes Care, 2013. **36**(4): p. 1033-46.

3. Danaei, G., et al., *National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants.* Lancet, 2011. **378**(9785): p. 31-40.

4. Mathers, C. and D. Loncar, *Projections of global mortality and burden of disease from 2002 to 2030.* PLoS medicine, 2006. **3**(11).

5. Lebovitz, H., *Type 2 diabetes: an overview.* Clinical chemistry, 1999. **45**(8 Pt 2): p. 1339-1345.

6. Ashcroft, F. and P. Rorsman, *Diabetes mellitus and the β cell: the last ten years.* Cell, 2012. **148**(6): p. 1160-1171.

7. Boden, G., X. Chen, and J. Urbain, *Evidence for a circadian rhythm of insulin sensitivity in patients with NIDDM caused by cyclic changes in hepatic glucose production.* Diabetes, 1996. **45**(8): p. 1044-1050.

8. Yudkin, J.S., et al., *C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue?* Arterioscler Thromb Vasc Biol, 1999. **19**(4): p. 972-8.

9. Trinh, I. and G. Boulianne, *Modeling obesity and its associated disorders in Drosophila.* Physiology (Bethesda, Md.), 2013. **28**(2): p. 117-124.

10. Nässel, D.R., *Insulin-producing cells and their regulation in physiology and behavior of Drosophila.* Canadian Journal of Zoology, 2012. **90**(4): p. 476-488.

11. Nassel, D.R., et al., *Factors that regulate insulin producing cells and their output in Drosophila.* Front Physiol, 2013. **4**: p. 252.

12. Haselton, A. and Y.-W.C. Fridell, *Adult Drosophila melanogaster as a model for the study of glucose homeostasis.* Aging, 2010. **2**(8): p. 523-526.

13. Lee, G. and J.H. Park, *Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster.* Genetics, 2004. **167**(1): p. 311-23.

14. Rajan, A. and N. Perrimon, *Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion.* Cell, 2012. **151**(1): p. 123-137.

15. Rajan, A. and N. Perrimon, *Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion.* Cell, 2012. **151**(1): p. 123-37.

16. Fernandez-Almonacid, R. and O.M. Rosen, *Structure and ligand specificity of the Drosophila melanogaster insulin receptor.* Mol Cell Biol, 1987. **7**(8): p. 2718-27.

17. van Heemst, D., *Insulin, IGF-1 and longevity.* Aging Dis, 2010. **1**(2): p. 147-57.

18. Clancy, D.J., et al., *Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein.* Science, 2001. **292**(5514): p. 104-6.

19. Papadopoulou, D., M.W. Bianchi, and M. Bourouis, *Functional studies of shaggy/glycogen synthase kinase 3 phosphorylation sites in Drosophila melanogaster.* Mol Cell Biol, 2004. **24**(11): p. 4909-19.

20. Hwangbo, D.S., et al., *Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body.* Nature, 2004. **429**(6991): p. 562-6.

21. Teleman, A., *Molecular mechanisms of metabolic regulation by insulin in Drosophila.* The Biochemical journal, 2010. **425**(1): p. 13-26.

22. Gems, D. and L. Partridge, *Insulin/IGF signalling and ageing: seeing the bigger picture.* Curr Opin Genet Dev, 2001. **11**(3): p. 287-92.

23. Bharucha, K.N., *The epicurean fly: using Drosophila melanogaster to study metabolism.* Pediatr Res, 2009. **65**(2): p. 132-7.

24. Kristensen, C., et al., *A single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the insulin receptor.* Biochem J, 1995. **305 (Pt 3)**: p. 981-6.

25. Reyes-DelaTorre A, Peña-Rangel MT, and Riesgo-Escovar JR, *Carbohydrate Metabolism in Drosophila: Reliance on the Disaccharide Trehalose*, in

Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology Chuan-Fa Chang, Editor. 2012, InTech.

26. De Luca, M., et al., *A conserved role for syndecan family members in the regulation of whole-body energy metabolism.* PloS one, 2010. **5**(6).

27. Tkachenko, E., J. Rhodes, and M. Simons, *Syndecans: new kids on the signaling block.* Circulation research, 2005. **96**(5): p. 488-500.

28. Manon-Jensen, T., Y. Itoh, and J.R. Couchman, *Proteoglycans in health and disease: the multiple roles of syndecan shedding.* Febs j, 2010. **277**(19): p. 3876-89.

29. Ramani, V.C., et al., *Heparan sulfate chains of syndecan-1 regulate ectodomain shedding.* J Biol Chem, 2012. **287**(13): p. 9952-61.

30. Spring, J., et al., *Drosophila syndecan: conservation of a cell-surface heparan sulfate proteoglycan.* Proc Natl Acad Sci U S A, 1994. **91**(8): p. 3334-8.

31. Cheng, J.Y., J. Whitelock, and L. Poole-Warren, *Syndecan-4 is associated with beta-cells in the pancreas and the MIN6 beta-cell line.* Histochem Cell Biol, 2012. **138**(6): p. 933-44.

32. Lowry, O.H., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-75.

33. Ja, W.W., et al., *Prandiology of Drosophila and the CAFE assay.* Proc Natl Acad Sci U S A, 2007. **104**(20): p. 8253-6.

34. Van Voorhies, W.A., A.A. Khazaeli, and J.W. Curtsinger, *Testing the "rate of living" model: further evidence that longevity and metabolic rate are not inversely correlated in Drosophila melanogaster.* J Appl Physiol (1985), 2004. **97**(5): p. 1915-22.

35. Birse, R., et al., *Regulation of insulin-producing cells in the adult Drosophila brain via the tachykinin peptide receptor DTKR.* The Journal of experimental biology, 2011. **214**(Pt 24): p. 4201-4208.

36. Yu, Y., et al., *Neuronal Cbl controls biosynthesis of insulin-like peptides in Drosophila melanogaster.* Mol Cell Biol, 2012. **32**(18): p. 3610-23.

37. Bai, H., P. Kang, and M. Tatar, *Drosophila insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain.* Aging Cell, 2012. **11**(6): p. 978-85.

38. Tettweiler, G., et al., *Starvation and oxidative stress resistance in Drosophila are mediated through the eIF4E-binding protein, d4E-BP.* Genes Dev, 2005. **19**(16): p. 1840-3.

39. Broughton, S.J., et al., *Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 3105-10.

40. Bilde, T., et al., *Sex differences in the genetic architecture of lifespan in a seed beetle: extreme inbreeding extends male lifespan.* BMC Evol Biol, 2009. **9**: p. 33.

41. Britton, J.S., et al., *Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions.* Dev Cell, 2002. **2**(2): p. 239-49.

42. Otonkoski, T., et al., *Unique basement membrane structure of human pancreatic islets: implications for beta-cell growth and differentiation.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 119-27.