HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2017 August 12.

Published in final edited form as:

Biochem Biophys Res Commun. 2016 August 12; 477(1): 103–108. doi:10.1016/j.bbrc.2016.06.027.

Knockdown expression of *Syndecan* in the fat body impacts nutrient metabolism and the organismal response to environmental stresses in *Drosophila melanogaster*

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Abstract

The heparan sulfate proteoglycan syndecans are transmembrane proteins involved in multiple physiological processes, including cell-matrix adhesion and inflammation. Recent evidence from model systems and humans suggest that syndecans have a role in energy balance and nutrient metabolism regulation. However, much remains to be learned about the mechanisms through which syndecans influence these phenotypes. Previously, we reported that *D. melanogaster Syndecan* (*Sdc*) mutants had reduced metabolic activity compared to controls. Here, we knocked down endogenous *Sdc* expression in the fat body (the functional equivalent of mammalian adipose tissue and liver) to investigate whether the effects on metabolism originate from this tissue. We found that knocking down *Sdc* in the fat body leads to flies with higher levels of glycogen and fat and that survive longer during starvation, likely due to their extra energy reserves and an increase in gluconeogenesis. However, compared to control flies, they are also more sensitive to environmental stresses (e.g. bacterial infection and cold) and have reduced metabolic activity under normal feeding conditions. Under the same conditions, fat-body Sdc reduction enhances expression of genes involved in glyceroneogenesis and gluconeogenesis and induces a drastic decrease in phosphorylation levels of AKT and extracellular signal regulated kinase 1/2 (ERK1/2). Altogether, these findings strongly suggest that Drosophila fat body Sdc is involved in a mechanism that shifts resources to different physiological functions according to nutritional status.

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Keywords

Fat body; syndecans; metabolism; survival; AKT; ERK1/2

Introduction

Excess body fat leads to obesity which has become a serious and increasingly costly worldwide public health problem due to its increased prevalence in adults, children, and adolescents [1]. The health consequences of excess fat are due to a strong correlation between adiposity and the metabolic syndrome, which is a cluster of cardio-metabolic alterations that increases the risk of type 2 diabetes mellitus, cardiovascular disease, and several cancers [2]. In recent years, *D. melanogaster* has emerged as a powerful model for studying human obesity and metabolic regulation [3]. To this end, earlier quantitative genetic studies performed in our laboratory pinpointed *Sdc* as a positional candidate gene affecting inter-individual differences in Drosophila fat storage [4]. Syndecans are type I transmembrane proteins that belong to the family of heparan sulfate proteoglycans and together with integrins are the major ECM adhesion receptors [5]. While the number of syndecan proteins varies across species (invertebrates have one, while humans have four encoded by four different genes, *SDC1-4*) their general structure appears to be conserved [5]. All syndecans are characterized by a core protein with extracellular attachment sites for glycosaminoglycan chains that mediate interactions with a wide array of ligands. Syndecans also contain a highly conserved transmembrane domain and a short cytoplasmic tail that binds to cytoskeleton proteins. This allows them to function independently and in synergy with integrin-mediated signaling to control cell adhesion, behavior, and fate [5, 6].

Our previous work showed that young flies homozygous for a hypomorphic *Sdc* mutation had lower levels of energy reserves, displayed lower metabolic activity, and reduced survival [4]. Human genetic studies performed in parallel also revealed that genetic polymorphisms in the *SDC4* gene were associated with adiposity, fasting plasma glucose, and resting energy expenditure in American children [4]. Studies in mice have also reported a role for members of the syndecan family in adipocyte differentiation and energy balance [7, 8]. However, little is still known about the mechanisms through which syndecans regulate these metabolic phenotypes. Here, we used RNA interference (RNAi) through a UAS-driven transgene (UAS-IR) to down-regulate *Sdc* expression in the Drosophila fat body. The insect fat body plays a crucial role in nutrient metabolism [9]. Additionally, nutrient-sensing signaling pathways that have been associated with survival are highly active in this tissue [9]. We, therefore, reasoned that by reducing the expression of *Sdc* specifically in the fat body we could gain insightful information into its role in body composition and metabolism regulation.

Materials and methods

Fly stocks and husbandry

We used the GAL4/UAS approach [10] to knock-down (KD) the expression of *Sdc* in the fat body using a *Larval serum protein 2* (*Lsp2*)-*Gal4* driver [11, 12]. We chose this driver

because the *Lsp2* gene, although highly expressed in third instar larvae and only moderately in adult flies, is a specific marker of the fat body [11, 12]. The *UAS-Sdc-IR* (Stock ID: v13322) and w^{1118} (Stock ID: 5905) lines were obtained from the Vienna Stock Center and the Bloomington Stock Center, respectively. We crossed *Lsp2-Gal4* male and *UAS-Sdc-IR* or w^{1118} female flies to generate the w^{1118}*; Lsp2-Gal4/+; UAS- Sdc-IR/+* (fat body *Sdc* KD, referred hereafter as lsp2>Sdc-IR) and w^{1118}*; Lsp2-Gal4/+; +/+* (control) genotypes. Four to six day old virgin flies of each genotype were assessed for the phenotypic assays described below. The w^{1118} genotype used in this study is the one in which the transgenic *UAS-Sdc-IR* stock was originally constructed (http://flystocks.bio.indiana.edu/Browse/VDRCtb.htm), thereby reducing differences in genetic background between lsp2>Sdc-IR and control flies that could affect the phenotypes. Virgin flies were used to minimize the influence of reproduction on energy metabolism. In our previous work we detected female-specific effects of the *Sdc* mutation on energy storage [4]; thus, only female flies were used in the present study. All flies were raised at 25°C on a standard cornmeal, molasses, and yeast medium.

Metabolic assays

TAG, glycogen, glucose, and protein levels were measured using protocols described in [13]. Free glucose concentration was subtracted from the total amount of glucose present in the sample after treatment with the enzyme amyloglucosidase in order to determine glycogen amounts. Trehalose was measured in hemolymph samples using the method described in [14].

Resting metabolic rate was measured as CO_2 production using a flow-through respirometry system (Qubit System Research, Kingston, Ontario, Canada) and the method described in [13].

Feeding assay

Food intake was assessed using the Capillary Feeder (CAFÉ) assay described in [15] with slight modifications. Groups of five flies were housed in vials containing 1% agar medium and a microcapillary tube filled with liquid food media made up of 5% glucose and 5% autolyzed yeast. Flies were first allowed to acclimate to the capillary system overnight. Food consumption was then quantified for the 12 hour day and night cycles as the change in height of the liquid in the microcapillary tube. Control vials containing no flies were included to estimate the amount of water in the food that evaporated.

Locomotor activity

Locomotor activity was measured with the Drosophila Activity Monitoring System (Trikinetics, Waltham, MA), which counts the number of times a given fly crosses an infrared beam during a specified time interval [4].

Starvation survival assay

Survival under starvation conditions was measured by placing 10 flies per genotype on 1.5% agarose medium and recording the number of flies alive at 8-h intervals until all were dead.

Immune response

Female flies were assayed for their ability to clear infection by *Escherichia coli* (HB101, a streptomycin resistant strain) using methods described in [16].

Chill-coma recovery

We used a chill-coma recovery assay to test cold tolerance. Groups of 25 females for each genotype were subjected to 0°C for 3 hours. Flies were then returned to room temperature and the time to restore locomotor function was measured.

Western Blot Analyses

Flies were dissected into phosphate-buffered saline and abdomens were homogenized in standard lysis buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Thermo Scientific, Rockford, USA), blocked in 5% BSA, and incubated with 1:1000 rabbit β-Actin (Cell Signaling # 4967); 1:1000 rabbit anti-Phospho-Drosophila Akt (Ser505) (Cell Signaling #4054) and rabbit anti-Akt (Cell Signaling #9272); 1:1000 rabbit anti-Phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling #9211) and rabbit anti-p38 MAPK (Cell Signaling #9212); 1:1000 rabbit anti-Phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling #4377) and rabbit anti-p44/42 MAPK (Cell Signaling #4695); and 1:1000 rabbit anti-Phospho-SAPK/JNK(Thr183/Tyr185) (Cell Signaling #4668) and rabbit anti-JNK (Santa Cruz Biotechnology, Inc. #sc-571). The primary antibodies were incubated overnight at 4°C. The secondary antibody was incubated with 1:5000 HRP-conjugated goat anti-rabbit or goat anti-mouse for 1 hour at room temperature and the signal developed using an ECL detection kit (Merk Millipore).

Quantitative (q)PCR analysis

qPCRs were performed using the protocol described in [4]. Primers used in the study are listed in Supplementary Table 1.

Statistical analyses

Cox regression as implemented by SAS (V9.1.3) was used to compare survival of lsp2 > Sdc-IR and control flies under starvation conditions. One-way analysis of variance and analysis of covariance (with body weight used as covariate) were used to assess differences between experimental and control groups in locomotor activity and metabolic assays, respectively. Data from the other experimental assays were analyzed by two-tailed Student's *t* test.

Results and Discussion

In the first series of experiments we verified that Sdc is localized to the plasma membrane of fat body cells in adult flies (Supplementary Fig. 1A). In contrast, no visible evidence of Sdc in the fat body of lsp2>Sdc-IR flies was observed by the immunohistochemical stain (Supplementary Fig. 1A). This result was corroborated by qPCR analysis, which showed a 60% reduction in *Sdc* expression in abdomens of lsp2>Sdc-IR flies compared to controls

(Supplementary Fig. 1B). Taken together, these results established that the RNAi approach produced the expected KD of *Sdc* in the adult fat body.

Next, we examined the effect of reducing Sdc in the fat body on energy stores. Contrary to observations in the *Sdc* mutant [4], fat-body specific KD of *Sdc* increased triacylglycerol (TAG) and glycogen contents by 27% and 37%, respectively (Fig. 1A). No difference in the levels of trehalose, the main circulating sugar, was observed (Fig. 1B). Because changes in energy balance can affect fat storage in flies [17], we then asked whether the increase in TAG content in lsp2>Sdc-IR flies might be due to an energy imbalance. There was no significant difference in locomotor activity between lsp2>Sdc-IR and control flies (Fig. 1C), but lsp2>Sdc-IR flies displayed a 16% reduction in metabolic rate (Fig. 1D), which could explain their higher TAG content. Interestingly, lsp2>Sdc-IR flies also ingested ~ 15% less food over a 24-hour window (Fig. 1E). To confirm these findings, we investigated two additional GAL4-driver lines - the Yolk-Gal4 and the drug (RU486) inducible S106-GS-Gal4 - that allow the expression of *Gal4* specifically in adult fat body. In both cases, as seen in lsp2>Sdc-IR flies, KD of *Sdc* expression led to increased metabolite storage (Supplementary Fig. 2A and 2B) and decreased resting metabolic rate (Supplementary Fig. 2C). Similarly, KD of *Sdc* by S106-GS-Gal4 in the presence of RU486 significantly reduced 24-hour food consumption (Supplementary Fig. 2D). Flies carrying Yolk-Gal4 and UAS-Sdc-IR did not display any difference in the amount of food consumed compared to controls (Supplementary Fig. 2D). However, given that Sdc is found in most tissues [18] and the Yolk-Gal4 line does not drive the expression of *Gal4* exclusively in the fat body [14], it is possible that this latter finding could be a compensatory effect generated from another tissue.

Next, we sought to determine whether the changes in metabolism observed in lsp2>Sdc-IR flies would alter their ability to respond to stress challenges, such as nutrient starvation, bacterial infection, and cold exposure. We observed that starved lsp2>Sdc-IR flies lived longer than controls (Fig. 2A), but they had significantly reduced immune function, with 34% higher bacterial loads than controls 24 hours after infection (Fig. 2B). Additionally, it took lsp2>Sdc-IR flies longer (on average four minutes) to recover from cold-induced paralysis (Fig. 2C) than control flies.

To verify whether starvation resistance resulted from utilization of the higher energy reserves, we measured glycogen levels in flies fasted for 24 and 30 hours. To maintain glucose homeostasis during starvation, wild-type flies use their stored glycogen after a 24 hour fast [19]. Consistent with this previous work, glycogen levels dropped significantly in 24-hour fasted lsp2 > Sdc-IR and control flies (Fig. 2D). However, while glycogen levels of control flies were almost depleted after a 30-hour fast, those of lsp2>Sdc-IR flies were 58% higher in comparison with the 24 hour fast (Fig. 2D). This suggests that the reduction of Sdc in the fat body enhances the gluconeogenic fasting response and promotes glucose storage to glycogen. Concurrently, 30-hour fasted lsp2 >Sdc-IR flies had significantly increased (54%) glucose levels than controls (Fig. 2E). The rate of gluconeogenesis is controlled by the enzyme phosphoenol-pyruvate carboxykinase (PEPCK), which is transcriptionally regulated by a number of physiological signals and plays a key role also in the glyceroneogenesis pathway [20]. *D. melanogaster* contains two genes encoding PEPCK, *CG10924* and *Pepck*

[21], thus we evaluated the expression of these two genes after a 30 hour fast. We found that the expression of *CG10924* and *Pepck* were significantly increased and decreased, respectively, in lsp2 > Sdc-IR flies compared to controls (Fig. 2F). Since *Pepck* has a more prominent role in Drosophila glycerol and triglyceride homeostasis than *CG10924* [21], these results corroborate the idea that lsp2 >Sdc-IR flies display enhanced gluconeogenesis under fasting conditions. Additionally, they suggest that KD of Sdc in the fat body might cause an attenuation of glyceroneogenesis to limit free-fatty acids re-esterification, which is a highly ATP-consuming process [22].

To gain insight into how KD of *Sdc* in the fat body affects nutrient metabolism and the response to environmental stresses under fed conditions, we analyzed the transcriptome profile of abdomens (where the fat body is more conspicuous) of lsp2 > Sdc-IR and control flies by RNA-sequencing (Supplementary Table 2) (GEO accession number: GSE67001). We then selected 22 transcripts among those with fold changes greater than 1.3 between genotypes (at an uncorrected *P*-value of <0.05) for validation by qPCR using independent mRNA samples. We chose transcripts expressed in the fat body with known biological functions relevant to the traits affected by fat body-specific Sdc reduction. For 11 of the 22 genes, we confirmed significant differences in expression levels between lsp2>Sdc-IR and control flies (Fig. 3). Genes significantly up-regulated in lsp2>Sdc-IR flies include those involved in glycerol and fat metabolism (*Pepck, Glycerol kinase-Gyk*, *Microsomal triacylglycerol transfer protein-Mtp,* and *Adipokinetic hormone receptor-AkhR*), gluconeogenesis and glycogen metabolic process (*CG10924* and *Glycogen binding subunit 70E-Gbs-70E*), response to oxidative stress (*Ecdysone-induced protein 28/29kD-Eip71CD* and *Glutathione S transferase E1-GstE1*), positive regulation of circadian sleep/wake and sleep (*Angiotensin-converting enzyme related-Acer*), and integrin-mediated cell adhesion (*Tiggrin-Tig*) [23]. qPCR analysis did not detect differential expression of NF-κB pathway controlled antimicrobial genes between lsp2 > Sdc-IR and control flies (Fig. 3). But, the expression of the gene *Listericin*, which encodes a protein that suppresses the growth of Gram-negative bacteria [24], was significantly reduced in lsp2 > Sdc-IR flies (Fig. 3) and so may contribute to the reduced bacterial clearance ability of these flies.

The transcriptional up-regulation of genes involved in gluconeogenesis under fed conditions raised the possibility that fat body-specific KD of *Sdc* might affect PI3K/AKT signaling. Thus, we assessed phosphorylation/activation of AKT (Ser505) in fly abdomens. As predicted, AKT phosphorylation levels were decreased by 43% in lsp2>Sdc-IR flies compared to control flies (Fig. 4). This finding echoes work in mammals showing that syndecan-4 activates the phosphorylation of AKT at Ser473 by regulating the assembly and plasma membrane recruitment of the mammalian Target of Rapamycin Complex 2 in endothelial cells [25].

The mammalian “stress regulated” mitogen-activated protein kinase (MAPK) cascades, which are activated by a variety of environmental stresses, interact at different levels with the PI3K/AKT pathway to control cell survival and proliferation [26]. Thus, we further investigated whether the phosphorylation status of p42/p44 MAPK, p38 MAPK, and JNK was altered in lsp2>Sdc-IR flies compared to controls. Whereas no change in phospho-p38 MAPK levels were observed between genotypes (Fig. 4), we detected a significant increase

(35%) in the phosphorylation of JNK (Fig. 4). Additionally, we found a dramatic decrease (75%) in the phosphorylation of p42/p44 MAPK (Fig. 4), which is consistent with the cross-inhibition between Ras-ERK and PI3-AKT pathways seen in mammals [27].

Altogether, our results indicate that reduced levels of Sdc in the fat body promotes an energy-saving mechanism for survival during periods of reduced food availability. We propose that this mechanism allows fat-body *Sdc* KD flies to allocate resources to storage and maintain circulating sugar (trehalose) homeostasis when food is available. This, in turn, triggers metabolic adaptations that lead to reduced metabolic activity, food intake, and ability to respond to other energy-demanding activities, such as immune function and cold tolerance. At the molecular level, this notion is supported by our finding of decreased phosphorylation of AKT and up-regulation of genes involved in glyceroneogenesis (*Gyk* and *Pepck),* gluconeogenesis (*CG10924*), and glycogen synthesis (*Gbs-70E*) in lsp2>Sdc-IR flies compared to controls under fed conditions. Of note, *Gbs-70E* encodes a protein similar to mammalian protein targeting to glycogen (PTG) whose overexpression in mouse liver has been recently reported to increase liver glycogen and reduce food intake [28]. Furthermore, we observed that fed lsp2>Sdc-IR flies had higher transcript levels of *Eip71CD* (encoding the Drosophila homolog of Methionine Sulfoxide Reductase A) than controls. Earlier work identified *Eip71CD* as a cold-responsive gene in two *D. virilis* groups [29], with expression levels of *Eip71CD* being consistently lower during acclimation to low temperature. Given that overexpression of *Eip71CD* also confers resistance to oxidative stress [30], it is possible that its higher expression levels in lsp2>Sdc-IR flies may be the result of an increase in oxidative stress in these flies. The trade-off, however, is an enhanced sensitivity to cold. The JNK signaling has been reported to trigger a stress-activated pathway that promotes Drosophila tolerance to oxidative stress and starvation [31]. Thus, the increase in JNK phosphorylation in lsp2>Sdc-IR flies further supports this view. These findings significantly increase our understanding of the role of syndecans in nutrient metabolism regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Michael Crowley and David K. Crossman at the UAB Heflin Center for Genomic Sciences for performing RNA-sequencing analysis. This work was supported by the National Institutes of Health (R01 DK084219) and UAB Diabetes Research Center Pilot & Feasibility Program (P30 DK079626) grants to MD.

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Highlights

- RNAi-mediated knockdown of Drosophila *Syndecan* in the fat body increases nutrient stores and reduces metabolic rate.
- Flies with decreased *Syndecan* expression in the fat body are more resistant to nutrient deprivation but show more sensitivity to bacterial infection and cold.
- Fat body specific-knockdown of *Syndecan* enhances the expression of genes related to glyceroneogenesis and glucose metabolism.
- Fat body specific-knockdown of *Syndecan* reduces the phosphorylation of AKT and ERK1/2.

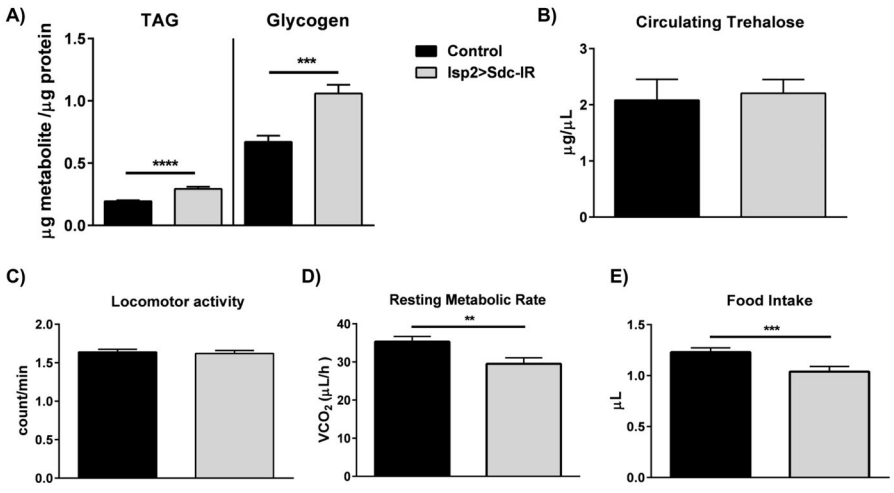

Figure 1. Fat body-specific *Sdc* KD alters energy balance and nutrient stores in adult flies

(A–B) Values represent the mean of TAG and glycogen stores (panel A) and circulating trehalose levels (panel B) in fat body *Sdc* KD flies (lsp2>Sdc-IR) and control flies from 10 independent biological replicates. TAG and glycogen measurements were normalized to total protein levels. (C) Values represent the mean number of locomotor activity counts per waking minute of two independent replicates of 16 flies. (D) Values represent the least-square means of whole-body CO_2 production, an index of resting metabolic rate, adjusted for live body weight (n = 10 independent replicates). (E) Values represent the average amount of food ingested by five housed flies from 10 independent replicates. In all panels, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ compared to control. Error bars represent SEM.

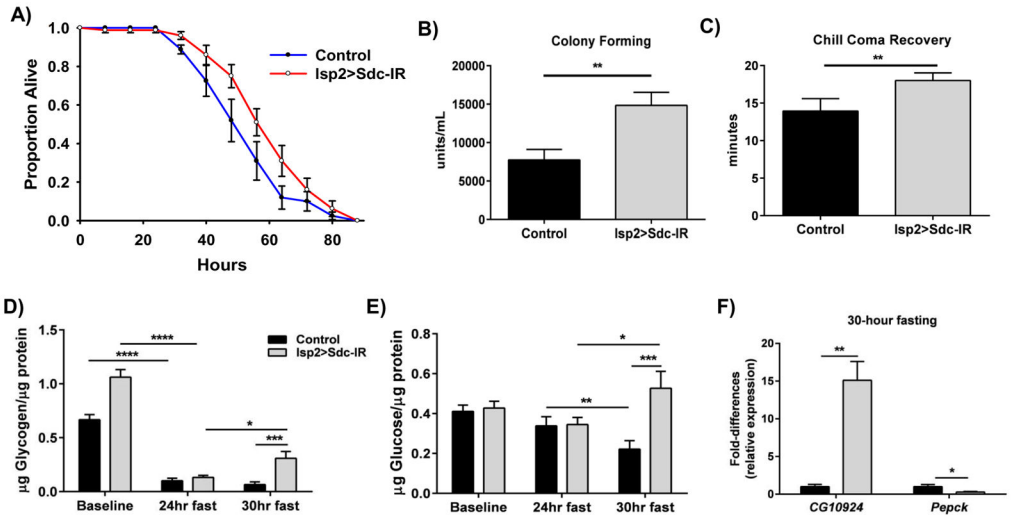

Figure 2. Fat body-specific *Sdc* KD promotes resistance to starvation, but increases sensitivity to microbial infection and low temperature

(A) Survival curves under water-only starvation conditions. The average survival time was significantly longer in fat body *Sdc* KD flies (lsp2>Sdc-IR) compared to controls (log-rank test, chi square statistic = 120.7, $P < 0.0001$). (B) Values represent the mean number of colonies grown in culture 24 hours after infecting flies with *E. coli*. A lower colony count indicates a better immune response. $n = 33–34$ independent biological replicates. (C) Values represent the average chill-coma recovery time in minutes of five independent replicates. (D–E) Values represent the mean of glycogen (panel D) and glucose (panel E) levels at baseline and after 24 and 30 hours of fasting from 10 independent replicates. Measurements were normalized to total protein levels. (F) Values represent the mean of the relative (fold change) gene expression from six independent replicates. The mRNA was isolated from abdomens of flies fasted for 30 hours. Transcript levels of each target gene were normalized to *rp49*. In all panels,* indicates $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ compared to control. Error bars represent SEM.

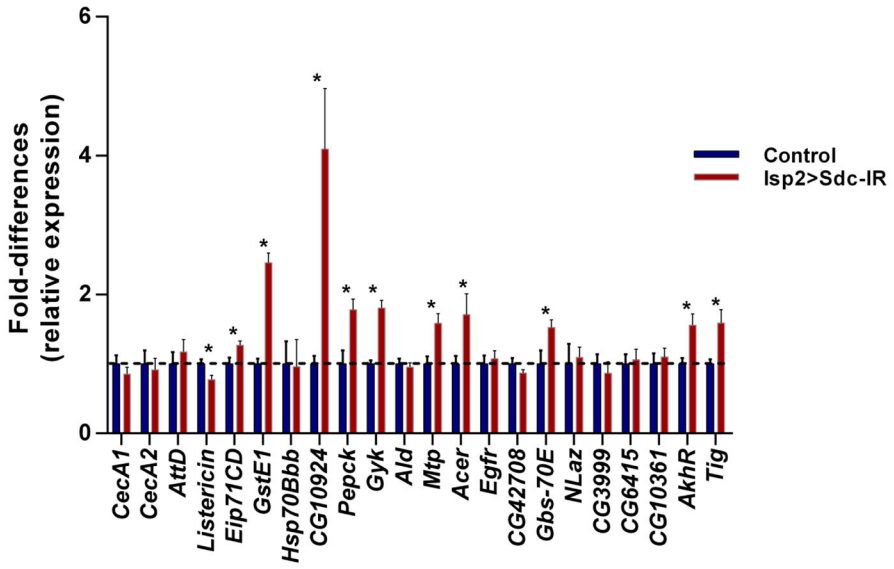

Figure 3. Fat body-specific *Sdc* KD induces changes in the expression of genes involved in lipid and glucose metabolism under fed conditions

Genes differentially expressed between fat body *Sdc* KD flies (lsp2>Sdc-IR) and control flies. Values represent the mean of the relative (fold change) gene expression from six independent replicates. Gene expression levels were measured by qPCR using mRNA isolated from abdomens. Transcript levels of each target gene were normalized to *rp49*. In all panels, * indicates $p \leq 0.05$ compared to control. Error bars represent SEM.

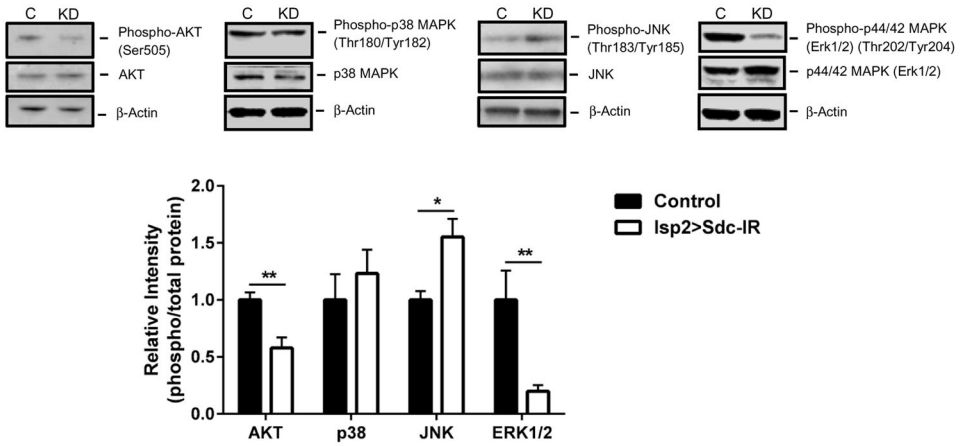

Figure 4. Fat body-specific *Sdc* KD reduces phosphorylation of AKT and ERK1/2, and increases phosphorylation of JNK under fed conditions

Upper level, representative images of western blots in Control (C) and Lsp2>Sdc-IR (KD) flies. Lower level, quantification of western blot results from four to six independent experiments. β-actin was used as loading control in all western blot experiments. *indicates $p \leq 0.05$ and ** $p \leq 0.01$ compared to control. Error bars represent SEM.