Original Article

MOLECULAR METABOLISM

An *in vivo* screen for neuronal genes involved in obesity identifies *Diacylglycerol kinase* as a regulator of insulin secretion

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ABSTRACT

Objective: Obesity is a complex disorder involving many genetic and environmental factors that are required to maintain energy homeostasis. While studies in human populations have led to significant progress in the generation of an obesity gene map and broadened our understanding of the genetic basis of common obesity, there is still a large portion of heritability and etiology that remains unknown. Here, we have used the genetically tractable fruit fly, *Drosophila melanogaster*, to identify genes/pathways that function in the nervous system to regulate energy balance.
Methods: We performed an *in vivo* RNAi screen in *Drosophila* neurons and assayed for obese or lean phenotypes by measuring changes in levels of stored fats (in the form of triacylglycerides or TAG). Three rounds of screening were performed to verify the reproducibility and specificity of the adiposity phenotypes. Genes that produced >25% increase in TAG (206 in total) underwent a second round of screening to verify their effect on TAG levels by retesting the same RNAi line to validate the phenotype. All remaining hits were screened a third time by testing the TAG levels of additional RNAi lines against the genes of interest to rule out any off-target effects.
Results: We identified 24 genes including 20 genes that have not been previously associated with energy homeostasis. One identified hit, *Diacylglycerol kinase (Dgk)*, has mammalian homologues that have been implicated in genome-wide association studies for metabolic defects. Downregulation of neuronal Dgk levels increases TAG and carbohydrate levels and these phenotypes can be recapitulated by reducing Dgk levels specifically within the insulin-producing cells that secrete *Drosophila* insulin-like peptides (dILPs). Conversely, overexpression of kinase-dead Dgk, but not wild-type, decreased circulating dILP2 and dILP5 levels resulting in lower insulin signalling activity. Despite having higher circulating dILP levels, *Dgk* RNAi flies have decreased pathway activity suggesting that they are insulin-resistant.
Conclusion: Altogether, we have identified several genes that act within the CNS to regulate energy homeostasis. One of these, Dgk, acts within the insulin-producing cells to regulate the secretion of dILPs and energy homeostasis in *Drosophila*.
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Keywords *Drosophila*; Obesity; Metabolism; CNS; Diacylglycerol kinase; Insulin secretion

1. INTRODUCTION

Obesity is the result of a disturbance to energy homeostasis, which is maintained by the central nervous system (CNS). Hormone, nutrient, and satiety signals generated by peripheral metabolic tissues convey the body's energy status to key brain areas. This information is processed to produce the appropriate autonomic, endocrine, and behavioral outputs both for long-term (body weight maintenance) and short-term (meal initiation and satiation) energy balance. Consistent with its pivotal role, about 25% of all susceptibility genes in the last Human Obesity Gene Map and nearly all genes implicated in monogenic obesity are expressed in the brain [1]. Furthermore, several genome-wide association studies in humans have also implicated single-nucleotide polymorphisms in several neuronal genes with predisposition to high BMI [2–4]. Therefore, the CNS is central to energy balance and further insights into its role could be valuable in understanding the pathological mechanisms and genetic susceptibilities underlying obesity and related metabolic disorders.
Family and twin studies have estimated the heritability of obesity to be between 40 and 70% [5–7]. However, all known risk variants can only account for ~3% of the variance in BMI [8,9]. Therefore, alternate methods are needed to identify additional risk factors. In recent decades, *Drosophila* have been increasingly used as a model for metabolic disorders and the ability to perform large-scale genetic screens in flies has accelerated the identification of potential susceptibility genes. The ability to perform these screens *in vivo* is a requirement to study

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Abbreviations: Dgk, *Diacylglycerol kinase*; dILPs, insulin-like peptides; TAGs, triacylglycerides

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physiological processes at the whole-organism level, such as energy homeostasis, which involve cross-talk between multiple tissues. *Drosophila* also possess many of the same basic metabolic functions as mammals including the ability to maintain carbohydrate homeostasis, storing and mobilizing energy stores, and modulating food intake in response to nutritional cues [10—14]. In addition, many of the molecular mechanisms that regulate these metabolic processes are largely conserved including the insulin, target of rapamycin, and leptin pathways [14—16].

We performed a large-scale *in vivo* genetic screen in neurons and assayed for alterations to the levels of stored lipids. After three rounds of screening to verify the reproducibility and specificity of the adiposity phenotypes, we identified 24 genes. These include genes that have been previously associated with energy homeostasis in flies including regulation of carbohydrate and lipid metabolism and starvation response as well as genes not previously linked with energy balance. One of the genes identified was *Diacylglycerol kinase* whose homologues have been associated with obesity in human populations and have been shown to regulate insulin secretion *in vitro*. We performed the first functional study of Dgk in flies and showed that it is involved in lipid and carbohydrate metabolism. Further, we show that Dgk is likely functioning within the insulin-producing cells to regulate dILP secretion and impacting systemic insulin pathway activity.

2. MATERIALS AND METHODS

2.1. Fly stocks and husbandry

The RNAi lines used in the first and second round of screening were generated by the Transgenic RNAi Project (TRiP) VALIUM10 library [17]. The RNAi lines used in the third round came from the TRiP VALIUM1 and VALIUM20 libraries and the Vienna *Drosophila* RNAi Center GD and KK libraries. Other lines used include: *fru-Gal4* from B. Dickson [18] and *dIlp2-Gal4* from E. Rulifson [19].

2.2. Generation of UAS-Dgk transgenics

Total RNA from w^{1118} flies was extracted using the High Pure RNA Isolation Kit (Roche). 5 µg of RNA was used to generate cDNA using Superscript First-Strand Synthesis System (Invitrogen). The Dgk-RF transcript was amplified using the primers: *Dgk.For* 5′-AGA AAC GGT CTT GAG TTC ATC AGT A-3′ and *Dgk.Rev* 5′-ATA CTC GTA CTT AGC CTA GGG CAT AAA A-3'.

The PCR product was run on a 0.8% agarose gel and the band was purified using the PureLink Quick Gel Extraction Kit (Invitrogen). Dgk-RF was then inserted into the pAc5.1/V5-His A vector (Invitrogen) in order to V5-tag the protein. EcoRI and XbaI restriction sites were added to flank the Dgk coding region by PCR using the primers: *EcoRI-Dgk.For* 5′-CCG GAA TTC CAC CAT GAA TAT TGG CAT CGC AGC-3′ (also adds Kozak sequence before ATG start codon) and *Dgk-XbaI.Rev* 5′-CCG GAA TTC CAC CAT GAA TAT TGG CAT CGC AGC-3′. The PCR product and vector were digested with EcoRI and XbaI (Thermo Scientific). The reactions were gel purified and ligated using T4 DNA ligase (Thermo Scientific). The resulting construct was designated pAc5.1-DgkRF.V5.

Next, the Dgk construct was inserted into the pUASTattB vector provided by K. Basler [20] to add a UAS sequence that permits Gal4 control of expression of the transgene and an attB sequence that allows for PhiC31-mediated transgenesis. A stop codon and NotI restriction site was added to the 3′ end of the V5 epitope in pAc5.1-DgkRF.V5 using the primers *EcoRI-Dgk.For* and *V5-NotI.Rev* 5′- ATA GTT TAG CGG CCG CTT ACG TAG AAT CGA GAC CGA GGA-3′. The PCR product and pUASTattB vector were digested with EcoRI and NotI (Thermo Scientific), gel purified and ligated together. The resulting construct was designated pUASTattB-DgkRF.V5.

The G509D point mutation was generated using the QuikChange Lightning Mutagenesis Kit (Agilent Technologies) and the primers: *DgkRF-G509D.For* 5′- AGC CGA CGG TGT CGT CGC CGC CA-3′ and *DgkRF-G509D.Rev* 5′- TGG CGG CGA CGA CAC CGT CGG CT-3′. The resulting construct was designated pUASTattB-DgkRF^{G509D}.V5.

Transgenesis of pUASTattB-DgkRF.V5 and pUASTattB-DgkRF^{G509D}.V5 constructs into y^1 *M{vas-int.Dm}ZH*-2A *w*; *M{3xP3-RFP.attP'}ZH*-68E (Bloomington Stock Centre stock #24485) was performed by Best Gene Inc.

2.3. TAG, glucose and glycogen assays

Ten 7—11 days old adult male flies were homogenized in 100 µL 0.5% Tween-20 using the Bullet Blender (Next Advance Inc.) for 3 min on Speed 8. The lysates were incubated at 70 °C for 5 min then spun down twice at 5000 rpm for 1 min. The supernatant was transferred to a fresh tube and stored at −20 °C.

To measure TAG levels, 2 µL of lysate was mixed with 40 µL Triglyceride Reagent and 160 µL Free Glycerol Reagent (Sigma Serum Triglyceride Determination Kit) and incubated at 37 °C for 30 min. Absorbance at 540 nm was measured using a VersaMax 190 Microplate Reader (Molecular Devices).

To measure glucose and glycogen levels, 5 µL of lysate was mixed with 10 µL of H_2O (glucose assay) or 2.5 µL of lysate was mixed with 10 µL of Starch Assay Reagent (glycogen assay, Sigma). The reactions were incubated at 60 °C for 15 min. The plate was cooled to room temperature before 200 µL of Glucose Assay Reagent (Sigma) was added. The reactions were incubated at room temperature for 15 min before measuring absorbance at 340 nm using a VersaMax 190 Microplate Reader (Molecular Devices).

Five biological replicates were tested for each genotype. All TAG, glucose, and glycogen levels were normalized to protein levels measured by BCA Protein Assay (Pierce). Statistical significance was determined by Student's t-test or one-way ANOVA and Holm-Sidak post hoc test using Sigma Plot software (RNAi screen).

2.4. Bioinformatics analyses

To identify screen hits with homologues involved in human obesity, the list of 510 hits after the first round of the screen was uploaded to the FlyMine Resource [21] and used to generate a list of mammalian homologues. This list was analyzed using the MetabolicMine resource [22] Gene–> GWAS hit template.

Determination of enriched functional annotations of RNAi screen hits was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource v6.7 [23]. The FlyBase IDs of the screen hits were entered as input and the 1748 genes tested in the screen were used as the background list for analyses.

2.5. Trehalose assay

Protocol was adapted from the method developed by Tennessen et al. [24]. Ten 7—11 days old adult males were homogenized in 100 µL of trehalase buffer pH 5.7 (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl). The lysates were incubated at 70 °C for 5 min. Two 45 µL aliquots of lysate were transferred into a 96-well assay plate. 45 µL of 3% porcine trehalase (Sigma) was added to one set of samples (+trehalase) while 45 µL of trehalase buffer was added to the other set (− trehalase). The reactions were incubated at 37 °C for 20 hours. 200 µL of Glucose Assay Reagent (Sigma) was added and the reactions were incubated at room temperature for 15 min before measuring absorbance at 340 nm.

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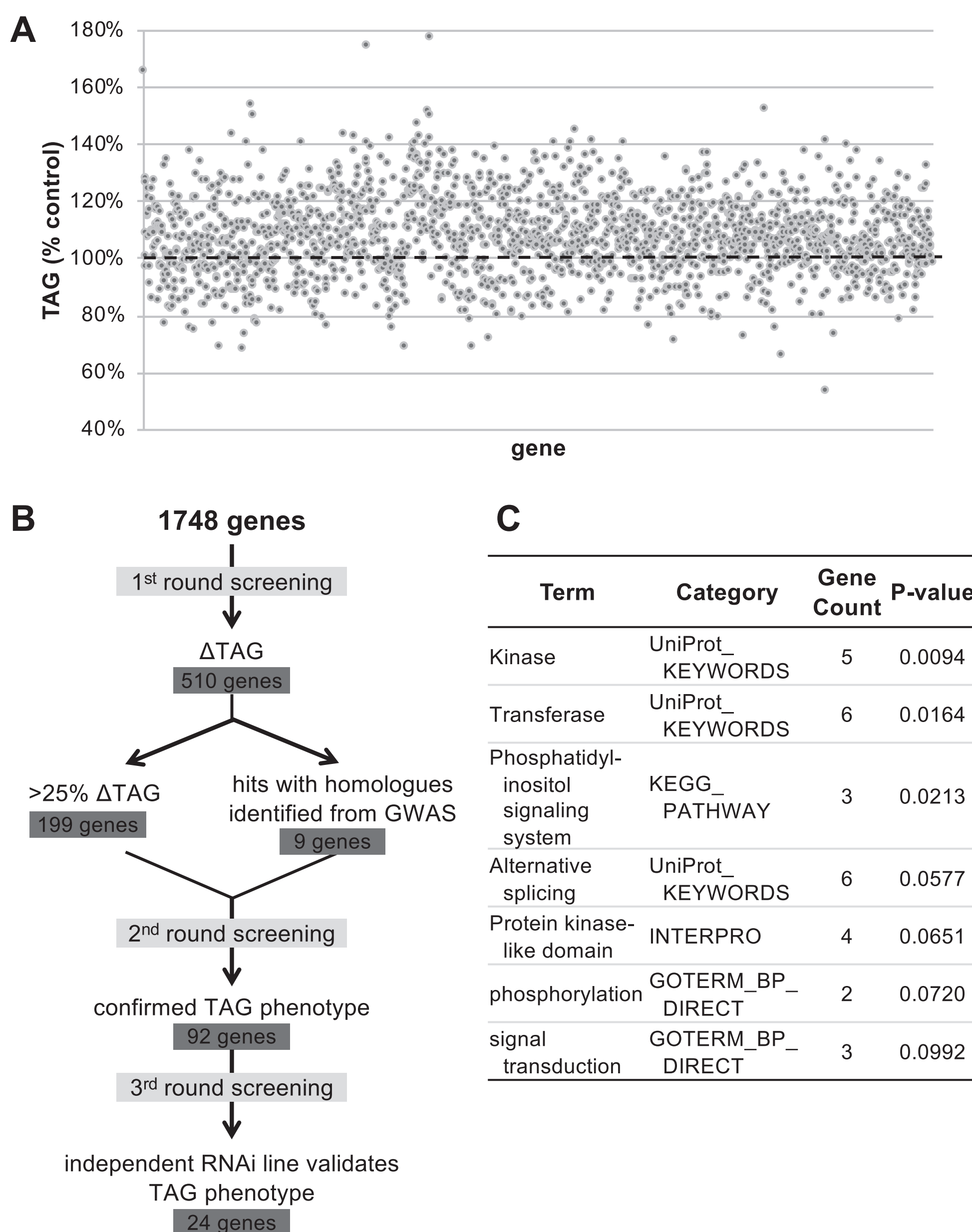
Trehalose levels were normalized to protein levels measured by BCA Protein Assay (Pierce).

2.6. Capillary feeder (CAFE) assay

Protocol is adapted from the method developed by Ja et al. [25]. Briefly, three adult males were transferred without anesthetization to a vial containing 1% agar. 30 replicates were tested for each genotype. A 5 μL microcapillary containing a solution of 5% sucrose, 5% yeast extract, 2% red food colouring was inserted into each vial. The flies are allowed to acclimatize for 24 hours after which the microcapillary is replaced with a new one containing exactly 5 μL of solution. After 24 hours the amount of solution missing from the microcapillary was measured. Empty vials without flies were used as evaporation controls.

2.7. Hemolymph extraction and dILP ELISA

Twenty 7–11-day old males were decapitated and placed in a 0.2 mL PCR tube with a hole punctured in the bottom. The tube was placed into another collection tube and centrifuged at 5000 rpm for 3 min at 4 °C. The extracted hemolymph was diluted with 100 μL cold PBS. 45 μL of the diluted hemolymph was coated on a MaxiSorp flat-bottom 96 well plate (Nunc) overnight at room temperature. The plate was incubated in block (0.02M $NaPO_4$ buffer pH 7.4, 150 mM NaCl,



Term	Category	Gene Count	P-value
Kinase	UniProt_ KEYWORDS	5	0.0094
Transferase	UniProt_ KEYWORDS	6	0.0164
Phosphatidyl-inositol signaling system	KEGG_ PATHWAY	3	0.0213
Alternative splicing	UniProt_ KEYWORDS	6	0.0577
Protein kinase-like domain	INTERPRO	4	0.0651
phosphorylation	GOTERM_BP_ DIRECT	2	0.0720
signal transduction	GOTERM_BP_ DIRECT	3	0.0992

Figure 1: RNAi screening procedure and results. (*A*) TAG levels of all 1748 genes tested in the first round of RNAi screen. Data is normalized to a *fru-Gal4/+* control. (*B*) Flowchart of RNAi screening procedure. 1748 genes were tested for a statistically significant change in triglyceride levels compared to control. Hits resulting in >25% ΔTAG or with homologues identified from GWAS underwent a second round of screening. Verified hits were then screened a third time using independent RNAi lines. 24 genes were confirmed by at least one independent RNAi line. (*C*) Enriched functional annotations among 24 final screen hits. GOTERM_BP, gene ontology: biological process; GOTERM_MF, gene ontology: molecular function.

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Table 1 — Screen hits with human homologues that came out of genome-wide association studies for obesity-related traits.

Gene	Human homologue(s)	GWAS phenotype	Reference(s)
Mdr65	*ABCB11*	Metabolic syndrome, fasting plasma glucose	[81,82]
cnc	*BACH2, NFE2L3*	Waist-hip ratio, Type 1 diabetes	[83–86]
Dgk	*DGKB, DGKG*	Body mass index, weight, fasting glucose-related traits	[3,41–43]
pan	*LEF1, TCF7L2*	Metabolic syndrome, Type 2 diabetes	[87–91]
CG4328	*LMX1B*	Body mass index	[92]
Mio	*MLXIPL*	Hypertriglyceridemia, Triglycerides	[93–96]
CG31646	*NEGR1*	Body mass index, obesity, overweight	[3,4,92,97]
Tom40, tomboy40	*TOMM40*	Triglycerides	[93]

1.27 mM EDTA, 1% BSA) for 1 hour at room temperature. The plate was washed twice with 0.05% Tween-20 in PBS then incubated with either rat anti-dILP2 (1:1000) or rabbit anti-dILP5 (1:2000) for 2 hours at room temperature. After three washes the plate was incubated with goat anti-rat HRP (1:2500, Santa Cruz) or donkey anti-rabbit HRP (1:2500, Santa Cruz) secondary antibody for 1 hour at room temperature. The plate was washed three times then incubated with 1X TMB ELISA substrate solution (eBioscience) for 15 min. The reaction was stopped with 1M phosphoric acid and the absorbance at 450 nm was measured using a VersaMax 190 Microplate Reader (Molecular Devices).
dILP2 and dILP5 levels were normalized to protein levels measured by BCA Protein Assay (Pierce). Anti-dILP2 and anti-dILP5 antibodies were provided by P. Leopold [26].

2.8. Quantitative PCR

The RNA from the heads of fifty 7–11 days old males was extracted using the High Pure RNA Isolation Kit (Roche). 500 ng of RNA was used to generate cDNA using the SensiFAST cDNA Synthesis Kit (Froggabio). The cDNA was diluted 1:1 and 1 μL was used in 10 μL qPCR reactions with the SensiFAST Probe Lo-ROX Kit (Froggabio). The Taqman probes used were: dILP2 - Dm01822534_g1, dILP3 - Dm01801937_g1, dILP5 - Dm01798339_g1 and Rp49 - Dm02151827_g1 (Life Technologies). Reactions were performed using a ViiA7 Real-Time PCR System (Applied Biosystems). Reaction conditions: 95 °C for 20s, 40 cycles - 95 °C for 1s, 60 °C for 20s.
Data are calculated from 3 technical and 3 biological replicates normalized to Rp49 transcript levels. Relative quantification of mRNA levels was calculated using the $\Delta\Delta C_T$ method.

2.9. Western blotting

Ten 7–11 days old adult males were homogenized in 100 μL of RIPA buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 1% Triton-X, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) plus protease and phosphatase inhibitors (Roche) and left on ice to lyse for 1 hour. The lysates were centrifuged at 14 000 rpm for 10 min at 4 °C. 100 μL of 2x SDS buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added before the lysates were heated to 95 °C for 5 min.
20 μL of each sample was run on a 10% polyacrylamide gel and transferred to PVDF membrane (Millipore). The membrane was incubated in block (5% BSA, 5% milk powder, 4% FBS, 2% NGS, 2% NDS in TBST) for 1 hour at room temperature before adding primary antibody and left at 4 °C overnight. Primary antibodies used: mouse anti-V5 (1:2000, Invitrogen), rabbit anti-Akt (1:500, Cell Signalling Technology), rabbit anti-phosphorylated Ser505 Akt (1:1000, Cell Signalling Technology) and mouse anti-actin (1:4000, Abcam). The membrane was washed three times with 1x TBST before incubating with HRP secondary antibody (1:10 000, Santa Cruz) for 2 hours at room temperature. After three washes, Western Lightning Plus-ECL substrate (Perkin Elmer) was added to the membrane for 2 min before it was exposed to film and developed.
The quantification of western blots was performed using ImageJ Software and represents three biological replicates for each genotype.

3. RESULTS

3.1. Neuronal mediators of adiposity

To identify genes that act in the CNS to regulate energy homeostasis, we selected an RNAi library which was specifically generated against genes known to be expressed in the nervous system [17]. To achieve neuron-specific knockdown we used *fruitless (fru)-Gal4* to drive expression of RNAi effectors in a widespread neuronal population in the late pupal and adult central and peripheral nervous systems [18]. Since this driver does not express during embryonic or larval stages, our screen allowed us to focus on metabolic effects by eliminating any confounding developmental effects. Importantly, this neuronal population been previously shown to affect adiposity, feeding and metabolic rate when their neuronal activity is altered [27].
We screened all 1748 genes targeted in our selected RNAi library and assayed for changes in levels of stored fats (in the form of triacylglycerides or TAG) (Figure 1A). Knockdown of 510 genes produced statistically significant changes in TAG levels compared to a *fru-Gal4/+*control (Figure 1B, Table S1). Human homologues of the 510 hits were analyzed using the MetabolicMine resource to identify any hits from obesity-related genome-wide association studies. Single-nucleotide polymorphisms near homologues of 9 hits were associated with obesity-related phenotypes including BMI, weight, metabolic syndrome, fasting glucose levels and triglyceride levels (Table 1). These 9 hits together with the genes that produced >25% increase in TAG (206 in total) underwent a second round of screening to verify their effect on TAG levels by retesting the same RNAi line to validate the phenotype (Figure 1B). 92 genes recapitulated the results from the first round (Table S2) and were screened a third time by testing the TAG levels of additional RNAi lines against the genes of interest to verify that the original RNAi line did not exhibit off-target effects. 24 hits had at least one independent line targeting a different part of the gene that gave rise to the same TAG phenotype thus confirming that the gene targeted by RNAi had a specific effect on TAG levels (Figure 1B, Table S3). Bioinformatic analysis of the hits to identify enriched gene ontology terms, pathways, and protein functions (Figure 1C) shows a clustering of annotations relating to kinases and phosphorylation.
Among the hits identified in the screen, several of the genes are known to function in energy homeostasis in flies including *Cyclic-AMP response element binding protein B* (*CrebB)* and *hemipterous* (*hep*), which are involved in carbohydrate and triglyceride metabolism [28,29]. *Slowpoke binding protein* (*Slob*) has been implicated in the response to starvation [22] and in the regulation of insulin-like peptide secretion [30,31]. In addition, *four wheel drive* (*fwd*) and *hep* are involved in determination of lifespan [32,33], which is linked to

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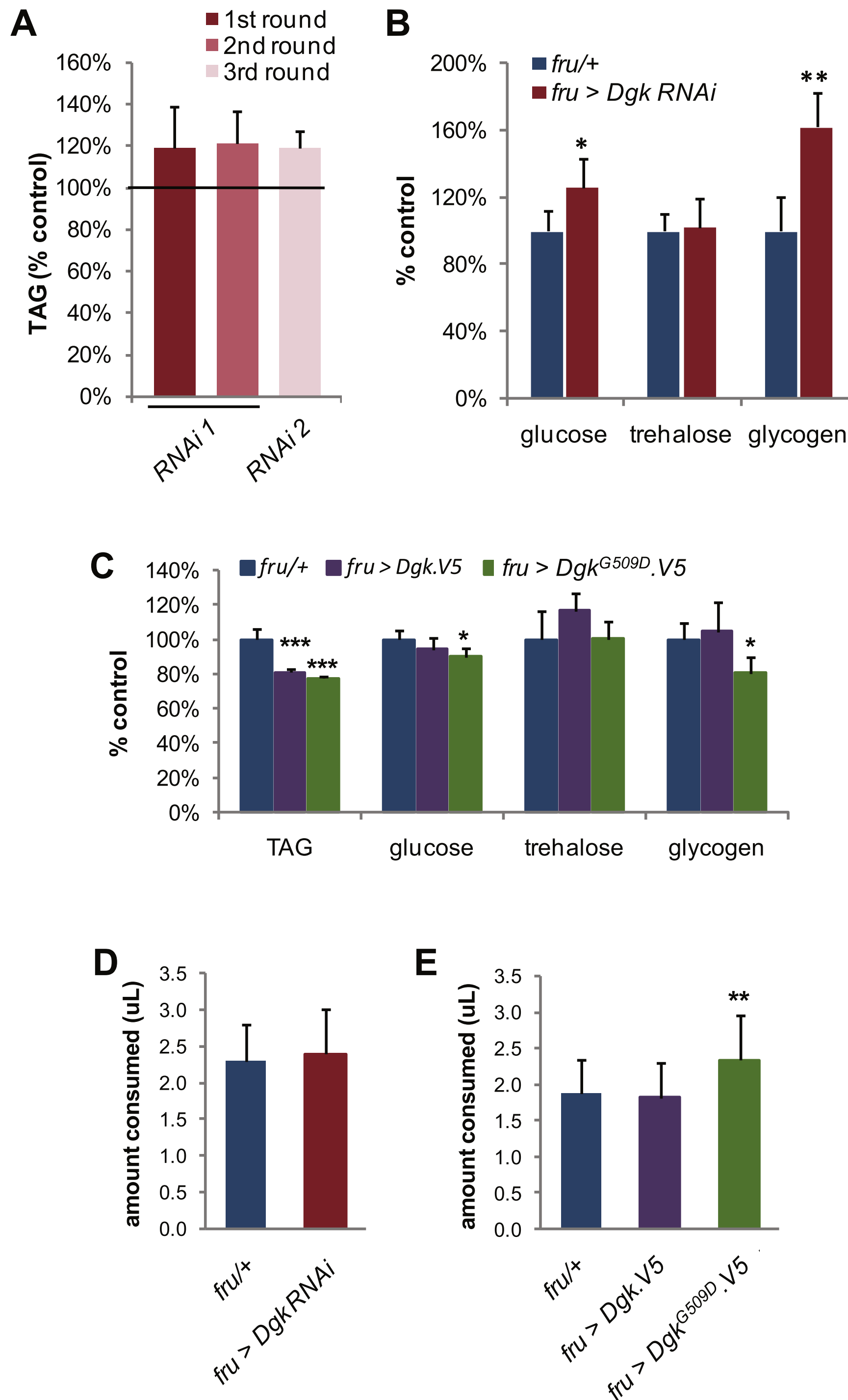

Figure 2: Dgk knockdown or overexpression using *fru-Gal4* alters lipid and carbohydrate homeostasis. (*A*) Knockdown of *Dgk* using two independent RNAi lines results in increased TAG. All bars are statistically significant by one-way ANOVA. (*B*) *fru* > *Dgk RNAi* flies have elevated glucose, glycogen but not trehalose levels. (*C*) Overexpression of either wild-type or kinase-dead Dgk results in lower TAG levels but does not alter trehalose levels. Kinase-dead Dgk but not wild-type Dgk also decreases glucose and glycogen levels. (*A*–*C*) Data is represented as percent of a *fru-Gal4/+* control ± SD, n = 5. (*D*–*E*) Knockdown or overexpression of wild-type Dgk does not affect food intake. Flies overexpressing kinase-dead Dgk are hyperphagic. Data are represented as volume consumed by 3 flies over a 24h period ±SD, n = 30. All assays were performed three times but results from only one representative assay are shown. Asterisks denote p-values based on Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001.

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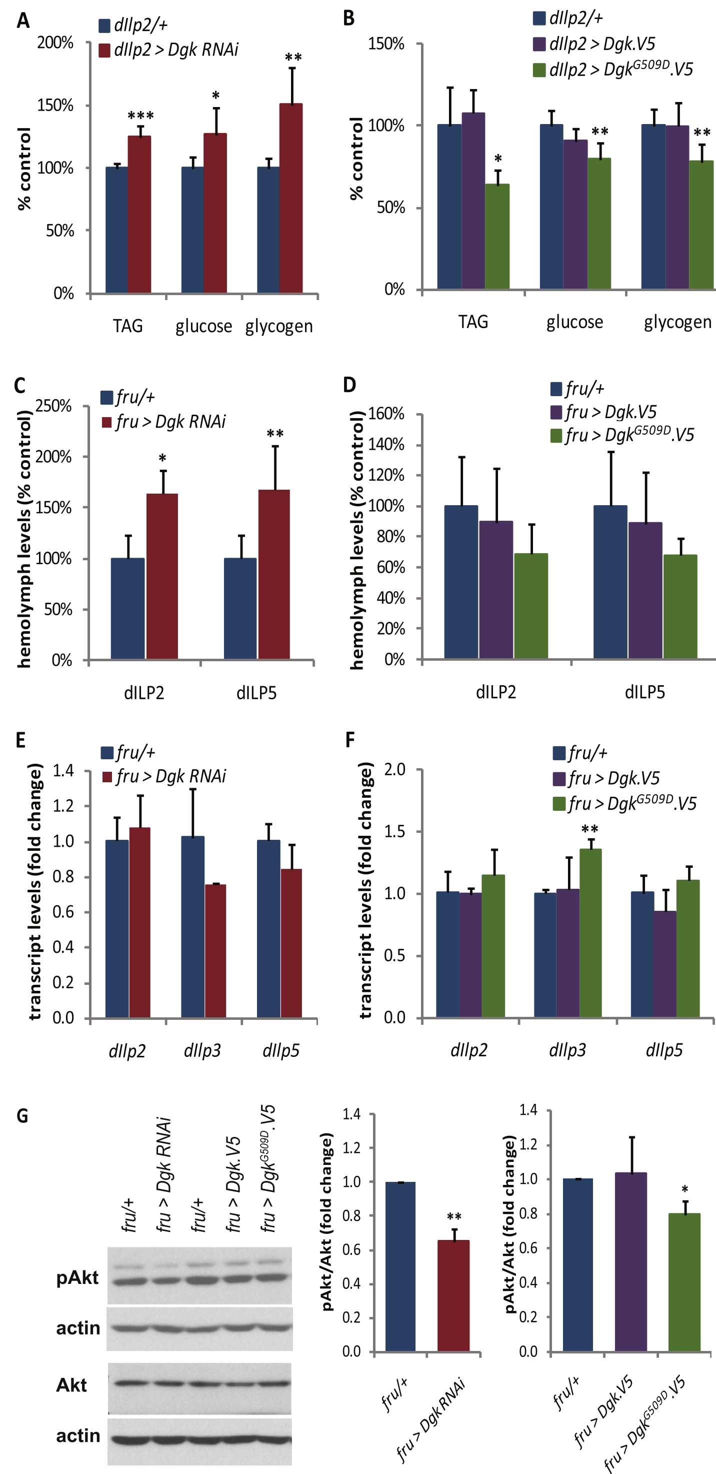

Figure 3: *Dgk* regulates dILP secretion and insulin signaling pathway activity. (*A*) Knockdown of Dgk using *dIlp2-Gal4* shows elevated TAG, glucose, and glycogen phenotypes. (*B*) *dIlp2-Gal4*-driven overexpression of kinase-dead Dgk but not wild-type Dgk also decreases glucose and glycogen levels. These phenotypes in (*A*–*B*) are similar to those seen using *fru-Gal4*. (*C*) *fru* > *Dgk RNAi* increases dILP2 and dILP5 levels in the hemolymph. (*D*) Overexpression of either Dgk.V5 or Dgk^{G509D}.V5 using *fru-Gal4* does not affect hemolymph dILP2 or dILP5 levels. (*A*–*D*) Data is represented as percent or fold-change relative to a *Gal4/+* control ± SD, n = 5. Assays were performed three times but results from only one representative assay are shown. (*E*–*F*) Knockdown or overexpression of Dgk with *fru-Gal4* doesn't affect *dIlp2* or *dIlp5* transcript levels. Overexpression of Dgk^{G509D}.V5 increases *dIlp3* levels. (*G*) *fru-Gal4*-driven Dgk RNAi or overexpression of Dgk^{G509D}.V5 result in lowered insulin pathway activity as measured by p-Ser505 Akt/total Akt levels. *fru* > *Dgk.V5* did not affect pAkt/Akt levels. Quantification is from 3 biological replicates and is normalized to a *fru-Gal4/+* control ±SD. Asterisks denote p-values based on student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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metabolism. Homologues of *CrebB* have also been shown to function in many aspects of mammalian glucose and lipid metabolism [34,35] and the homologues of *dunce* and *n-synaptobrevin* are involved in the regulation of insulin secretion [36–38].
Altogether, the RNAi screen identified and confirmed 24 genes that act in neurons to regulate adiposity. Several of these genes or their homologues have been previously known to affect energy homeostasis thereby validating that the screen was able to identify relevant genes in flies and mammals. We also identified 20 genes that have not been associated with metabolism in *Drosophila* and could prove to be novel neuronal mediators of energy homeostasis.

3.2. Dgk regulates lipid and carbohydrate homeostasis

One of the genes identified in our screen, *Diacylglycerol kinase* (*Dgk*) encodes a member of the diacylglycerol kinase (DGKs) family of intracellular lipid kinases that phosphorylate diacylglycerol (DAG) to form phosphatidic acid (PA). *Dgk* is highly expressed in the nervous system [39,40] but its function in flies is not known. Dgk is classified as a Type I DGK, which includes three mammalian members: DGKα, DGKβ, and DGKγ. There have been few studies of *Dgk* or its homologues in the context of energy homeostasis; however, single nucleotide polymorphisms (SNPs) near two of the *Dgk* homologues have been identified as risk variants by genome-wide association studies for metabolism: *DGKG* is associated with BMI and weight [3] while *DGKB* is implicated in metabolic syndrome [41] and fasting glucose-related traits [42,43]. In addition, D-α-tocopherol which is used in the treatment of diabetic nephropathy exerts its function through the regulation of DGKα translocation and activation thereby preventing glomerular dysfunction [44]. Hence, analysis of *Drosophila Dgk* and its homologues could have implications for metabolic disorders in humans.
To further determine the role of *Dgk* in regulating energy homeostasis, we performed a phenotypic analysis of *Dgk* knockdown and overexpression using the *fru-Gal4* driver. In the screen, knockdown of *Dgk* using two independent RNAi lines resulted in increased TAG levels (Figure 2A). Since the TAG phenotypes were similar, we only used the *Dgk RNAi 1* line for subsequent experiments. *Fru* $>$ *Dgk RNAi* flies also exhibit elevated glucose and glycogen levels (Figure 2B). Conversely, overexpression of either wild-type or kinase-dead (Dgk^{G509D}) Dgk resulted in lower TAG levels (Figure 2C). In addition, overexpression of Dgk^{G509D}.V5 but not wild-type Dgk, produced decreases in glucose and glycogen levels (Figure 2C), which are not due to reduced expression of wild-type Dgk protein (Supplemental Fig. 1). None of the manipulations of Dgk levels seemed to affect the levels of trehalose (the main circulating sugar in flies) (Figure 2B, C); however, this may reflect a limitation of the assay method since trehalose levels from whole-fly lysates could be overpowered by glucose levels. The sensitivity of this assay might be enhanced by measuring trehalose levels in the hemolymph and where changes in the circulating levels of trehalose would more accurately reflect any defects in carbohydrate homeostasis. Since *fru-Gal4* is expressed in the CNS we also assayed for any effects of *Dgk* knockdown on feeding behaviour. Measurement of food consumption over a 24-hour period found that only overexpression of kinase-dead Dgk but not wild-type or knockdown of Dgk (Figure 2D, E) had an effect.

3.3. Dgk regulates insulin-like peptide secretion and function

The insulin/insulin-like growth factor (IGF) signalling (IIS) pathway and its many physiological functions are conserved in *Drosophila* including regulation of metabolism, growth, reproduction and longevity [14,45,46]. DGKα and γ have been shown to play a role in the regulation of insulin secretion from mammalian pancreatic β-cells *in vitro* and *ex vivo* [47,48]. Whether this also occurs *in vivo* and the physiological consequences of loss of DGK activity and dampening of insulin secretion is unknown. Therefore, it is possible that Dgk could be involved in the regulation of insulin secretion in *Drosophila*.
Of the eight *Drosophila* insulin-like peptides (dILPs), dILP2, 3, and 5 and are thought to mediate most metabolic functions in the fly [14,49] and are regulated by nutritional cues [50,51]. These dILPs are expressed in the insulin-producing cells (IPCs), a set of neurosecretory neurons in the brain [19,51,52]. Ablation of the IPCs gives rise to adult flies with elevated glycogen and triglyceride levels [53–55]. These phenotypes are consistent with the lipid and carbohydrate defects seen in our manipulation of neuronal Dgk levels, suggesting that the effect of Dgk on energy homeostasis may be mediated through dILPs and the IIS pathway.
To determine whether Dgk is involved in dILP secretion, we examined the effect of knockdown or overexpression of *Dgk* using *dIlp2-Gal4* that expresses in the IPCs and found that it gives rise to similar TAG, glucose and glycogen phenotypes as seen using *fru-Gal4* (Figures 3A, B and 2A–C). This result, combined with the fact that *fru-Gal4* is also expressed in the IPCs [27] suggests that the Dgk phenotypes seen using *fru-Gal4* are due to Dgk's function within the IPCs.
To directly measure if *Dgk* affects dILP secretion, hemolymph was extracted and levels of circulating dILP2 and dILP5 were measured by ELISA. *Fru-Gal4*-mediated knockdown of Dgk increases both dILP2 and dILP5 levels (Figure 3C) while overexpression of kinase-dead Dgk produces lower levels but is not statistically significant (Figure 3D). These effects are not due to changes in mRNA expression levels of dILPs since quantitative PCR from head extracts did not show a difference in *dIlp2* or *dIlp5* transcript levels (Figure 3E, F).
To determine whether the changes in circulating dILP levels alters insulin signalling, we quantified the levels of phosphorylated Akt Ser505 (corresponding to Ser473 in mammalian Akt), which is a marker for pathway activity [56]. We found that Dgk RNAi flies have decreased pathway activity (Figure 3G) despite having higher circulating dILP levels suggesting that they are insulin-resistant. Kinase-dead Dgk flies have lower levels of pathway activation (Figure 3G) consistent with their lower hemolymph dILP levels.

4. DISCUSSION

While studies in human populations have led to significant progress in the generation of an obesity gene map and broadened our understanding of the genetic basis of common obesity, there is still a large portion of heritability and etiology that remains unknown. An alternative approach to hasten the rate of gene discovery is to use the genetically tractable fruit fly. While there have been previous genetic screens in flies to identify metabolic regulators [57–60], this study is the first to examine the role of specific genes in the central nervous system and to uncover a role for Dgk in energy homeostasis and insulin secretion.
To identify neuronal genes involved in energy homeostasis in *Drosophila*, we systematically screened 1748 genes for effects on stored TAG levels when knocked down using a neuron-specific driver. After three rounds of screening, we identified 24 hits including several previously known to play a role in lipid and carbohydrate metabolism, response to starvation, dILP secretion and lifespan. Some of the hits also have homologues that are involved in mammalian energy homeostasis thereby validating the approach and the ability to identify factors relevant to energy balance in flies and mammals.
Interestingly, all 24 genes identified resulted in increased TAG levels when knocked down in neurons using the *fru-Gal4* driver. The fact that we did not identify any genes with decreased TAG levels could be

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attributed to the fact that silencing of Fru-Gal4-expressing neurons results in increased TAG levels whereas hyperactivation reduces levels [27] and knockdown of genes in the nervous system is more likely to result in a decrease rather than an increase in neuronal function. Thus, the screen seems to be unintentionally biased towards genes that negatively regulate TAG (and therefore would result in increased TAG when knocked out) and may have missed genes that positively regulate lipid storage.

Diacylglycerol kinase was one of the hits identified from the RNAi screen. Diacylglycerol kinases are conserved across a diversity of species with more complex, multicellular organisms possessing several DGKs with differing protein domains, expression patterns and functions [61–63]. Some of these functions include metabolically-relevant ones including central control of energy homeostasis and insulin resistance [64–66]. For example, type I DGKs have been associated with insulin secretion from pancreatic β-cells *in vitro* and SNPs near *DGKB* and *DGKG* have been associated with obesity related-measures by genome-wide association studies. However, little is known about the *in vivo* role of type I DGKs in metabolism.

In *Drosophila*, there is a single type I DGK whose function is largely uncharacterized. Using multiple lines of evidence, we found that Dgk plays an essential role in regulating energy homeostasis by acting within the IPCs in the CNS to regulate secretion of dILP2 and dILP5. First, the TAG, glucose, and glycogen phenotypes seen with knockdown or overexpression of Dgk using *fru-Gal4* can be recapitulated with *dIlp2-Gal4*. These findings are consistent with the association of SNPs near *Dgk* homologues with BMI, weight and fasting blood glucose levels. Furthermore, manipulations of Dgk levels affect dILP2 and dILP5 levels in the hemolymph at the level of secretion since *dIlp2* and *dIlp5* transcript levels are unchanged. However, it is possible that Dgk could affect dILP protein stability or trafficking into secretory vesicles and remains to be measured. This Dgk-mediated disturbance in dILP secretion alters insulin signalling activity, which is likely responsible for the changes in lipid and sugar energy stores. While a similar function has been suggested for its mammalian homologues in cultured pancreatic β-cells, our results constitute the first *in vivo* confirmation of this function as well as its physiological consequences.

The mechanism through which Dgk affects dILP secretion still needs to be determined. The large majority of functions attributed to DGKs are dependent on their kinase function and its regulation of cellular DAG and PA levels [61–63], both of which are important signalling molecules. There are a few studies that implicate DAG and PA in the regulation of insulin secretion: DAG activates PKCs [67–69] and Munc13, a synaptic protein that regulates vesicle release [70], while PA increases insulin granule trafficking and exocytosis [71,72]. It is therefore possible, that Dgk functions to regulate the cellular levels of these two signalling molecules in the insulin-producing cells which in turn, modulate dILP secretion. However, a possible kinase-independent function of Dgk in energy homeostasis is supported by our results from overexpression of wild-type and kinase-dead Dgk. With the exception of low TAG levels, overexpression of wild-type Dgk did not exhibit any defects in the other measured phenotypes. Instead, overexpression of kinase-dead Dgk^{G509D} gave many phenotypes that might be expected with wild-type Dgk i.e. opposing phenotypes compared to *Dgk* RNAi. These differences are not due to reduced expression of wild-type Dgk protein. There have been several instances of other DGKs where the enzymatic activity is not required for its function in specific contexts [73–77]. Thus, the exact mechanism through which Dgk regulates dILP secretion in *Drosophila* remains to be determined.

Interestingly, despite high circulating dILP2 and dILP5 levels in *fru > Dgk RNAi flies*, overall insulin signalling pathway activity was attenuated suggesting that these flies are insulin resistant. These results are consistent with previous studies that showed that flies, like mammals, develop insulin resistance in conjunction with increased TAG and sugar levels [78–80].

Altogether, further study of Dgk and the other RNAi screen hits could lead to an expansion to our understanding of energy homeostasis in flies and mammals as well as the etiological mechanisms and genetic susceptibilities underlying human metabolic disorders.

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CONFLICTS OF INTEREST

There are no known conflicts of interest associated with the research outlined in this publication.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2018.10.006.

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