A Drosophila In Vivo Screen Identifies Store-Operated Calcium Entry as a Key Regulator of Adiposity

Jens Baumbach, Petra Hummel, Iris Bickmeyer, Katarzyna M. Kowalczyk, Martina Frank, Konstantin Knorr, Anja Hildebrandt, Dietmar Riedel, Herbert Jäckle, and Ronald P. Kühnlein

INTRODUCTION

Energy homeostasis is an evolutionarily conserved regulatory process that is essential for all animal organisms including human. Consistent with its ancient genetic basis, cellular lipid storage regulators are conserved from yeast to mammals (Czaban et al., 2007; Goodman, 2009; Kohlwein, 2010; Murphy, 2012; Walther and Farese, 2012; Zechner et al., 2012). Regulator mutants, which give rise to obese or lean individuals, have been identified in animal species as diverse as nematodes, insects, fish, mice, and man (Grönke et al., 2005; Haemmerle et al., 2006; Halaas et al., 1995; Mak et al., 2006; McMenamin et al., 2013; Montague et al., 1997; Suh et al., 2007). The understanding of organismal body fat storage control is most advanced in mammals, in particular because the human obesity pandemics spurred an intensive search for obesity-associated genes (Sandholt et al., 2012). Yet, the identified obesity gene variants explain only less than 2% of the interindividual variation in body mass index (Speliotes et al., 2010). This in comprehen sive identification of the genetic factors underlying adiposity can be explained by the polygenic nature of obesity (Choquet and Meyre, 2011), by the heterogeneity of genetic background within human populations, and by the complex geneenvironment interactions that strongly influence disease expressivity (McAlister et al., 2009). Genetic screens in invertebrate model organisms such as yeast (Daum et al., 1999; Natter et al., 2005), C. elegans (Ashrafi et al., 2003), and Drosophila (Beller et al., 2008; Guo et al., 2008; Pospisil et al., 2010) were successfully used to overcome some of these limitations and identified numerous genes that can affect organismal fat storage. Moreover, they revealed that many of these genes are conserved in evolution. However, none of these screens aimed yet at the identification of genes that function in an organ-specific manner in differentiated fat storage tissues of adult animals or in interorgan communication processes underlying adiposity.

We performed a large-scale Drosophila screen based on in vivo RNAi gene knockdowns, specifically in the fat storage tissue of the adult fly (i.e., the fat body and parts of the midgut) (Sieber and Thummel, 2009). We used a transgenic RNAi line collection enriched for orthologs of human genes and asked whether the corresponding gene knockdowns affect the accumulation of body fat in adult flies (i.e., resulted in obese or lean individuals). By screening almost half of all Drosophila genes specifically in adult fat storage tissue. This approach identified 77 genes, which affect the body fat content of the fly, including 58 previously unknown obesity-associated genes. These genes function in diverse biological processes such as lipid metabolism, vesicle-mediated trafficking, and the universal store-operated calcium entry (SOCE). Impairment of the SOCE core component Stromal interaction molecule (Stim), as well as other components of the pathway, causes adiposity in flies. Acute Stim dysfunction in the fat storage tissue triggers hyperphagia via remote control of the orexigenic short neuropeptide F in the brain, which in turn affects the coordinated lipogenic and lipolytic gene regulation, resulting in adipose tissue hypertrophy.

SUMMARY

To unravel the evolutionarily conserved genetic network underlying energy homeostasis, we performed a systematic in vivo gene knockdown screen in Drosophila. We used a transgenic RNAi library enriched for fly orthologs of human genes to functionally impair about half of all Drosophila genes specifically in adult fat storage tissue. This approach identified 77 genes, which affect the body fat content of the fly, including 58 previously unknown obesity-associated genes. These genes function in diverse biological processes such as lipid metabolism, vesicle-mediated trafficking, and the universal store-operated calcium entry (SOCE). Impairment of the SOCE core component Stromal interaction molecule (Stim), as well as other components of the pathway, causes adiposity in flies. Acute Stim dysfunction in the fat storage tissue triggers hyperphagia via remote control of the orexigenic short neuropeptide F in the brain, which in turn affects the coordinated lipogenic and lipolytic gene regulation, resulting in adipose tissue hypertrophy.

REFERENCES

1Abteilung Molekulare Entwicklungbiologie
2Forschungsgruppe Molekulare Physiologie
3IT und Elektronik Service
4Facility für Elektronenmikroskopie
5Max-Planck-Institut für biophysikalische Chemie, 37077 Göttingen, Germany
6Abteilung Molekulare Entwicklungbiologie, Forschungsgruppe Molekulare Organogenese, Max-Planck-Institut für biophysikalische Chemie, 37077 Göttingen, Germany
7Present address: Institut für Zoologie, Tierärztliche Hochschule Hannover, 30173 Hannover, Germany
8Present address: Abteilung Molekulare Entwicklungbiologie, Forschungsgruppe Molekulare Organogenese, Max-Planck-Institut für biophysikalische Chemie, 37077 Göttingen, Germany
9Correspondence: rkuhnl@gwdg.de

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Genetic Screen for Fly Adiposity Identifies SOCE

A. Conditional fat storage driver + nuclear lacZ:eGFP reporter

I. Repressed (18°C)

II. Active (30°C)

B. Storage fat

I. Repressed (18°C) DAPI

II. Active (30°C) DAPI

C. Gene knockdown in fat storage tissue

Conditional driver

X

RNAi effectors

Obesity/Anti-Obesity Genes

D. RNAi effector lines (sorted by stock number)

Z score (class I candidate genes)

Z score (class II candidate genes)

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store-operated calcium entry (SOCE), which acts as an adiposity regulator. Genetic interventions that reduce or increase the intracellular Ca\textsuperscript{2+} concentration in fat storage tissue rapidly cause obesity and leanness, respectively. To address the mechanisms underlying StimKD-dependent adiposity progression in the fly, we took advantage of the switchable knockdown system to monitor the sequence of regulatory events involved in interorgan communication. Early Stromal interaction molecule (Stim) impairment in fly fat storage tissue induces the orexigenic short neuropeptide F (sNPF) gene in the brain and causes hyperphagia, which, in turn, feeds back in adipose tissue by inducing an obesogenic gene transcription response, resulting in severe adiposity.

**RESULTS AND DISCUSSION**

**Obesity-Associated Genes Acting in the Adult Drosophila Fat Storage Tissues**

*Drosophila* offers unique options for the control of combined tissue- and time-specific gene expression control in a large-scale genetic screen format. We used the temperature-controlled in vivo TARGET system (McGuire et al., 2003) to perform gene knockdown experiments by RNAi transgene expression specifically in the two fat storage organs of the adult fly (i.e., the fat body and parts of the midgut) (Sieber and Thummel, 2009). Reporter gene expression (i.e., nuclear \(\beta\)-galactosidase:eGFP) shows that this conditional driver system (see Experimental Procedures) causes transgene expression exclusively in the fat storage organs at 30°C (active condition), but not at 18°C (repressed condition) (Figures 1A and 1B). To perform large-scale in vivo gene knockdowns, we combined this conditional driver system with individual transgenic RNAi effector fly lines (Dietzl et al., 2007). Flies were crossed to obtain offspring, which contained the conditional driver system combined with a single RNAi effector transgene. To prevent the functional impairment of the targeted genes prior to adulthood, the flies were raised under repressed condition. After hatching of the adults, the RNAi-dependent gene knockdown was induced in the fat storage tissues by shifting the flies to the active condition for 6 days, and the body fat content was determined (Figure 1C). Gene knockdowns that caused flies with low or high body fat content (called “lean” or “obese” flies in the following), compared to the average body fat content of all flies tested, were selected as body fat regulator candidates. These candidates were restested to confirm the results of the primary screen. We used either the average body fat content of the primary screen candidates (class I; Figure 1D, left) or control flies, which carry the RNAi effector transgene but no driver transgene (class II; Figure 1D, right) as a reference for the body fat content. To minimize false positive identifications, we excluded candidate fly lines expressing RNAi transgenes with low target specificity, and we confirmed the candidates by experimental validation using alternative fat body driver lines and/or independent transgenic RNAi effector transgenes targeting the same gene (for details on the selection strategy, see Experimental Procedures).

We screened 7,524 RNAi effectors fly strains, which target a total of 6,796 individual genes, corresponding to 49% of all protein-coding *Drosophila* genes. After retesting and validation, we obtained 77 (1%) body fat regulator genes. Forty-seven of them cause obese flies (called “antiobesity genes” in the following), and 30 cause lean flies (called “obesity genes” in the following). The majority of the 77 identified genes (64; 83%) possess a human ortholog, and 58 genes (75%) have not been previously associated with body fat control in flies (Figures 2A and 2B; Table S1 available online).

In order to test whether the effect on body fat regulation of the genes is specific for adult fat storage tissue, we expressed the 77 RNAI transgenes in the developing fat storage tissue of larvae and pupae. Developmental impairment of almost half of the identified genes (35; 45%) caused preadult lethality (Table S1). This result suggests that the newly identified adult obesity and antiobesity genes carry developmentally relevant functions in the fly. Since the corresponding individuals never develop into adult flies, these genes would have escaped the identification as body fat regulators by conventional mutant analysis.

In order to uncover the possible biological function of the identified obesity and antiobesity genes, we employed gene ontology (GO) analysis. Fifty-five genes (71%) had at least one GO term (“Biological function”; Table S1) and could be assigned to distinct molecular and cellular processes, such as lipid metabolism, vesicle-mediated transport, and calcium signaling (Figures 2A and 2B; Table S1). Genes involved in the lipid metabolism of the fly include previously identified key regulators of glycerolipid homeostasis, such as *Drosophila* diacylglycerol O-acyltransferase (DmDGAT1), encoded by the midway (*mdy*) gene (Buszczak et al., 2002), and the triacylglycerol (TAG) lipase, encoded by *brummer* (*bmm/DmATGL*) (Grönke et al., 2005), an ortholog of the mammalian adipose triglyceride lipase (Zimmermann et al., 2004) (Figures 2 and S1). The body fat storage phenotypes of the *mdy* and *bmm* conditional RNAI knockdown flies and the respective mutants are indistinguishable.
### Functional class

**Anti-Obesity gene/short name or Obesity gene/short name (human ortholog)**

#### Lipid metabolism
- Adipokinetic hormone receptor/AkhR (GNRHR), brummer/bmm (ATGL), Phosphoethanolamine cytidylyltransferase/Pecq (PCYT2)

#### Vesicle-mediated transport
- ADP-ribosylation factor 79F/Arf79F (ARF1), sec71 (ARFGEF1/2), stenosis/sten (SEC24C), CG5484 (YF1B), Ykt6

#### Calcium signaling
- Calmodulin/Cam (CALM), purity of essence/poe (UBR4), Stromal interaction molecule/Stim (STIM1)

#### GPCR signaling
- G protein γ49B/Ga49B (GNAQ), G protein γ1/Gy1 (GNG12), Leucine-rich repeat-containing G protein-coupled receptor 1/Lgr1 (LHCGR)

#### Kinase/Phosphatase signaling
- multiple ankyrin repeats single KH domain/mask (ANKHD1), punt/put (ACVR2), CG16903 (CCNL1)

#### Ubiquitin system/Proteasome/Autophagy
- COP9 complex homolog subunit 4/COP9 (COPS4), Aut1 (ATG3), Ecdysone-induced protein 74EF/Eip74EF
- Proteasome 5 subunit/Pros5 (PSMA5), Proteasome 3 subunit/Pros3 (PSMB3), Regulatory particle non-ATPase 6/Rpn6 (PSMD11), Regulatory particle non-ATPase 7/Rpn7 (PSMD6), Regulatory particle non-ATPase 8/Rpn8 (PSMD7), Pox5 (PBXW9)

#### Transcriptional/Translational regulation
- bsp2 (TAF3), Retinal Homeobox/Rx, Rpd3 (HDAC2), split ends/spen (SPEN), Br140 (BRD1), CG6727 (CEBPG), CG5937 (MKI67IP)

#### General metabolism
- antdh (DHRS11), Carbonic anhydrase 2/CAH2, Tyramine β hydroxylase/Tbh (DBH), CG10116 (DBP1), CG15890, CG11915 (COLGALT2), CG9940 (NADSYN1)

#### Mitochondrial proteins
- tamas/tam (POLG), CG4743 (SLC25A26), CG3214 (NDUFA12)

#### Nuclear transport
- Cullin-4/Cul-4 (CUL4B), CAS/CSE1 segregation protein/Cas (CSE1L), Megator/Mtor (TPR), Nuclear transport factor-2/Mtf-2 (NUTF2)

#### Miscellaneous
- Na,K-ATPase interacting/NKAIN, Related to the N terminus of the oncogene/RN-ire (TEC1Q3), Vacular H+ ATPase M8.9 accessory subunit/Vhama8.9 (ATP6A2P), Vacular H+ ATPase subunit 16-1/Vha16-1 (ATP6VC), CG7379 (ING2), CG7770 (PFK68), unc-45 (UNC45B), CG8750 (EMC3), CG14210

#### Unknown function
- CG15142, lethal (2) 05714, lethal (3) 05822, CG14270 (C19orf52), CG3500 (TEX261), CG15618 (THADA)

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These observations confirm the tissue-specific role of the genes in adult flies and emphasize the validity of the screening approach.

In addition to glycerolipid metabolism genes, we also identified a key regulator of the phospholipid biosynthesis, Pect (also referred to as DmPCYT2), as an antiobesity gene (Figures 2B and S1D). Pect encodes phosphoethanolamine (PE) cytidylyltransferase, the fly homolog of mammalian PCYT2. It catalyzes the rate-limiting step in the CDP-ethanolamine pathway of PE biosynthesis (Fullerton et al., 2009), which requires diacylglycerol (DAG), an intermediate of the Kennedy pathway required for TAG biosynthesis. Thus, Pect links the phospholipid and the glycerolipid metabolism (Figure S1D). Consistent with this functional assignment, Pect knockdown in adult fat storage tissue increases the TAG and DAG contents (Figures S1E–S1G) and enhances mdy/DmDGAT1 gene expression (Figure S1H). Moreover, Pect knockdown flies display the transcriptional signature of increased lipogenesis by upregulating the Fatty acid synthase (Fas), Acetyl-CoA carboxylase (ACC), and Acetyl Coenzyme A synthase (ACS) genes (Figure S1H). This finding supports earlier studies, showing that an impairment of PE biosynthesis (i.e., by mutations in the ethanolamine kinase gene easily shocked or due to global Pect knockdown) causes lipotoxic cardiomyopathy and obesity in flies (Lim et al., 2011).

The coupling of the phospholipid and glycerolipid metabolisms appears to be conserved between flies and mammals, since heterozygous PCYT2 knockout mice develop obesity (Fullerton et al., 2009), and hepatocyte-specific PCYT2 knockout mice suffer from liver steatosis (Leonardi et al., 2009). In both cases, lipogenic genes are upregulated (Fullerton et al., 2009; Leonardi et al., 2009), as observed in flies.

GO analysis indicates that genes involved in vesicle-mediated transport between endoplasmic reticulum (ER) and Golgi participate in adiposity control (Figure 2). Among those genes, ADP-ribosylation factor 79F (Art79F) and sec71 act as antiobesity genes (Figures S1I–S1K). ARF79F is the fly ortholog of mammalian ARF1, a small GTPase required for Golgi integrity. ARF1 acts as key regulator of COPII-mediated retrograde transport between Golgi and ER and is regulated by the guanine nucleotide exchange factors (GEFs) ARFGEF1 and ARFGEF2 at the trans-Golgi. Impairment of the Drosophila ARFGEF1/2 homolog encoded by sec71 in the fat body causes adiposity in flies (Figures S1I–S1K), as do knockdowns of the COPII-dependent vesicle trafficking component SEC24 encoded by the stenosis (or ghost) gene and the R/v-SNARE Ykt6, which functions in ER-Golgi trafficking (McNew et al., 1997). Ykt6 orthologs participate in macroautophagy (Nair et al., 2011). In fact, macroautophagy of lipid droplets has been described as a key process in regulating the cellular fat storage homeostasis in mammals (Singh et al., 2009). Furthermore, vesicle-mediated trafficking regulators are known to participate in cellular lipid accumulation (Beller et al., 2008; Guo et al., 2008). The identification of already known and additional vesicle-mediated trafficking regulators in our screen emphasizes the importance of cellular trafficking processes in adipocytes for the organismal fat storage homeostasis.

Body Fat Control by SOCE

Regulation of ion transport is the most prominent GO term among the newly identified adiposity genes (Table S1), including genes that encode core components of the SOCE (Figure 2). Canonical SOCE (Figure 3A) is initiated by the activation of the inositol 1,4,5-trisphosphate (IP3) receptor (ITP-R83A/DmITPR1) at the ER. This activation causes a primary Ca2+ efflux from the ER to the cytoplasm, which in turn activates the ER calcium sensor encoded by the Stim gene. Activated STIM then interacts with the plasma membrane Ca2+-channel OLF186-F/DmORAI to further elevate the cytoplasmic calcium concentrations from extracellular pools. High intracellular Ca2+ (iCa2+) concentrations trigger a plethora of downstream effectors, such as Calmodulin (CAM). The Ca2+ efflux from the ER is counteracted by the calcium pump Ca-P60A/DmSERCA and accordingly helps to terminate the SOCE activity (Soboloff et al., 2012).

To in vivo visualize cytoplasmic iCa2+ concentrations of adult fat body cells, we used the CaLexA system. It translates the iCa2+ concentrations to transcriptional activity of a GFP reporter gene via the Ca2+-dependent nuclear import of a synthetic transcription factor (Masuyama et al., 2012). Figure 3B shows a reduction of plasma-membrane-targeted GFP in fat body cells after the Stim gene knockdown, indicating a depletion of iCa2+ in response to the SOCE impairment. Genetic manipulations of SOCE genes, which decrease the iCa2+ concentration in fat storage cells, such as knockdown of Stim or ltp-r83A and the overexpression of a dominant-negative form of Itp-r83A, cause a massive increase of the body fat content (50%–150%) and a corresponding accumulation of subcuticular body fat in flies (Figures 3C–3E and S2). Conversely, an increased iCa2+ concentration in fat storage cells caused by the knockdown of Ca-P60A or the targeted overexpression of ofl186-F and Stim, respectively, results in the reduction of the body fat content of the flies by up to 85% and depletes the subcuticular abdominal lipid stores (Figures 3C, 3D, S2A, and S2B). A recent study showing that the obese phenotype of ltp-r83A mutants can be partially rescued by Itp-r83A expression in the fat body (Subramanian et al., 2013) supports the participation of SOCE genes in adiposity regulation. Knockdown of the SOCE-downstream mediator Cam gene increases the total body fat stores by more than 50% (Figure 3C). These findings support the conclusion that the SOCE machinery, of which the key components were identified in our screen, carries an important role in the control of adiposity.
Genetic Screen for Fly Adiposity Identifies SOCE
Adiposity by SOCE Dysfunction in Fat Storage Tissue Involves Orexigenic Brain sNPF Signaling

In order to examine the role of SOCE in adiposity, we focused on the characterization of Stim gene function. After 6 days of Stim gene knockdown in the fat storage tissue (Stim RNAi ON; the corresponding flies are called “StimKD” in the following), the Stim protein and the iCa\textsuperscript{2+} concentration in the fat storage cells are significantly reduced (Figures 3B, 3C, and S3A). The body fat of these flies is more than doubled, and subcuticular fat stores are increased as compared to the control flies (Stim RNAi OFF) (Figures 3C–3E). At the cellular level, the lipid droplet size, the total lipid droplet area, and the overall size of fat body cells are significantly increased (Figures 3F–3H). StimKD-dependent obesity can be triggered in both mature adult male and female flies of different ages by different Stim RNAi transgenes and also in response to different transgene systems, including a temperature-independent switchable driver in adipose tissue (Figures 3C and S3B). In contrast, carbohydrate homeostasis is not affected by the Stim impairment, since the hemolymph concentrations of the circulating sugars trehalose and glucose (Figure 4A), the body content of the storage carbohydrate glycogen, and the starvation-induced glycogen mobilization profile of the StimKD-dependent obese flies did not differ from control flies (Figure 4B). Normal dietary sugar responsiveness of the StimKD-dependent adiposity adds further support to the conclusion that the carbohydrate metabolism is fully functional in StimKD flies (i.e., these flies accumulate more body fat in response to increasing dietary sugar concentrations compared to controls) (Figure 4C). Thus, the StimKD in the fat storage tissue impairs only the lipid metabolism component of energy homeostasis control. In contrast to mature adult flies (6 days after hatching), however, the Stim knockdown in the fat storage tissues of third instar larvae or immature adult flies (4 hr after hatching) has no significant effect on the body fat content (Figure S3C). This observation indicates that Stim has a specific function in regulating the energy intake and/or expenditure of the adult fly, likely related to the adult lifestyle.

To address this point, we profiled the physiological basis of obesity progression in mature adult StimKD flies. While StimKD flies develop severe adiposity, their locomotor activity was unchanged when compared to the control flies (Figure 4D). Moreover, measurements of CO\textsubscript{2} production of control flies indicate that the metabolic rate of obese StimKD flies did not differ from control flies (Figure S3D). Thus, reduced energy expenditure can be excluded as a major cause of StimKD-dependent obesity. We observed, however, that the body fat content increases rapidly after StimKD induction (Figure 4D), and flies become hyperphagic as early as 2 days after StimKD induction (Figure 4E). Under ad libitum feeding conditions, the food intake increases steadily up to more than twice over control values from day four onward. However, when StimKD flies were pair-fed (i.e., that they were restricted to the food intake of normophagic control flies), StimKD flies accumulate only slightly more body fat than control flies (Figure 4F). These results support the argument that the adiposity of StimKD flies is driven by hyperphagia.

Hyperphagia indicates the lack of proper food intake control by the central nervous system. We therefore set out to reveal the regulatory events underlying adiposity progression in flies, where Stim activity was impaired in the fat storage tissue. We first addressed the transcriptional profile of the lipid metabolism effectors mdy/DmDGAT1 and bmm/DmATGL in fat storage tissue and the orexigenic sNPF gene in the brain. sNPF is a functional homolog of mammalian orexigenic neuropeptide Y (Nässel and Wegener, 2011), and its overexpression in sNPF-producing neurons causes hyperphagia and body fat accumulation in flies (Figure 4G). This observation suggests sNPF as a plausible candidate factor to relay StimKD-dependent food intake control. As shown in Figure 4H, the expression levels of sNPF, mdy, and bmm are not different compared to controls prior to StimKD induction. However, as early as 1 day after functional impairment of Stim in the fat storage tissue, sNPF is upregulated by 42% in the brain, while fat body mdy and bmm expression are still unchanged. Even 10 hr later, sNPF continues to be upregulated in the brain, but the fat storage tissue now shows an obesogenic transcriptional response (i.e., the transcript abundance of mdy is increased by 178%, whereas bmm is decreased by 84%). During further progression of adiposity until day six, when the body fat content of StimKD flies has more than doubled as compared to control flies (Figure 4D), sNPF is still upregulated in the brain. However, the StimKD-dependent effects on mdy and bmm expression are less pronounced than 1 day after Stim impairment. The temporal sequence of transcriptional regulation suggests that Stim dysfunction in the fat storage tissue controls sNPF activity in the brain, which in turn causes hyperphagia, resulting in the obesogenic transcriptional response back in the fat body. In fact, sNPF overexpression in the brain of control flies has the same effect on mdy and bmm expression as observed with StimKD flies (Figure 4I). Conversely, the obesogenic transcriptional response in the fat body is blunted in pair-fed StimKD flies, which accumulate only slightly more body fat.
compared to controls (i.e., the bmm gene expression is unaffected, and mdy is only weakly upregulated) (Figure 4H). Collectively, these data suggest that StimKD in the fat body upregulates sNPF in the brain, which triggers hyperphagia, leading to mdy and bmm gene regulation back in the fat storage tissue. Since the fat-storage-tissue-targeted knockdown of Cam alters the sNPF, mdy, and bmm expression as observed with StimKD flies (Figure 4J), it is likely that Cam participates in the interorgan feedback control via sNPF signaling.

To further address the functional implication of the sNPF, mdy, and bmm gene regulation in StimKD-dependent adiposity, we first analyzed the body fat content of flies when these three genes were altered according to the effects observed in StimKD flies. Overexpression of sNPF in the brain, overexpression of mdy in the fat body, and bmm gene knockdown in the fat body cause adiposity, as observed with StimKD flies (Figures 5A–5C). In order to test whether StimKD-dependent transcriptional dysregulation is indeed mediated by sNPF in the brain, we simultaneously impaired sNPF and Stim in brain and fat body. In such flies, StimKD-dependent adiposity is reduced (Figure 5A). Furthermore, simultaneous knockdown of both mdy and Stim in the adipose tissue, which counteracts the StimKD-induced upregulation of mdy, largely represses StimKD-dependent adiposity (Figure 5B). Moreover, overexpression of bmm in the adipose tissue of StimKD flies, which counteracts the StimKD-induced downregulation of bmm, reduces StimKD-dependent adiposity (Figure 5C).

These results indicate that the StimKD-dependent regulation of mdy and bmm expression in fat storage tissue requires sNPF activity in the brain and that Stim does, at least in part, act in a non-tissue-autonomous manner in body fat storage control (Figure 5A). The data are consistent with a model suggesting that an impairment of Stim in the fat storage tissue initiates sNPF upregulation in the central nervous system and

Figure 4. Physiology, Hyperphagia, and Fat Body—Brain Organ Communication in StimKD-Dependent Obesity
(A–C) Normal carbohydrate metabolism in obese StimKD flies. Euglycemia (A), normal glycogen storage and glycogen mobilization (B), and normal body fat response to dietary sugar (C) in obese StimKD flies, compared to controls. Shown are circulating hemolymph sugar (glucose and trehalose) levels (A) and total body fat content (C) of adult male flies on diets of varying sugar concentrations. Total body glycogen content and mobilization in (B) was determined for adult male flies subject to fat-storage-tissue-targeted individual mdy gene overexpression and knockdown, respectively.

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These results indicate that the StimKD-dependent regulation of mdy and bmm expression in fat storage tissue requires sNPF activity in the brain and that Stim does, at least in part, act in a non-tissue-autonomous manner in body fat storage control (Figure 5A). The data are consistent with a model suggesting that an impairment of Stim in the fat storage tissue initiates sNPF upregulation in the central nervous system and

Figure 5. Transgenic Correction of sNPF, mdy, and bmm Gene Dysregulation Suppresses StimKD-Dependent Adiposity
Shown are changes in total body fat content of male flies.

(A) Attenuated adiposity in StimKD flies in response to simultaneous sNPF knockdown in the sNPF-positive neurons. Individual gene knockdown of sNPF in the brain causes leanness. No effect on body fat upon Stim knockdown in sNPF-positive neurons or sNPF knockdown in the fat storage tissue. FB-Gal4 indicates fat-body-Gal4.

(B) Strong suppression of StimKD-dependent adiposity by simultaneous mdy gene knockdown in the fat storage tissue. Adiposity and leanness of flies subject to fat-storage-tissue-targeted individual mdy gene overexpression and knockdown, respectively.

(C) Attenuated onset of StimKD-dependent adiposity by simultaneous bmm gene overexpression (gof) in the fat storage tissue. bmm gene overexpression causes leanness in control flies. Note that fly body fat content was determined after constitutive (A), conditional 6-day (B) or conditional 34-hr (C) knockdown of the respective genes. Note that a control (UAS-GFP) was used to match the number of effector transgenes. Error bars represent SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. n.s. indicates “not significant.” See also Figure S4.
thereby causes hyperphagia. In turn, hyperphagia then triggers an obesogenic program in the fat storage tissue.

Interestingly, mammalian Stim1 has been shown to suppress the differentiation of 3T3-L1 preadipocytes (Graham et al., 2009). Conversely, pharmacological elevation of the iCa\textsuperscript{2+} concentrations, either in response to the activated transient receptor potential vanniloid-1 calcium channel or by the SERCA inhibitor thapsigargin, inhibits the differentiation of 3T3-L1 and human preadipocytes into functional adipocytes (Ntambi and Takova, 1996; Zhang et al., 2007; Shi et al., 2000). However, elevation of iCa\textsuperscript{2+} has several effects described for mammalian adipocytes. It promotes human adipocyte maturation during late differentiation phases (Shi et al., 2000), inhibits lipolysis in human adipocytes (Xue et al., 1998), and increases lipid storage by upregulation of lipogenic genes in mature 3T3-L1 adipocytes (Jones et al., 1996). Given those cell-autonomous roles of iCa\textsuperscript{2+} in the mammalian fat storage cells, the possible evolutionarily conserved role of SOCE members in flies and mammals in the regulation of fat storage control has to await future studies addressing the function in mammalian organism rather than in tissue culture. It will also be of interest to elucidate the mechanisms by which interorgan communication in StimKD-dependent adiposity is mediated and to identify the components involved in the signaling from adipose tissue to the brain and vice versa.

In Drosophila, fat body signaling to the brain integrates information on nutrition, metabolism, and systemic growth (Britton and Edgar, 1998; Colombani et al., 2003; Géménard et al., 2009). In this interactive pathway, the fat-body-expressed cytokine Unpaired 2 (Upd2) acts as a functional homolog of mammalian leptin, which signals the “fed state” to the central nervous system via remote control of Drosophila insulin-like peptide (DILP) release in the brain (Rajan and Perrimon, 2012). Flies subjected to an upd2 knockdown in the fat body are normophagic, hyperglycemic, and lean and accumulate DILP2 protein in the brain insulin-producing cells (IPCs), suggesting reduced systemic insulin signaling in such flies (Rajan and Perrimon, 2012). In the abdomen of obese StimKD flies, where most fat body tissue resides, upd2 is downregulated close to half (Figure S4A). In contrast to upd2 knockdown flies, StimKD flies are hyperphagic, euglycemic, and have significantly lower DILP2 accumulation in IPCs compared to controls (Figures S4B and S4C). Hence, upd2 is not the mediator of interorgan information between fat body and brain of StimKD flies. The factor(s) and mechanisms underlying this process in StimKD-dependent adiposity are currently unknown.

Similar to its mammalian homolog NPY, sNPF is a critical regulator of food intake, acting in the central nervous system of the fly. Upregulation of sNPF gene expression in the brain of StimKD flies (Figure 4H) or by starvation of wild-type flies (Hong et al., 2012) increases the food intake leading to body fat accumulation under ad libitum feeding conditions (Figure 4G). Conversely, downregulation of the gene in sNPF-positive neurons reduces food intake (Lee et al., 2004), increases starvation sensitivity (Kahsai et al., 2010), and causes lean flies (Figure 5A). Interestingly, the delicate control of the sNPF gene expression level is subject to an evolutionarily conserved autoregulatory loop in the brain (Hong et al., 2012). Murine NPY or fly sNPF signaling upregulates the dual specificity tyrosine-phosphorylation-regulated kinase 1 (Dyrk1). Dyrk1 activates the transcription factor FOXO by sirtuin-dependent deacetylation, which in turn increases NPY and sNPF expression in the brain. Thus, it is conceivable that aspects of StimKD-dependent adiposity regulation that include interorgan communication are conserved up to mammals.

Our screen was designed to identify body fat regulators required in the fat storage tissue of adult flies. We isolated a number of already known fly antiobesity and obesity genes, providing the proof of concept for the validity of the screening approach taken. Importantly, we found 58 previously unknown body fat regulator genes, including 46 (79%) which are sequence conserved in evolution up to humans. In addition to enzymes acting in lipid metabolism and components of the intracellular vesicle trafficking, our screen also identified the key components of the SOCE machinery, which controls the iCa\textsuperscript{2+} homeostasis. Calcium signaling was previously shown to participate in cell-autonomous processes of mammalian lipid metabolism. Our results provide evidence that in the fly organism, changes in the SOCE-dependent iCa\textsuperscript{2+} levels in fat storage cells act in a non-tissue-autonomous manner by using an as-yet-unknown interorgan communication pathway to enhance sNPF expression in the brain. Since the energy expenditure of such flies does not change, it is conceivable that the excess food intake causes the observed changes in gene activities, leading to a rapid increase of fat storage. Our identification of previously unknown obesity and antiobesity genes of the fly, and the non-cell-autonomous function of the SOCE machinery, now pave the way to address key questions regarding the orchestration of energy homeostasis in flies, to elucidate the fat-tissue-brain communication process, and to address questions concerning the evolutionary conservation of the factors and mechanisms underlying fat storage control in animal organisms.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks and Husbandry**

Transgenic RNAi fly strains used in this study were received from either the VDRC GD and KK library; Dietzl et al., 2007; http://stockcenter.vdrc.at/) or the BDSC (Harvard T3RF library; www.flyma.org). Unless stated differently, the flies were propagated as described (Grönke et al., 2005). If not noted otherwise, transgene ON versus OFF conditions refer to flies carrying the effector transgene in the presence and absence of a driver transgene, respectively. For details on all fly stocks and fly husbandry see Supplemental Experimental Procedures.

**Histology, Microscopy, and Image Analysis**

Bright-field and epifluorescence microscopies of adult fly guts were done on a Zeiss Axioskop equipped with a ProgRes 3012 camera or a Zeiss Axiosvert 200M with a Hamamatsu ORCA ER camera. A Zeiss LSM 780 was used for confocal fluorescence microscopy on adult fat body tissue, for DILP2 (Géménard et al., 2009) immunocytochemistry, and for scoring of the CalLexA (Masuyama et al., 2012) reporter system. Images were analyzed with ZenLite2011 and ImageJ and assembled with Adobe Photoshop CS3. Cryosections of adult males were done on a Leica CM 3050 S cryostat and stained with oil red O. Electron microscopy of adult male flies was done on a Hitachi H-7650. For details, see Supplemental Experimental Procedures.

**Selection Scheme for Obesity and Antiobesity Genes**

In the primary screen, VDRC GD library transgenic RNAi lines were crossed (in cohorts of 200 lines) against a temperature-sensitive fat storage
tissue-specific driver line (ts-FB-Ga4; Beller et al., 2010) and raised under gene-knockdown-repressed conditions (18°C). Male fly progeny (females for X chromosome RNAi transgene integrations) were kept under gene-knockdown-active conditions (30°C) for 6 days, and subsequently the body fat content (FC) was determined in duplicate groups of five flies each. Primary candidate (c) lines, which gave rise to the most obese and most lean flies within their cohort, (criteria were as follows: $FC_C <$ average $FC_{cc} - 1.5 \times SD$ $FC_{cc}$ for obesity gene candidates, and $FC_C >$ average $FC_{cc} + 1.5 \times SD$ $FC_{cc}$ for obesity gene candidates) were retested in additional rounds of screening and scored as class I or II candidates according to the following criteria. Regulator candidates consist of the most lean or most obese flies compared to the average of the primary screen candidates ($Z$ score $\leq$ 1.5 or $\geq$ 1.5; tested in cohorts of 100 lines; class I). To account for the observed transgene integration effects on body fat content, candidate regulators that cause substantial relative body fat increase or decrease, respectively, compared to control flies carrying the same RNAi effector transgene but no driver transgene, were identified ($Z$ score $\geq$ 2.06 or $\geq$ 1.49 for males; $Z$ score $\geq$ 1.45 or $\geq$ 1.19 for females; class II). Most class I and II regulators (87%) were validated by restesting their effect on body fat storage in two independent experiments using (i) the primary screen RNAi effector fly line expressed in response to an alternative adult fat body driver (to-Ga4 for autosomal and yolk-Ga4 for X chromosome GD lines) and/or (ii) by using a second transgenic RNAi line (VDRC KK or Harvard TRIP collection) targeting the same gene to avoid false positive identifications by RNAi off-target effects. Gene knockdowns, which changed the body fat content by more than 25% compared to controls lacking the driver transgene, received a positive validation score. Candidate genes that (i) reached a validation score of $\geq$ 2 (out of up to four independent experiments), (ii) were represented by at least one high-quality RNAi transgenic lines ($Z$ score $\geq$ 0.79 and CAN repeats $< 7$), and (iii) were not predicted to function in general RNA interference qualified as confirmed fly obesity or antiobesity genes (Figure 2; Table S1).

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.12.004.

AUTHOR CONTRIBUTIONS
The study was designed by R.P.K. and J.B. The authors J.B., I.B., K.K., M.F., K.M.K., and R.P.K. performed the genetic screen. P.H. contributed the bioinformatics analysis; D.R. performed the electron microscopy, and A.H. performed the TLC analyses. All other experiments were performed by J.B. and analyzed by J.B and R.P.K. The authors R.P.K., J.B. and H.J. wrote the manuscript.

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REFERENCES

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Murphy, D.J. (2012). The dynamic roles of intracellular lipid droplets: from archaea to mammals. Protoplasma 249, 541–585.


