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ORIGINAL RESEARCH

Gαq, *Gγ1* and *Plc21C* Control *Drosophila* Body Fat Storage☆

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ABSTRACT

Adaptive mobilization of body fat is essential for energy homeostasis in animals. In insects, the adipokinetic hormone (Akh) systemically controls body fat mobilization. Biochemical evidence supports that Akh signals *via* a G protein-coupled receptor (GPCR) called Akh receptor (AkhR) using cyclic-AMP (cAMP) and Ca^{2+} second messengers to induce storage lipid release from fat body cells. Recently, we provided genetic evidence that the intracellular calcium (iCa^{2+}) level in fat storage cells controls adiposity in the fruit fly *Drosophila melanogaster*. However, little is known about the genes, which mediate Akh signalling downstream of the AkhR to regulate changes in iCa^{2+}. Here, we used thermogenetics to provide *in vivo* evidence that the GPCR signal transducers G protein α q subunit (Gαq), G protein γ1 (Gγ1) and Phospholipase C at 21C (Plc21C) control cellular and organismal fat storage in *Drosophila*. Transgenic modulation of *Gαq*, *Gγ1* and *Plc21C* affected the iCa^{2+} of fat body cells and the expression profile of the lipid metabolism effector genes *midway* and *brummer*, which results in severely obese or lean flies. Moreover, functional impairment of *Gαq*, *Gγ1* and *Plc21C* antagonised Akh-induced fat depletion. This study characterizes *Gαq*, *Gγ1* and *Plc21C* as anti-obesity genes and supports the model that Akh employs the *Gαq/Gγ1/Plc21C* module of iCa^{2+} control to regulate lipid mobilization in adult *Drosophila*.

KEYWORDS: *Plc21C*; *Gαq*; *Gγ1*; Calcium; Fat storage; Adipokinetic hormone

INTRODUCTION

Fat storage is a universal mechanism of energy homeostasis control in animal organisms. An essential component of this energy homeostasis is lipid mobilization from body fat stores to match the organismal demand in periods of negative energy balance. The *Drosophila* model organism has been successfully used to identify key regulator genes of storage fat mobilization, some of which are evolutionarily conserved (Pospisilik et al., 2010; Baumbach et al., 2014). Among them are the lipogenic *midway* (*mdy*) gene (Buszczak et al., 2002; Beller et al., 2010) and the lipolytic *brummer* (*bmm*) gene (Grönke et al., 2005). *mdy* encodes *Drosophila* diacylglycerol O-acyltransferase 1 (*Dm*DGAT1) and *bmm* encodes a triglyceride lipase (*Dm*ATGL), a member of the patatin-domain containing lipase gene family, which controls storage lipid mobilization from yeast to men (Haemmerle et al., 2006; Kurat et al., 2006; Fischer et al., 2007). While *bmm* mutant *Drosophila* are obese, the overexpression of the gene results in lean animals (Grönke et al., 2005). We have shown earlier that *bmm* is one part of a dual control mechanism affecting fat storage and mobilization in *Drosophila* (Grönke et al., 2007). Brummer interacts with adipokinetic hormone (Akh) signalling, which controls a complementary pathway of lipid mobilization in *Drosophila*.

Akh activity has been first described to mobilize lipids for sustained flight in locusts (Beenakkers, 1969; Mayer and Candy, 1969). Structurally, *Locusta migratoria* Akh (Stone et al., 1976) represents the founding member of the arthropod Akh family of short neuropeptides, which are

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phylogenetically related to vertebrate gonadotropin-releasing hormones (Lindemans et al., 2011). Next to storage lipid mobilization, Akhs serve pleiotropic metabolic functions, e.g. in carbohydrate mobilization and in locomotion control. Accordingly, Akhs are considered to act as central coordinators of energy homeostasis in insects (Lorenz and Gäde, 2009).

Storage lipid mobilization by canonical Akh signalling starts by secretion of the peptide from the neurohemal organ called corpora cardiaca (cc) into the hemolymph. Circulating Akh binds to specific G protein-coupled receptors (GPCRs) called Akh receptors (AkhRs) of fat body cells (Ziegler et al., 1995). Binding of Akh to AkhR triggers a complex cAMP and intracellular Ca^{2+} (iCa^{2+}) second messenger response in the insect fat storage tissue, called the fat body (Vroemen et al., 1995; Arrese et al., 1999). This second messenger response results in Akh-dependent storage lipid mobilization in a variety of insects such as locusta [*Schistocerca gregaria* (Ogoyi et al., 1998), *L. migratoria* (Auerswald and Gade, 2006), and *Gryllus bimaculatus* (Anand and Lorenz, 2008)], lepidoptera [*Manduca sexta* (Arrese et al., 1996)], coleoptera [*Pachnoda sinuata* and *Zophobas atratus* (Auerswald et al., 2005; Slocinska et al., 2013)] and diptera [*Drosophila melanogaster* (Lee and Park, 2004; Grönke et al., 2007)]. However, species- and developmental stage-specific differences in the physiological response to Akh have been documented, implying that insect fat body cells interpret the intracellular signalling response to the Akh hormone signal in a context-dependent manner. Accordingly, the biochemistry of Akh signalling in the insect fat body has attracted significant research attention in various insect species (Gäde and Auerswald, 2003). Functional studies of AkhR in heterologous tissue culture systems recently identified new downstream factors of Akh/AkhR signalling such as the extracellular signal-regulated kinase 1/2 (Zhu et al., 2009, Huang et al., 2010). However, little is known about the identity of the genes which relay Akh/AkhR signalling to iCa^{2+} increase in fat body cells in the context of storage lipid mobilization.

We and others have recently shown a reverse correlation between the iCa^{2+} in fat body cells and the body fat content in adult flies (Subramanian et al., 2013; Baumbach et al., 2014). Obese flies resulted from the functional impairment of any of the core component of the so-called store-operated calcium entry (SOCE). Canonical SOCE stimulation increases iCa^{2+} *via* a calcium release-activated calcium plasma membrane channel called ORAI or Olf186-F in flies (Cahalan, 2009). This channel interacts with the endoplasmic reticulum (ER) calcium sensor Stromal interaction molecule (Stim) in response to a depletion of the ER Ca^{2+} stores. ER Ca^{2+} depletion, in turn, is triggered by binding of inositol-1,4,5-trisphosphate (IP3) to its cognate receptor Itp-r83A. One way of generating the IP3 second messenger is by Phospholipase Cβ (Plcβ) activation in response to GPCR signalling, which involves the trimeric G protein Gα and Gγ subunits.

A recent genetic screen identified the G protein Gαq and Gγ subunits encoded by *G protein α q* (*Gαq*) and *G protein γ1* (*Gγ1*), respectively, as candidate body fat regulators acting in the adult fly fat body (Baumbach et al., 2014). Here, we tested the hypothesis whether *Gγ1*, *Gαq* and the Plcβ encoded by *Phospholipase C at 21C* (*Plc21C*) act upstream of SOCE and therefore possibly link AkhR signalling to iCa^{2+}-dependent body fat control in adult *Drosophila*.

Our study provides evidence that functional impairment of *Plc21C*, *Gγ1*, *Gαq*, *Stim* and *AkhR* lowers the iCa^{2+} concentration in adult fat body cells *in vivo*. Modulation of the *Plc21C*, *Gγ1* and *Gαq* gene activities caused the same changes to cellular and organismal fat storage as does the repression or overexpression of the SOCE member *Stim* or of *Akh/AkhR*. Moreover, changes in *Plc21C*, *Gγ1*, *Gαq*, *Stim* and *AkhR* gene activities in the adult fat body resulted in a similar but not identical transcriptional response of the lipid metabolism effector genes *mdy* and *bmm*. Finally, the functional impairment of *Plc21C*, *Gγ1*, *Gαq* and *Stim* suppressed the body fat depletion caused by hyperactivation of the Akh pathway in adult *Drosophila*. Collectively, our data provide strong *in vivo* support for *Gαq/Gγ1/Plc21C/Stim* to act as a functional module in GPCR-dependent body fat control *via* iCa^{2+} in *Drosophila* flies, which can be employed by the Akh/AkhR signalling pathway.

RESULTS

Control of *Drosophila* fat storage by *in vivo* modulation of *Gαq*, *Gγ1* and *Plc21C*

To address the specific role of *Gαq*, *Gγ1* and *Plc21C* in lipid metabolism control, we first assayed changes of the fly body fat content upon functional impairment of these genes. We employed conditional *in vivo* modulation of gene functions to compare the impact on lipid storage in flies of the GPCR signal transducers *Gαq*, *Gγ1*, *Plc21C* and of *Stim* to the corresponding changes in lipid storage upon modulation of *Akh/AkhR*. Combination of a fat body-specific driver (Beller et al., 2010) of the binary UAS-GAL4 expression system (Brand and Perrimon, 1993) with the switchable ubiquitous repressor system TARGET (Mcguire, 2003) allowed for organ- and time-specific expression control of RNAi-mediated knockdown or overexpression of transgenes in adult flies, respectively. The gene knockdown and overexpression efficiencies are shown in Fig. S1A.

Adult male flies, which were subjected to fat body-specific *Stim* knockdown for six days, become massively obese (up to 180% more body fat; Fig. 1A and Baumbach et al., 2014). Similarly, the fly body fat content was more than doubled compared to controls in response to functional impairment of the *Gαq*, *Gγ1* and *Plc21C* genes (Fig. 1A). This adiposity matched to the obese phenotype of *AkhR* gene knockdown flies (body fat increased by 143%) or *AkhR* deletion mutants [body fat increased by 172%; Fig. 1A, Grönke et al., 2007; Bharucha et al., 2008]. Moreover, chronic RNAi-mediated depletion of the Akh peptide in the neuroendocrine corpora cardiaca cells (Fig. S1B) almost doubled the body fat content of flies (Fig. 1A). Conversely, *Akh* or *AkhR* overexpression

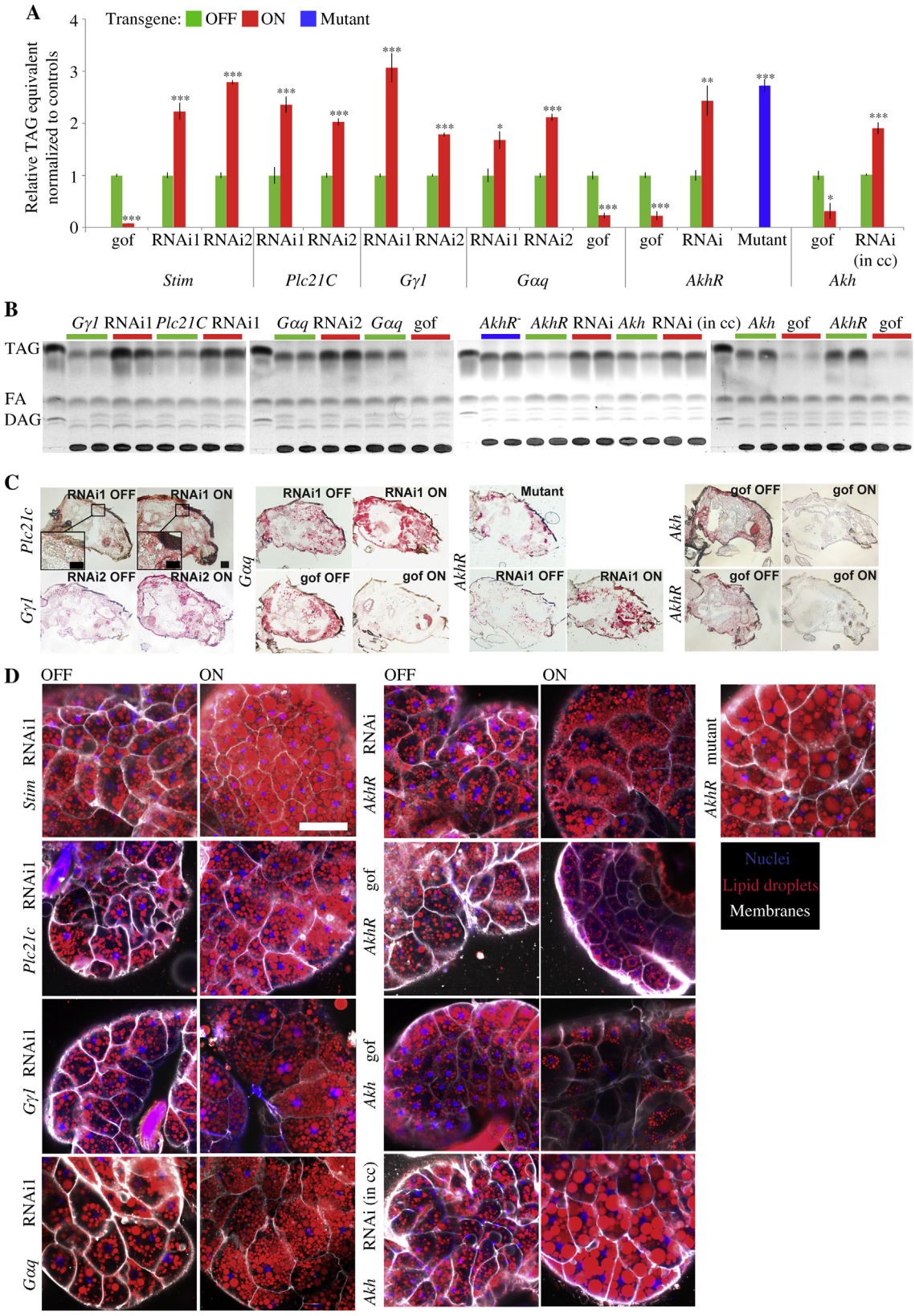

Fig. 1. Body fat storage control by modulation of *Stim*, *Plc21C*, *Gγ1*, *Gαq*, *AkhR* and *Akh* gene activities.
Comparison of adiposity in response to RNAi-mediated gene knockdown of *Stim*, *Plc21C*, *Gγ1*, *Gαq* and *AkhR* (in the adult fat storage tissue) or of the *Akh* gene (in corpora cardiaca (cc) cells) with the *AkhR*$^{-}$ deletion mutant. Overexpression (gof) of *Stim*, *Gαq* and *Akh* causes leanness in flies. Changes of body fat content was assayed by total body fat analysis (**A**), by thin layer chromatography (**B**), by Oil Red O staining on sagittal cryosections of adult male fly abdomen (**C**) and by confocal fluorescence microscopy imaging of *in vivo* adult virgin female fat body tissue (**D**; lipid droplets stained with LD540 in red, nuclei stained with DAPI in blue, membranes stained with CellMask in white). Image details in **C** show magnifications of the subcuticular fat body of *Plc21C* knockdown flies and controls. Statistical significance: $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$. Scale bar represents 100 μm (50 μm for image details) in **C** and 50 μm in **D**.

(referred to as gain-of-function abbreviated gof) in the adult fat body caused lean flies (body fat reduced by 70%; Fig. 1A). Similar lean flies resulted from overexpression of *Gαq* or *Stim* in adult fat body (body fat reduced by 77% and 93%, respectively; Fig. 1A).

Separation of neutral lipid classes by thin layer chromatography (TLC) confirmed, that all the described differences in total fly body fat content reflect changes in neutral storage lipids, i.e. triacylglycerides (TAGs) (Fig. 1B). Moreover, Oil Red O staining of lipids in histological sections showed that the lipid storage distribution in the abdominal fat body tissue corresponds to the total body fat and the TAG quantifications (Fig. 1C). These data suggest that the changes in the organismal fat content largely reflect the lipid loading of the fat body cells. To directly address this question, we imaged adult fat body tissue of virgin female flies after functional impairment of the *Gαq*, *Gγ1*, *Plc21C* and *Stim* genes and compared the effects to the modulation of *Akh* and *AkhR* gene activities (Fig. 1D). RNAi-mediated *in vivo* knockdown of the *Gαq*, *Gγ1*, *Plc21C* and *Stim* genes increased cellular lipid loading in fat body cells compared to the tissue from control flies. Similarly, lipid droplets (LDs) accumulated in fat body cells of obese *AkhR* deletion mutants and in flies subjected to RNAi-mediated knockdown of *AkhR* in the fat body or *Akh* in the neuroendocrine cells, respectively. Conversely, LDs were largely depleted from fat body cells of lean flies, which were subjected to *Akh* or *AkhR* overexpression.

Collectively, these findings showed that the GPCR signal transducers *Gαq*, *Gγ1* and *Plc21C* exert comparable effects on body fat control at the cellular and organismal level as does *Stim*. Similar changes in body fat content also resulted from the modulation of *Akh and AkhR* gene activities. These findings support the possibility that *Gαq*, *Gγ1*, *Plc21C* and *Stim* relay *Akh/AkhR* signalling in adult *Drosophila* to changes in the iCa^{2+} concentration of fat body cells. In support of this, *Akh/AkhR* has been demonstrated to signal *via* iCa^{2+} in other insect species such as the tobacco hornworm *M. sexta* (Arrese et al., 1999).

Gαq, *Gγ1* and *Plc21C* control iCa^{2+} concentrations in adult fat body cells

If body fat control by *Akh/AkhR* signalling in *Drosophila* would involve calcium second messenger regulation controlled by *Gαq*, *Gγ1*, *Plc21C* and *Stim*, the activities of all of these factors are expected to influence the iCa^{2+} concentrations in adult fat body cells. To image the iCa^{2+} concentrations of explanted adult fat body cells, we used the CaLexA system, which translates the iCa^{2+} concentrations to green fluorescence protein (GFP) reporter expression *via* the Ca^{2+}-dependent nuclear import of a synthetic transcription factor (Masuyama et al., 2012). As expected from a core gene of SOCE, the fat body iCa^{2+} concentration was decreased in response to the functional impairment of *Stim* (Fig. 2A, Baumbach et al., 2014). Similarly, RNAi-mediated gene knockdown of the *Gαq*, *Gγ1*, *Plc21C* and *AkhR* genes strongly reduced the adult fat body cell iCa^{2+} concentration compared to controls (Fig. 2).

Taken together, these findings showed that *Gαq*, *Gγ1* and *Plc21C* are involved in the control of the iCa^{2+} concentration and they support a reverse correlation between the iCa^{2+} concentration and the cellular lipid accumulation in adult fly fat body cells. Moreover, the data indicate that *AkhR* signals, at least in part, *via* the iCa^{2+} second messenger in the *Drosophila* adult fat body.

Gαq, *Gγ1* and *Plc21C* control the expression of the lipid metabolism effectors *midway* and *brummer*

It has been shown that short-term functional impairment of *Stim* causes an obesogenic transcriptional response in the adult fly fat body (Baumbach et al., 2014). At the onset of adiposity caused by RNAi-mediated *Stim* gene knockdown in the fat body (total body fat increased by 22%; Fig. 3A), the expression of the lipogenic gene *mdy*, which encodes *Drosophila* diacylglycerol O-acyltransferase 1, was significantly up-regulated by 52%. Concurrently, the lipolytic *bmm* gene was down-regulated by 37% (Fig. 3B). The same obesogenic pattern of gene regulation also resulted from the RNAi-mediated knockdown of *Gαq*, *Plc21C* or *Gγ1* in adult fat storage tissue. At the onset of *Plc21C*-dependent adiposity (body fat increased by 43%; Fig. 3A), the *mdy* gene expression was increased and the *bmm* gene expression decreased by 68% and 76%, respectively (Fig. 3B). Similarly, a *Gγ1*-dependent body fat increase by 34% (Fig. 3A) correlated with an almost doubled expression of the lipogenic *mdy* gene and the reduction of *bmm* gene expression by 57% (Fig. 3B). Also, at the beginning of *Gαq*-dependent storage fat accumulation by 29% (Fig. 3A), *mdy* gene expression was increased by 102% and *bmm* gene expression was reduced by 32% (Fig. 3B). Conversely, short-term overexpression of *Gαq* in the fat body caused lean flies (body fat decreased by 69%; Fig. 3A), which resulted in a lipolytic gene expression profile, i.e. the reduction of *mdy* by 40% and the up-regulation of *bmm* by 57% (Fig. 3B).

Taken together, the functional impairment of *Stim*, *Plc21C*, *Gγ1* and *Gαq* caused a comparable transcriptional response of the lipid metabolism regulators *mdy* and *bmm*, which is compatible with the hypothesis that all these genes act in the same signal transduction pathway. Notably, short-term gene knockdown of *Calmodulin* in the adult fat storage tissue evokes a similar transcriptional response of the *mdy* and *bmm* genes compared to *Gαq*, *Gγ1*, *Plc21C* and *Stim* (Baumbach et al., 2014). Since Calmodulin is regulated by iCa^{2+}, the observed obesogenic transcriptional regulation of *mdy* and *bmm* might represent a transcriptional readout of calcium signalling controlled by *Gαq*, *Gγ1*, *Plc21C* and *Stim*. Similar to these genes, the short-term functional impairment of *AkhR* caused an increase of 27% in body fat (Fig. 3A) and of 65% in *mdy* gene expression but *bmm* gene expression was unchanged compared to controls (Fig. 3B). Thus, *AkhR* signalling might employ the *Gαq/Gγ1/Plc21C/Stim* module of GPCR-dependent calcium signalling to control *mdy* gene transcription, but additionally uses other pathways for body fat storage control.

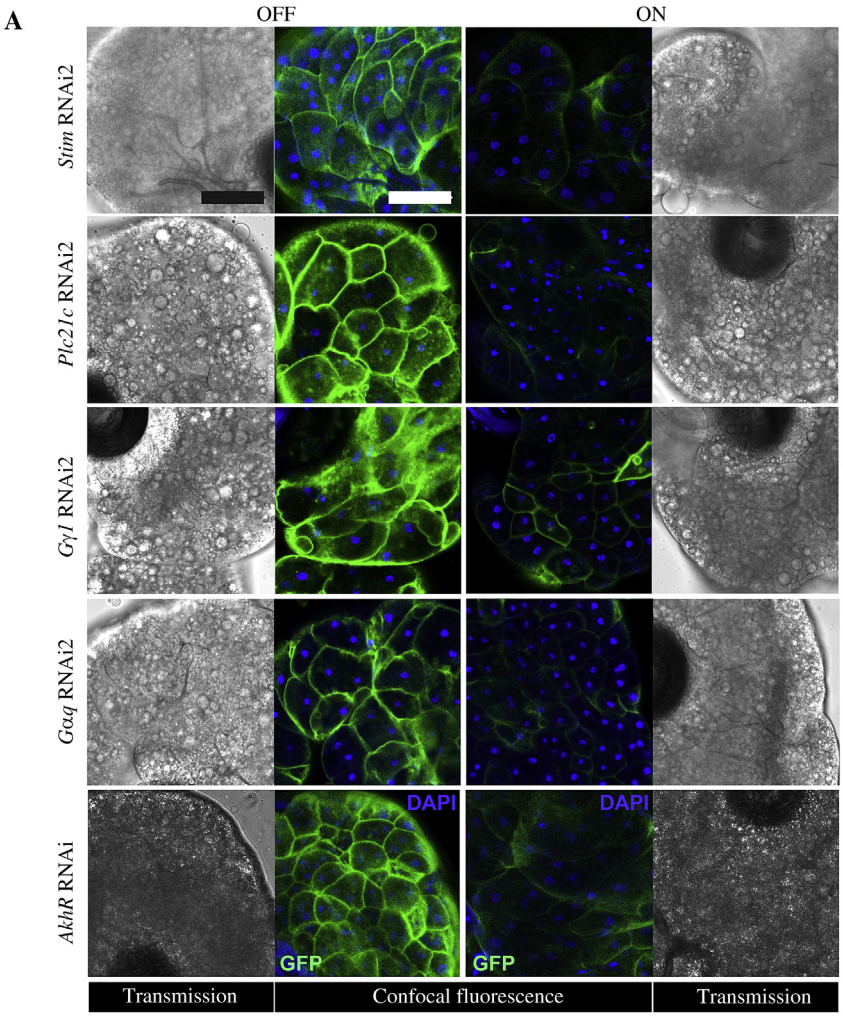

Fig. 2. Low iCa^{2+} concentration in adult virgin female fly adipose tissue upon RNAi-mediated gene knockdown of *Stim*, *Plc21C*, *Gγ1*, *Gαq* and *AkhR*.
A: Transmission microscopy images (outer panels) and confocal fluorescence microscopy images (inner panels) of *in vivo* fat body cells using the CaLexA system, which is based on the iCa^{2+}-dependent transcription of membrane-targeted GFP reporter protein (green). Nuclei are stained with DAPI (blue). Scale bar is 50 μm.
B: Image-based GFP signal quantification of the genotypes shown in **A**.

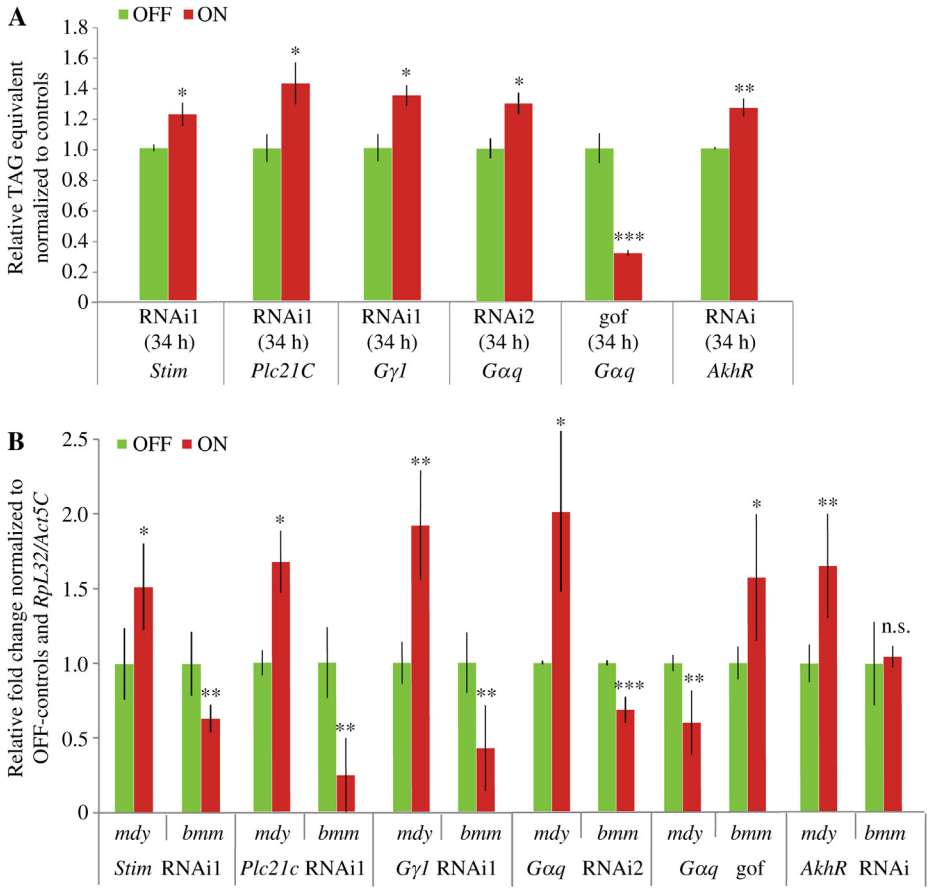

Fig. 3. Transcriptional regulation of the lipogenic *mdy* and the lipolytic *bmm* genes in response to body fat changes caused by short-term modulation of *Stim*, *Plc21C*, *Gγ1*, *Gαq* and *AkhR*.

A: Comparable increase (22%–43%) of total body fat in male flies subjected to a short-term gene knockdown of *Stim*, *Plc21C*, *Gγ1*, *Gαq* and *AkhR* in the adult fat storage tissue. Total body fat stores were depleted by 69% in response to a short-term overexpression (gof) of *Gαq* in the adult fat storage tissue. **B:** Transcriptional regulation of the lipogenic *mdy* and the lipolytic *bmm* genes in obese or lean flies in response to the modulation of *Stim*, *Plc21C*, *Gγ1*, *Gαq* and *AkhR* gene activities described in **A**. Shown are relative *mdy* and *bmm* transcript abundances in total fly abdomen compared to control flies lacking a driver transgene (OFF) assayed by qRT-PCR. n.s., non-significant, $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$.

Impairment of *Gαq*, *Gγ1* and *Plc21C* in the fat body suppresses storage lipid depletion by enhanced Akh signalling

Functional impairment of *Gαq*, *Gγ1* or *Plc21C* in the adult fat body leads to adiposity in flies (Fig. 1). Conversely, chronic activation of the *Akh/AkhR* signalling pathway by overexpression of *Akh* depletes the body fat stores (Grönke et al., 2007; Fig. 1). We performed genetic interaction experiments to address whether the knockdown of the *Gαq*, *Gγ1* or *Plc21C* genes would suppress the *Akh*-dependent body fat depletion in flies. The body fat content of flies subjected to simultaneous overexpression of *Akh* and down-regulation of *Stim* was more than doubled as compared to controls (Fig. 4). This fat accumulation was as severe as the obese phenotype of flies subjected to *Stim* single gene knockdown (Fig. 1A). In addition, the functional impairment of any of the *Gαq*, *Gγ1* and *Plc21C* genes suppressed the lean phenotype of *Akh*-overexpressing flies compared to control flies (Fig. 4). In particular, the gene knockdown of *Gαq* and *Plc21C* in *Akh*-overexpressing flies moderately increased the total body fat content by 34% and 47%, respectively. The functional impairment of *Gγ1* normalized the body fat content of flies to the level of control flies.

Collectively, the results of the genetic interaction analysis are compatible with the hypothesis that *Gαq*, *Gγ1*, *Plc21C* and *Stim* act as downstream factors of *Akh/AkhR* signalling in adult

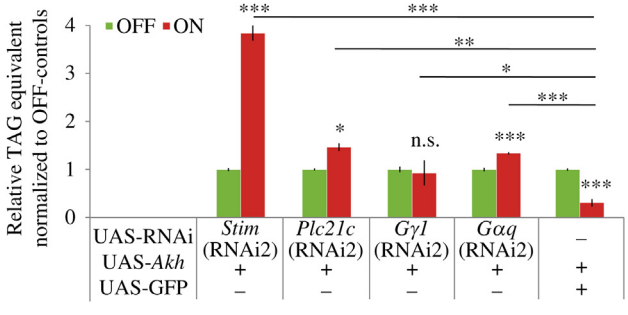

Fig. 4. Reversion of *Akh*-induced body fat depletion by fat storage tissue-targeted *Stim*, *Plc21C*, *Gγ1* and *Gαq* gene knockdown.

Shown is the total body fat content of male flies carrying an *Akh*-overexpression effector and RNAi effector transgenes in the presence (ON) or absence (OFF) of the adult fat body-specific driver. UAS-GFP was used to match the total number of UAS transgenes in all experiments. n.s., non-significant, $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$.

Drosophila fat storage tissue. Next to AkhR, other GPCRs might also employ *Gαq*, *Gγ1* and *Plc21C* to increase fat body cell iCa^{2+} levels in a *Stim*-dependent manner, which eventually results in storage lipid mobilization from the fly fat storage tissue.

DISCUSSION

Our study presents genetic evidence that *Gαq*, *Gγ1* and *Plc21C* act in body fat storage control of adult flies. Moreover, the finding that impairment of any of these genes lowered the iCa^{2+} levels in fat body cells supports the model that they couple GPCR signalling to calcium-dependent body fat mobilization in *Drosophila*. Our *in vivo* findings complement a wealth of biochemical data from non-dipteran insects, which demonstrate that iCa^{2+} is involved in Akh-mediated storage lipid mobilization. For example, in locusts, the *in vitro* lipid mobilization from *S. gregaria* fat body by corpora cardiaca extract was shown to depend on both cAMP and extracellular Ca^{2+} (Spencer and Candy, 1976). Similarly, in adult *L. migratoria*, the injection of Ca^{2+} ionophores mimics the Akh effect on lipid mobilization (Lum and Chino, 1990) and Akh signalling requires the influx of extracellular Ca^{2+} (Vroemen et al., 1995). Interestingly, studies in lepidoptera and locusta confirm that extracellular Ca^{2+} is necessary for the cAMP-dependent response to Akh (Wang et al., 1990; Arrese et al., 1999), which suggests a crosstalk between the cAMP and the Ca^{2+} branches of Akh signalling in storage fat mobilization. In support of this, pharmacological elevation of the iCa^{2+} in *M. sexta* fat body cells increases the activity of the cAMP-dependent protein kinase A (PKA) (Arrese et al., 1999), which in turn phosphorylates the LD-associated protein Perilipin 1 (Plin1) in *Manduca* and *Drosophila* (Patel et al., 2005; Arrese et al., 2008). This Plin1 phosphorylation has been proposed to increase the LD accessibility for TAG lipases, which facilitates lipid mobilization (Patel et al., 2005). Consistently, *Drosophila plin1* mutants are obese (Beller et al., 2010) as are flies with impaired *Gαq*, *Gγ1* or *Plc21C* function. Next to this post-translational regulation of lipolysis, transcriptional regulation is likely to contribute to Akh-dependent lipid mobilization. In *Drosophila*, comparable body fat accumulation results from overexpression of a dominant-negative version of CrebB, the cAMP response element binding protein B, or from the inactivation of *AkhR* (Iijima et al., 2009). Recently, tissue culture studies demonstrated that Akh-dependent CrebB activity requires not only cAMP but also $iCa2^{+}$ second messenger signalling including the calcium-controlled protein phosphatase called Calcineurin (Yang et al., 2013). Calcineurin, in turn, is controlled by Calmodulin, which we have previously shown to control body fat accumulation and transcriptional activation of the lipogenic *mdy* gene (Baumbach et al., 2014). The *in vivo* modulation of the *Gαq*, *Gγ1* or *Plc21C* genes now allows to address whether iCa^{2+} capacitates the cAMP-dependent transcriptional and posttranslational changes in response to Akh signalling in *Drosophila*.

Little is known about the transcriptional targets of Akh signalling. In this study, we demonstrated that the functional impairment of *Gαq*, *Gγ1* and *Plc21C* or *AkhR* in the fly fat body increased the transcription of the lipogenic *mdy* gene. Conversely, *mdy* expression was reduced upon fat body-targeted overexpression of *Gαq*, which mimics the lean phenotype of Akh pathway hyperactivation. Since hypomorphic *mdy* mutant flies are also lean (Beller et al., 2010), this gene is a candidate factor to transmit the Akh-induced lipogenesis inhibition observed in non-dipteran insects such as *S. gregaria* and *G. bimaculatus* (Gokuldas et al., 1988; Lorenz, 2001). In contrast to *mdy*, the transcription of *bmm* depends on *Gαq*, *Gγ1*, *Plc21C* and *Stim* but not on *AkhR*, in support of our earlier finding that *Akh/AkhR* and *bmm* act in different but cross-communicating lipid mobilization pathways in the fly (Grönke et al., 2007).

Besides storage lipid mobilization in the fat body, Akh signalling affects complex behaviours including locomotion and food intake control in a variety of insects (Lorenz et al., 2004; Wicher et al., 2006; Katewa et al., 2012; Konuma et al., 2012). Therefore, systemic Akh coordinates organismal functions, which necessarily involve the crosstalk between various organs. It remains challenging to differentiate which of these functions are a direct consequence of Akh binding to AkhR-expressing target organs and which are secondary consequences of inter-organ communication processes. Our data support that Akh/AkhR signalling employs the Ca^{2+} second messenger in the adult *Drosophila* fat body. Decreasing the fat body iCa^{2+} level by functional impairment of *Stim* caused obesity (Fig. 1), which depends on hyperphagia. This *Stim*-dependent hyperphagia involves the remote up-regulation of the orexigenic short neuropeptide F (*sNPF*) gene in the central nervous system (Baumbach et al., 2014). Notably, hyperphagia has also been reported in response to *AkhR* gene knockdown in *G. bimaculatus* (Konuma et al., 2012). In *Drosophila*, *AkhR* knockdown reduced fat body iCa^{2+} (Fig. 2A). Therefore, a potential feedback regulation between fat body and brain during the Akh-induced lipid mobilization deserves future research attention.

In this study, thermogenetic conditional gene knockdown was exploited to identify and characterize *Gαq*, *Gγ1* and *Plc21C* as novel body fat storage regulators using the *Drosophila* model system. This unique approach holds great promise for future insights into organ-autonomous functions as well as inter-organ communication processes underlying organismal storage lipid homeostasis in insects.

MATERIALS AND METHODS

Fly stocks and husbandry

Transgenic RNAi fly strains used in this study were made available by either the VDRC [GD and KK library, (Dietzl et al., 2007) and www.vdrc.at] or the BDSC (Harvard TRiP library, www.flyrnai.org). Unless stated differently, the flies were propagated as described (Grönke et al., 2007). The fly stocks used in this study are listed in Table S1.

Young flies (24–48 h after hatching) were kept for 5–6 days on standard food (20 L H_2O, 125 g agar, 360 g dry yeast, 200 g

soy flour, 440 g treacle, 1.6 kg cornmeal, 1.6 kg malt, 125 mL propionic acid and 30 g nipagine). Fly progeny containing (ON) or lacking (OFF) the ts-FB-Gal4 driver was raised under repressed conditions (18°C) until hatching and then induced on active conditions (30°C) for six days. Alternatively, young flies (24–48 h) were kept for six additional days on repressed conditions and exposed to active conditions for short-term (34 h, Fig. 3) or long-term (six days, Fig. 4). Flies containing the non-conditional FB-Gal4 fat body driver (ON) were raised at 25°C until hatching and afterwards kept either at 18°C for 6–7 days prior to CaLexA GFP reporter imaging or at 25°C for 5 days prior to fat body imaging (*Akh* RNAi). Control flies (OFF) were generated by crossing the respective transgenic effector lines against *w*-control flies.

For conditional transgene induction using the SWITCH system (Roman et al., 2001), flies containing the *da*-GS driver (Tricoire et al., 2009) and UAS-RNAi constructs were raised on standard food at 25°C until one day after hatching and afterwards kept for three days on standard food containing either 200 μmol/L RU486 (mifepristone; M8046, Sigma, USA; stock solution 10 mmol/L in 80% ethanol; ON-condition) or solvent only (OFF-condition).

Histology, microscopy and image analysis

Cryosections were done on adult male progeny of flies carrying effector transgenes crossed against ts-FB-Gal4 or *w*-control flies, raised under repressed conditions and fed for six days under active conditions. Wings were removed and whole flies were incubated in OCT medium (Leica, Germany) for 6 h prior to freezing on dry ice. Sections of 18 μm at −16°C/−13°C chamber/object temperature were done on a Leica CM 3050 S cryostat (Leica, Germany) and transferred on coated slides (Superfrost Plus; Thermo Fisher Scientific, USA). Sections were dried for 5 min at 30°C prior to staining. For Oil Red O staining, cryosections were fixed in 5% paraformaldehyde/1 × PBS for 30 min. Specimens were washed three times in 1 × PBS and covered with propylene glycol (W294004, Sigma, USA) for 10 min. Sections were stained in 0.5% Oil Red O (O0625, Sigma, Germany) in propylene glycol for 3 h at room temperature. Afterwards, the staining solution was replaced by propylene glycol for 10 min and the propylene glycol removed by dipping three times in 1 × PBS. Sections were mounted and imaged on a Zeiss Axiophot microscope (Zeiss, Germany) in bright field mode equipped with a ProgRes 3012 camera (Jenoptik, Germany).

For adult fat body imaging by confocal microscopy, transgenic flies with (ON) or without (OFF) ts-FB-Gal4 driver were aged six days under active conditions, while *Akh* RNAi flies were crossed against *Akh*-Gal4 flies or *w*-controls and virgin female progeny was kept for five days on 25°C before imaging. Abdominal deep fat body tissue was manually dissected and embedded in 1 × PBS containing the following dyes: 5 μg/mL LD540 (Spandl et al., 2009) for lipid droplets, 1 μg/mL DAPI (D1306, Invitrogen/Thermo Fisher Scientific, USA) for nuclei and 0.5 μg/mL CellMask (C10046, Invitrogen/Thermo Fisher Scientific) for membranes. Fat body tissue was imaged on a Zeiss LSM-780 (Zeiss, Germany) at 25× magnification in fixed gain mode.

For imaging of the iCa^{2+} concentration using the CaLexA system (Masuyama et al., 2012), abdominal deep fat body tissue of 6−7-day-old virgin progeny (at 18°C) from the CaLexA reporter flies crossed against FB-Gal4 flies or FB-Gal4 UAS-RNAi flies was dissected as described above, embedded in 1 × PBS containing DAPI and imaged as described. Image areas of 134 × 134 μm abdominal deep fat body tissue from at least five females per genotype were used for CaLexA reporter GFP intensity quantifications using ImageJ (NIH, USA). Intensity values were normalized to intensities of RNAi OFF control genotypes. Confocal fluorescence image channel splitting and merging was done with ImageJ software.

Lipid analysis

Organismal fat content (expressed as total glycerides) of 6-day-old male flies was quantified by a coupled colorimetric assay as described (Grönke et al., 2003; Hildebrandt et al., 2011). Depicted are representative experiments with average values of triplicate measurements (on five flies per replicate) and corresponding standard deviations. Experiments were repeated at least twice.

For TLC analysis, fly lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959). Transgenic flies with (ON) or without (OFF) the ts-FB-Gal4 driver were raised under repressed conditions (18°C) and induced for 6 days under active conditions (30°C) directly after hatching. Five flies per replicate (two replicates) were homogenized in a 0.5 mL screw cap tube containing 150 μL of methanol, 75 μL of chloroform and 60 μL of water and ten 1.4 mm ceramic beads (Peqlab, Germany) using a peqlab Precellys 24 instrument (10 s, 5000 r/min; Peqlab). Lipids were extracted for 1 h in a water bath at 37°C before adding 75 μL of chloroform (mixed well by vortexing) and 75 μL of 1 mol/L KCl (mixed thoroughly). Separation of phases was achieved by centrifugation at 1000 *g* for 2 min and the chloroform phase (lower phase) was transferred into a new Eppendorf tube. Solvent was evaporated in a SpeedVac concentrator and the lipid pellets were resuspended in 100 μL of chloroform/methanol (1:1, v/v). Lipid equivalent to one fly (20 μL) was separated on high performance TLC plates (105633, Merck, Germany) using n-hexane/diethyl ether/acetic acid (70:30:1, v/v/v; Merck). The following standard lipids were loaded: 40 μg of trioleoylglycerol (TAG standard; T7140, Sigma, USA), 4 μg of pentadecanoin (DAG standard; D8508, Sigma) and 4 μg of oleic acid (FA standard; 4954, Calbiochem, Germany). Plates were air dried, dipped into 8% (w/v) H_3PO_4 containing 10% (w/v) copper (II) sulphate pentahydrate and charred at 180°C for 5 min.

Quantitative RT-PCR

Quantitative RT-PCR analysis was performed as described (Grönke et al., 2009). Flies were snap frozen in liquid nitrogen and RNA of whole abdomen was extracted using the TRIzol reagent (15596026, Ambion/Thermo Fisher Scientific, USA)

and the Direct-zol RNA MiniPrep Kit (Zymo Research, USA) according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR analysis was performed on a Qiagen Rotor-Gene Q using the Rotor-Gene SYBR Green PCR Kit (204174, Qiagen) with Ribosomal protein L32 (*RpL32*) and Actin 5C (*Act5C*) as reference genes for normalization. Quantifications of mRNA abundance from 2 to 3 biological independent samples are shown with standard deviations and significances. Primer sequences are listed in Table S2.

Statistical analysis

If not stated otherwise, error bars represent standard deviations SD among replicate experiments and the statistical significance of differences between datasets was analysed using the unpaired *t*-test and expressed as *P*-values.

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SUPPLEMENTARY DATA

Fig. S1. *Stim*, *Plc21C*, *Gγ1*, *Gαq*, *AkhR and Akh* gene regulation in response to transgenic gene knockdown or gene overexpression at the transcriptional and protein level.

Table S1. List of fly stocks.

Table S2. List of oligonucleotide primers.

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REFERENCES

Anand, A.N., Lorenz, M.W., 2008. Age-dependent changes of fat body stores and the regulation of fat body lipid synthesis and mobilisation by adipokinetic hormone in the last larval instar of the cricket, *Gryllus bimaculatus*. J. Insect Physiol. 54, 1404−1412.

Arrese, E., Rojas-Rivas, B., Wells, M., 1996. The use of decapitated insects to study lipid mobilization in adult *Manduca sexta*: effects of adipokinetic hormone and trehalose on fat body lipase activity. Insect Biochem. Mol. Biol. 26, 775−782.

Arrese, E.L., Flowers, M.T., Gazard, J.L., Wells, M.A., 1999. Calcium and cAMP are second messengers in the adipokinetic hormone-induced lipolysis of triacylglycerols in *Manduca sexta* fat body. J. Lipid Res. 40, 556−564.

Arrese, E.L., Rivera, L., Hamada, M., Mirza, S., Hartson, S.D., Weintraub, S., Soulages, J.L., 2008. Function and structure of lipid storage droplet protein 1 studied in lipoprotein complexes. Arch. Biochem. Biophys. 473, 42−47.

Auerswald, L., Gäde, G., 2006. Endocrine control of TAG lipase in the fat body of the migratory locust, *Locusta migratoria*. Insect Biochem. Mol. Biol. 36, 759−768.

Auerswald, L., Siegert, K., Gäde, G., 2005. Activation of triacylglycerol lipase in the fat body of a beetle by adipokinetic hormone. Insect Biochem. Mol. Biol. 35, 461−470.

Baumbach, J., Hummel, P., Bickmeyer, I., Kowalczyk, K.M., Frank, M., Knorr, K., Hildebrandt, A., Riedel, D., Jäckle, H., Kühnlein, R.P., 2014. A *Drosophila in vivo* screen identifies store-operated calcium entry as a key regulator of adiposity. Cell Metab. 19, 331−343.

Beenakkers, A.M., 1969. The influence of Corpus Allatum and Corpus Cardiacum on lipid metabolism in *Locusta migratoria*. Gen. Comp. Endocrinol. 13, 492.

Beller, M., Bulankina, A.V., Hsiao, H.-H., Urlaub, H., Jäckle, H., Kühnlein, R.P., 2010. PERILIPIN-dependent control of lipid droplet structure and fat storage in *Drosophila*. Cell Metab. 12, 521−532.

Bharucha, K.N., Tarr, P., Zipursky, S.L., 2008. A glucagon-like endocrine pathway in *Drosophila* modulates both lipid and carbohydrate homeostasis. J. Exp. Biol. 211, 3103−3110.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911−917.

Brand, A., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401−415.

Buszczak, M., Lu, X., Segraves, W.A., Chang, T.Y., Cooley, L., 2002. Mutations in the midway gene disrupt a *Drosophila* acyl coenzyme A: diacylglycerol acyltransferase. Genetics 160, 1511−1518.

Cahalan, M.D., 2009. STIMulating store-operated Ca^{2+} entry. Nat. Cell Biol. 11, 669−677.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., Dickson, B.J., 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature 448, 151−156.

Fischer, J., Lefèvre, C., Morava, E., Mussini, J.-M., Laforêt, P., Negre-Salvayre, A., Lathrop, M., Salvayre, R., 2007. The gene encoding adipose triglyceride lipase (PNPLA2) is mutated in neutral lipid storage disease with myopathy. Nat. Genet. 39, 28−30.

Gäde, G., Auerswald, L., 2003. Mode of action of neuropeptides from the adipokinetic hormone family. Gen. Comp. Endocrinol. 132, 10−20.

Gokuldas, M., Hunt, P.A., Candy, D.J., 1988. The inhibition of lipid synthesis *in vitro* in the locust, *Schistocerca gregaria*, by factors from the corpora cardiaca. Physiol. Entomol. 13, 43−48.

Grönke, S., Beller, M., Fellert, S., Ramakrishnan, H., Jäckle, H., Kühnlein, R.P., 2003. Control of fat storage by a *Drosophila* PAT domain protein. Curr. Biol. 13, 603−606.

Grönke, S., Bickmeyer, I., Wunderlich, R., Jäckle, H., Kühnlein, R.P., 2009. Curled encodes the *Drosophila* homolog of the vertebrate circadian deadenylase Nocturnin. Genetics 183, 219−232.

Grönke, S., Mildner, A., Fellert, S., Tennagels, N., Petry, S., Müller, G., Jäckle, H., Kühnlein, R.P., 2005. Brummer lipase is an evolutionary conserved fat storage regulator in *Drosophila*. Cell Metab. 1, 323−330.

Grönke, S., Müller, G., Hirsch, J., Fellert, S., Andreou, A., Haase, T., Jäckle, H., Kühnlein, R.P., 2007. Dual lipolytic control of body fat storage and mobilization in *Drosophila*. PLoS Biol. 5, e137.

Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., Heldmaier, G., Maier, R., Theussl, C., Eder, S., Kratky, D., Wagner, E.F., Klingenspor, M., Hoefler, G., Zechner, R., 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 312, 734−737.

Hildebrandt, A., Bickmeyer, I., Kühnlein, R.P., 2011. Reliable *Drosophila* body fat quantification by a coupled colorimetric assay. PLoS ONE 6, e23796.

Huang, H., He, X., Deng, X., Li, G., Ying, G., Sun, Y., Shi, L., Benovic, J.L., Zhou, N., 2010. *Bombyx* adipokinetic hormone receptor activates extracellular signal-regulated kinase 1 and 2 *via* G protein-dependent PKA and

PKC but β-arrestin-independent pathways. Biochemistry 49, 10862−10872.
Iijima, K., Zhao, L., Shenton, C., Iijima-Ando, K., 2009. Regulation of energy stores and feeding by neuronal and peripheral CREB activity in *Drosophila*. PLoS ONE 4, e8498.
Katewa, S.D., Demontis, F., Kolipinski, M., Hubbard, A., Gill, M.S., Perrimon, N., Melov, S., Kapahi, P., 2012. Intramyocellular fatty-acid metabolism plays a critical role in mediating responses to dietary restriction in *Drosophila melanogaster*. Cell Metab. 16, 97−103.
Konuma, T., Morooka, N., Nagasawa, H., Nagata, S., 2012. Knockdown of the adipokinetic hormone receptor increases feeding frequency in the two-spotted cricket *Gryllus bimaculatus*. Endocrinology 153, 3111−3122.
Kurat, C., Natter, K., Petschnigg, J., Wolinski, H., Scheuringer, K., Scholz, H., Zimmermann, R., Leber, R., Zechner, R., Kohlwein, S., 2006. Obese yeast: triglyceride lipolysis is functionally conserved from mammals to yeast. J. Biol. Chem. 281, 491−500.
Lee, G., Park, J.H., 2004. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics 167, 311−323.
Lindemans, M., Janssen, T., Beets, I., Temmerman, L., Meelkop, E., Schoofs, L., 2011. Gonadotropin-releasing hormone and adipokinetic hormone signaling systems share a common evolutionary origin. Front. Endocrinol. 2, 16.
Lorenz, M., 2001. Synthesis of lipids in the fat body of *Gryllus bimaculatus:* age- dependency and regulation by adipokinetic hormone. Arch. Insect Biochem. Physiol. 47, 198−214.
Lorenz, M.W., Gäde, G., 2009. Hormonal regulation of energy metabolism in insects as a driving force for performance. Integr. Comp. Biol. 49, 380−392.
Lorenz, M.W., Zemek, R., Kodrík, D., Socha, R., 2004. Lipid mobilization and locomotor stimulation in *Gryllus bimaculatus* by topically applied adipokinetic hormone. Physiol. Entomol. 29, 146−151.
Lum, P.Y., Chino, H., 1990. Primary role of adipokinetic hormone in the formation of low density lipophorin in locusts. J. Lipid Res. 31, 2039−2044.
Masuyama, K., Zhang, Y., Rao, Y., Wang, J.W., 2012. Mapping neural circuits with activity-dependent nuclear import of a transcription factor. J. Neurogenet. 26, 89−102.
Mayer, R.J., Candy, D.J., 1969. Control of haemolymph lipid concentration during locust flight: an adipokinetic hormone from the corpora cardiaca. J. Insect Physiol. 15, 611−620.
Mcguire, S.E., 2003. Spatiotemporal rescue of memory dysfunction in *Drosophila*. Science 302, 1765−1768.
Ogoyi, D.O., Osir, E.O., Olembo, N.K., 1998. Fat body triacylglycerol lipase in solitary and gregarious phases of *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae). Comp. Biochem. Physiol. 119B, 163−167.
Patel, R.T., Soulages, J.L., Hariharasundaram, B., Arrese, E.L., 2005. Activation of the lipid droplet controls the rate of lipolysis of triglycerides in the insect fat body. J. Biol. Chem. 280, 22624−22631.
Pospisilik, J.A., Schramek, D., Schnidar, H., Cronin, S.J.F., Nehme, N.T., Zhang, X., Knauf, C., Cani, P.D., Aumayr, K., Todoric, J., Bayer, M., Haschemi, A., Puviindran, V., Tar, K., Orthofer, M., Neely, G.G., Dietzl, G., Manoukian, A., Funovics, M., Prager, G., Wagner, O., Ferrandon, D., Aberger, F., Hui, C.-C., Esterbauer, H., Penninger, J.M., 2010. *Drosophila* genome-wide obesity screen reveals hedgehog as a determinant of brown *versus* white adipose cell fate. Cell 140, 148−160.
Roman, G., Endo, K., Zong, L., Davis, R.L., 2001. P[Switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 98, 12602−12607.
Slocinska, M., Antos-Krzeminska, N., Golebiowski, M., Kuczer, M., Stepnowski, P., Rosinski, G., Jarmuszkiewicz, W., 2013. UCP4 expression changes in larval and pupal fat bodies of the beetle *Zophobas atratus* under adipokinetic hormone treatment. Comp. Biochem. Physiol. A 166, 52−59.
Spandl, J., White, D.J., Peychl, J., Thiele, C., 2009. Live cell multicolor imaging of lipid droplets with a new dye, LD540. Traffic 10, 1579−1584.
Spencer, I.M., Candy, D.J., 1976. Hormonal control of diacyl glycerol mobilization from fat body of the desert locust, *Schistocerca gregaria*. Insect Biochem. 6, 289−296.
Stone, J.V., Mordue, W., Batley, K.E., Morris, H.R., 1976. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. Nature 263, 207−211.
Subramanian, M., Metya, S.K., Sadaf, S., Kumar, S., Schwudke, D., Hasan, G., 2013. Altered lipid homeostasis in *Drosophila* InsP3 receptor mutants leads to obesity and hyperphagia. Dis. Model. Mech. 6, 734−744.
Tricoire, H., Battisti, V., Trannoy, S., Lasbleiz, C., Pret, A.M., Monnier, V., 2009. The steroid hormone receptor EcR finely modulates *Drosophila* lifespan during adulthood in a sex-specific manner. Mech. Ageing Dev. 130, 547−552.
Vroemen, S.F., van Marrewijk, W.J., Schepers, C.C., van der Horst, D.J., 1995. Signal transduction of adipokinetic hormones involves Ca^{2+} fluxes and depends on extracellular Ca^{2+} to potentiate cAMP-induced activation of glycogen phosphorylase. Cell Calcium 17, 459−467.
Wang, Z., Hayakawa, Y., Downer, R.G., 1990. Factors influencing cyclic AMP and diacylglycerol levels in fat body of *Locusta migratoria*. Insect Biochem. 20, 325−330.
Wicher, D., Agricola, H., Sohler, S., Gundel, M., Heinemann, S., Wollweber, L., Stengl, M., Derst, C., 2006. Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. J. Neurophysiol. 95, 2314−2325.
Yang, H., He, X., Yang, J., Deng, X., Liao, Y., Zhang, Z., Zhu, C., Shi, Y., Zhou, N., 2013. Activation of cAMP-response element-binding protein is positively regulated by PKA and calcium-sensitive calcineurin and negatively by PKC in insect. Insect Biochem. Mol. Biol. 43, 1028−1036.
Zhu, C., Huang, H., Hua, R., Li, G., Yang, D., Luo, J., Zhang, C., Shi, L., Benovic, J.L., Zhou, N., 2009. Molecular and functional characterization of adipokinetic hormone receptor and its peptide ligands in *Bombyx mori*. FEBS Lett. 583, 1463−1468.
Ziegler, R., Jasensky, R.D., Morimoto, H., 1995. Characterization of the adipokinetic hormone receptor form the fat body of *Manduca sexta*. Regul. Pept. 57, 329−338.