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

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Received 31 October 2013; revised 26 February 2014; accepted 9 March 2014

Available online 27 March 2014

**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 (Gγ1), 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 (*DmDGAT1*) and *bmm* encodes a triglyceride lipase (*DmATGL*), 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
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 Ca²⁺ (iCa²⁺) 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 iCa²⁺ increase in fat body cells in the context of storage lipid mobilization.

We and others have recently shown a reverse correlation between the iCa²⁺ 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 iCa²⁺ 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 Ca²⁺ stores. ER Ca²⁺ 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 Gz and Gy subunits.

A recent genetic screen identified the G protein Gzq and Gy subunits encoded by G protein α q (Gaq) 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, Gaq and the Plcβ encoded by Phospholipase C at 21C (Plc21C) act upstream of SOCE and therefore possibly link AkhR signalling to iCa²⁺-dependent body fat control in adult Drosophila.

Our study provides evidence that functional impairment of Plc21C, Gγ1, Gaq, Stim and AkhR lowers the iCa²⁺ concentration in adult fat body cells in vivo. Modulation of the Plc21C, Gγ1 and Gaq gene activities caused the same changes to cellular and organisal fat storage as does the repression or overexpression of the SOCE member Stim or of Akh/AkhR. Moreover, changes in Plc21C, Gγ1, Gaq, 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, Gaq 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 Gaq/Gy1/Plc21C/Stim to act as a functional module in GPCR-dependent body fat control via iCa²⁺ in Drosophila flies, which can be employed by the Akh/AkhR signalling pathway.

RESULTS

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

To address the specific role of Gaq, 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 Gaq, 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 Gaq, 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, Gaq and AkhR activities.

Comparison of adiposity in response to RNAi-mediated gene knockdown of Stim, Plc21C, Gγ1, Gaq 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, Gaq and Akh causes leaness 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 ≤ 0.05, **P ≤ 0.01 and ***P ≤ 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 Gaq 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 organ- ismal 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 impair- ment of the gene knockdown in the fat body or 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 Gaq, 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 genes activities. These findings support the possibility that Gaq, Gγ1, Plc21C and Stim relay Akh/AkhR signalling in adult Drosophila to changes in the iCa2+ concentration of fat body cells. In support of this, Akh/AkhR has been demonstrated to signal via iCa2+ in other insect species such as the tobacco hornworm M. sexta (Arrese et al., 1999).

**Gaq, Gγ1 and Plc21C control iCa2+ concentrations in adult fat body cells**

If body fat control by Akh/AkhR signalling in Drosophila would involve calcium second messenger regulation controlled by Gaq, Gγ1, Plc21C and Stim, the activities of all of these factors are expected to influence the iCa2+ concentrations in adult fat body cells. To image the iCa2+ concentrations of explanted adult fat body cells, we used the CaLexA system, which translates the iCa2+ concentrations to green fluorescence protein (GFP) reporter expression via the Ca2+- dependent nuclear import of a synthetic transcription factor (Masuyama et al., 2012). As expected from a core gene of SOCE, the fat body iCa2+ concentration was decreased in response to the functional impairment of Stim (Fig. 2A, Baumbach et al., 2014). Similarly, RNAi-mediated gene knockdown of the Gaq, Gγ1, Plc21C and AkhR genes strongly reduced the adult fat body cell iCa2+ concentration compared to controls (Fig. 2).

Taken together, these findings showed that Gaq, Gγ1 and Plc21C are involved in the control of the iCa2+ concentration and they support a reverse correlation between the iCa2+ 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 iCa2+ second messenger in the Drosophila adult fat body.

**Gaq, 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 Gaq, 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 Gaq-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 Gaq 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 Gaq 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 Gaq, Gγ1, Plc21C and Stim (Baumbach et al., 2014). Since Calmodulin is regulated by iCa2+, the observed obesogenic transcriptional regulation of mdy and bmm might represent a transcriptional readout of calcium signalling controlled by Gaq, 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 Gaq/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\textsuperscript{2+} concentration in adult virgin female fly adipose tissue upon RNAi-mediated gene knockdown of Stim, Plc21C, G\gamma1, G\alphaq 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\textsuperscript{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.
Impairment of \textit{Gaq}, \textit{Gg1} and \textit{Plc21C} in the fat body suppresses storage lipid depletion by enhanced Akh signalling

Functional impairment of \textit{Gaq}, \textit{Gg1} or \textit{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 \textit{Akh} depletes the body fat stores (Gronke et al., 2007; Fig. 1). We performed genetic interaction experiments to address whether the knockdown of the \textit{Gaq}, \textit{Gg1} or \textit{Plc21C} genes would suppress the \textit{Akh}-dependent body fat depletion in flies. The body fat content of flies subjected to simultaneous overexpression of \textit{Akh} and down-regulation of \textit{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 \textit{Stim} single gene knockdown (Fig. 1A). In addition, the functional impairment of any of the \textit{Gaq}, \textit{Gg1} and \textit{Plc21C} genes suppressed the lean phenotype of \textit{Akh}-overexpressing flies compared to control flies (Fig. 4). In particular, the gene knockdown of \textit{Gaq} and \textit{Plc21C} in \textit{Akh}-overexpressing flies moderately increased the total body fat content by 34% and 47%, respectively. The functional impairment of \textit{Gg1} 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 \textit{Gaq}, \textit{Gg1}, \textit{Plc21C} and \textit{Stim} act as downstream factors of Akh/AkhR signalling in adult flies.

Fig. 3. Transcriptional regulation of the lipogenic \textit{mdy} and the lipolytic \textit{bmm} genes in response to body fat changes caused by short-term modulation of \textit{Stim}, \textit{Plc21C}, \textit{Gg1}, \textit{Gaq} and \textit{AkhR}.

A: Comparable increase (22%–43%) of total body fat in male flies subjected to a short-term gene knockdown of \textit{Stim}, \textit{Plc21C}, \textit{Gg1}, \textit{Gaq} and \textit{AkhR} in the adult fat storage tissue. Total body fat stores were depleted by 69% in response to a short-term overexpression (gof) of \textit{Gaq} in the adult fat storage tissue. B: Transcriptional regulation of the lipogenic \textit{mdy} and the lipolytic \textit{bmm} genes in obese or lean flies in response to the modulation of \textit{Stim}, \textit{Plc21C}, \textit{Gg1}, \textit{Gaq} and \textit{AkhR} gene activities described in A. Shown are relative \textit{mdy} and \textit{bmm} transcript abundances in total fly abdomen compared to control flies lacking a driver transgene (OFF) assayed by qRT-PCR. n.s., non-significant, *\textit{P} \leq 0.05, **\textit{P} \leq 0.01 and ***\textit{P} \leq 0.001.

Fig. 4. Reversion of \textit{Akh}-induced body fat depletion by fat storage tissue-targeted \textit{Stim}, \textit{Plc21C}, \textit{Gg1} and \textit{Gaq} gene knockdown.

Shown is the total body fat content of male flies carrying an \textit{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, *\textit{P} \leq 0.05, **\textit{P} \leq 0.01 and ***\textit{P} \leq 0.001.
**Drosophila** fat storage tissue. Next to AkhR, other GPCRs might also employ Gaq, Gγ1 and Plc21C to increase fat body cell iCa²⁺ 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 Gaq, Gγ1 and Plc21C act in body fat storage control of adult flies. Moreover, the finding that impairment of any of these genes lowered the iCa²⁺ 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 iCa²⁺ 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 Ca²⁺ (Spencer and Candy, 1976). Similarly, in adult *L. migratoria*, the injection of Ca²⁺ ionophores mimics the Akh effect on lipid mobilization (Lum and Chino, 1990) and Akh signalling requires the influx of extracellular Ca²⁺ (Vroemen et al., 1995). Interestingly, studies in lepidoptera and locusta confirm that extracellular Ca²⁺ 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 Ca²⁺ branches of Akh signalling in storage fat mobilization. In support of this, pharmacological elevation of the iCa²⁺ 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 Gaq, 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 Gaq, Gγ1 or Plc21C genes now allows to address whether iCa²⁺ 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 Gaq, 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 *Gaq*, 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 *Gaq*, 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 employing the Ca²⁺ second messenger in the adult *Drosophila* fat body. Decreasing the fat body iCa²⁺ 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 iCa²⁺ (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 Gaq, 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 H₂O, 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 (Ahk 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 (00625, 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 Axioptik 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) 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) H3PO4 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.

ACKNOWLEDGMENTS

We thank Regina Krügener for technical assistance and the Vienna Drosophila RNAi Center (VDRC), the Bloomingtom Drosophila Stock Center (BDSC), and TriP at Harvard Medical School (NIH/NIARDS R01GM084947) for fly stocks. Dr. Seung Kim is acknowledged for anti-Akh antisemur, Dr. Christoph Thiele for LD540 lipid dye and Dr. Thomas Riemensperger for help with the immunohistochemistry. This work was supported by the Max Planck Society and by the German research foundation (No. DFG KU 2866/1-1 to R.P.K.).

SUPPLEMENTARY DATA

Fig. S1. Stim, Plc21C, Gγ1, Gaq, Akt1R and Areh 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.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2014.03.005.

REFERENCES


