Dual Lipolytic Control of Body Fat Storage and Mobilization in Drosophila

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Energy homeostasis is a fundamental property of animal life, providing a genetically fixed balance between fat storage and mobilization. The importance of body fat regulation is emphasized by dysfunctions resulting in obesity and lipodystrophy in humans. Packaging of storage fat in intracellular lipid droplets, and the various molecules and mechanisms guiding storage-fat mobilization, are conserved between mammals and insects. We generated a lipodystrophy in humans. Packaging of storage fat in intracellular lipid droplets, and the various molecules and mobilization. The importance of body fat regulation is emphasized by dysfunctions resulting in obesity and lipodystrophy in humans. Packaging of storage fat in intracellular lipid droplets, and the various molecules and mechanisms guiding storage-fat mobilization, are conserved between mammals and insects. We generated a lipodystrophy in humans. Packaging of storage fat in intracellular lipid droplets, and the various molecules and

Introduction

Tightly regulated storage-fat accumulation and mobilization are a central characteristic of organismal energy homeostasis. In organisms as different as flies and man, body fat reserves are primarily stored as triacylglycerol (TAG) in lipid droplets [1,2], which are intracellular organelles most prominent in specialized storage tissues such as insect fat body or mammalian adipose tissue. A finely tuned balance between lipid synthesis (lipogenesis) and lipid mobilization (lipolysis) adjusts the fat storage level within cells.

In humans, the disruption of this balance is linked to complex metabolic disorders such as obesity and type II diabetes, and is causative for monogenic neutral lipid storage diseases (NLSD) such as Chanarin-Dorfman Syndrome (CDS) [3,4]. The accumulation of TAG-containing lipid droplets in multiple tissues characteristic of CDS has been linked to impaired lipolysis caused by mutations in comparative gene identification 58 (CGI-58; also called ABHD5) [5]. CGI-58 acts as a coactivator of adipose triglyceride lipase (ATGL) [6], also called TTS, desnutrin [7], calcium-independent phospholipase A2 [8], or patatin-like phospholipase domain-containing protein 2 [9]. Recently, it was shown that patients carrying ATGL gene mutations suffer from increased systemic TAG accumulation (so called NLSD with myopathy, [10]), supporting the idea that the impaired activation of ATGL contributes to the pathogenesis of CDS. The findings that polymorphisms in human ATGL are associated with plasma levels of TAG and free fatty acids (FFA) [11] and that ATGL knockout mice are obese [12] further underscores the central role of ATGL in mammalian lipolysis.

ATGL is ubiquitously expressed; however, it is strongly enriched in adipose tissue, where it acts in concert with hormone-sensitive lipase (Hsl) to execute lipolysis at the lipid droplet surface (for review, see [13,14]). Initiation of lipid mobilization is controlled by lipolytic hormones that act via β-adrenergic receptor signaling. According to the current model, β-adrenergic receptor stimulation activates protein kinase A (PKA), which subsequently phosphorylates Hsl and the lipid droplet scaffold protein perilipin. PKA activation promotes the translocation of cytoplasmic Hsl to the lipid droplet surface in a manner dependent on the phosphorylation state of perilipin (for review, see [15]). ATGL activity is indirectly activated by PKA signaling via the phosphorylation-triggered release of its perilipin-bound activator CGI-58 [16,17]. Notably, starvation-induced increase of ATGL transcript levels is dependent on glucocorticoid, but not on cyclic adenosine monophosphate (cAMP)/PKA signaling [7], suggesting that ATGL activity is controlled by various regulatory inputs. ATGL function is highly conserved during evolution; ATGL-related proteins have been identified as key regulators of yeast, plant, and insect lipometabolism (Saccharomyces cerevisiae Tgl4 [18,19], Arabidopsis thaliana SUGAR-DEPENDENT1 [20], and Drosophila melanogaster Brummer [21]).

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Abbreviations: Adh, Alcohol dehydrogenase; AKH, adipokinetic hormone; AKHR, adipokinetic hormone receptor; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification 58; DAG, diacylglycerol; Hsl, hormone-sensitive lipase; LSD, lipid storage droplet; PKA, protein kinase A; TAG, triacylglycerol; UAS, upstream activation sequence

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**Author Summary**

The amount of body fat that an animal stores is a critical parameter for its survival. Although under-storage of fat creates risk during periods of famine, over-storage also impairs fitness—obesity in humans is associated with severe health threats, such as cardiovascular disease, type II diabetes, and cancer. A delicate balance between two antagonistic processes adjusts body fat storage. Lipogenesis produces fat stores, and lipolysis mobilizes fat. It is unclear, however, how many regulatory systems orchestrate lipolysis in animals, whether these systems are evolutionarily conserved, and to what extent impaired lipolytic regulation contributes to excessive body fat accumulation. We show that in the fruit fly Drosophila, lipolysis is under dual control. Inactivation of either of the two control pathways generates flies with excessive fat accumulation and limited fat-mobilization capability. Mutant flies simultaneously lacking key genes of both lipolytic systems, however, are extremely obese and completely blocked in body fat mobilization even when fully fed. Interestingly, our study reveals that key components and regulatory mechanisms of lipolysis are evolutionarily conserved between insects and mammals, making the fruit fly a valuable model system for research on lipid metabolism.

Despite their anatomical and physiological differences, there is remarkable evolutionary conservation of lipolytic factors and mechanisms between mammals and insects. Like the ATGL knockout mice, brummer (bmm) mutant flies are obese and impaired in acute lipid mobilization [12,21]. Comparably with β-adrenergic signaling in mammalian adipose tissue, initiation of storage-fat mobilization in the insect fat body relies on hormonal signaling via the adipokinetin hormone (AKH) pathway (for review, see [22,23]). Starvation-induced release of AKH from neuroendocrine corpora cardiaca cells of the ring gland triggers signaling via G protein–coupled AKH receptor (AKHR) [24], activates PKA, and controls both Drosophila hemolymph sugar homeostasis [25,26] and larval lipolysis [25,27]. The primary target of AKH-dependent PKA phosphorylation in the tobacco hornworm Manduca sexta is the perilipin homolog LSDP-1 (a synonym for lipid storage droplet-1 [LSD-1]) [28]. Activation of lipid droplets by phosphorylation of LSDP-1 mediates most of the AKH-induced lipolysis in Manduca [29]. In Drosophila, another perilipin relative called LSD-2 has been demonstrated to be crucial for fat-storage regulation [30,31]. It is currently unknown what the identity of the TAG lipase(s) executing the AKH-induced fat-mobilization program in the fly is. Recently, Drosophila CG8552, the homolog of the Manduca sexta TG-lipase, has been proposed to implement AKH-dependent lipolysis [32], but its in vivo role has still to be analyzed. Remarkably, starvation also stimulates lipid mobilization by an uncharacterized, AKH-independent mechanism in adult Manduca [33], suggesting that, like in mammals, insect lipolysis is under control of multiple regulatory systems.

To address the question of how many lipocatabolic systems orchestrate acute lipolysis in response to energy shortage in animals and to what extent chronic dysregulation of inducible lipolytic systems contributes to fat-storage diseases, we analyzed the function of the Drosophila AKHR in vivo. Here we show that AKHR mutant flies become obese and are impaired in storage-fat mobilization. Flies lacking AKHR and Brummer lipase activity demonstrate that acute storage-fat mobilization in Drosophila is coordinated by two regulatory systems, which may communicate in a compensatory manner to ensure lifelong fat-storage homeostasis.

**Results/Discussion**

Expression studies in a heterologous tissue culture system [24] and in Xenopus oocytes [34] identified AKH-responsive G protein–coupled receptors in Drosophila, such as the one encoded by the AKHR (or CG11325) gene (FlyBase name: Gonadotropin-releasing hormone receptor [GRHR]). AKHR is expressed during all ontogenetic stages of the fly ([35] and unpublished data). It consists of seven exons, which encode a predicted protein of 443 amino acids (Figure 1A and [36]). In late embryonic and larval stages, AKHR is expressed in the fat body (Figure 1C and 1E), and this results in consistent with its predicted role as transmitter of the lipolytic AKH signal in this organ.

In order to examine the effect of AKHR signaling on fat storage and mobilization in vivo, two different P element–insertion mutants were used, CG11188A1332 and AKHRG6244, which are located close to and within the AKHR gene, respectively. CG11188A1332 and AKHRG6244 flies carrying the transposable element integration designated A1332 allow for the transcriptional activation of the adjacent AKHR gene (Figure S1A and unpublished data). This ability was used for AKHR gain-of-function studies by overexpression of AKHR in the fat body of flies. As shown in Figure S1B, overexpression of AKHR in response to a fat body–specific Gal4 inducer causes dramatic reduction of organismal fat storage. This finding could be recapitulated by fat body–targeted AKHR expression from a cDNA-based upstream activation sequence (UAS)-driven AKHR transgene (Figure S1B). These gain-of-function results suggest a critical role for AKHR in storage-lipid homeostasis of the adult fly.

Flies of strain AKHRG6244, which carry a P element integration in the AKHR untranslated leader region, were used to generate the AKHR deletion mutants AKHR1 and AKHR2, as well as the genetically matched control AKHR(w). These mutants lack AKHR allele (Figure 1A). As exemplified for embryonic and larval stages (Figure 1D and 1F), AKHR1 mutants lack AKHR transcript. Adult–fed flies without AKHR function are viable, fertile, and have a normal lifespan (unpublished data). However, such flies accumulate lipid storage droplets in the fat body and have 65%–127% more body fat than the controls (Figures 2A, 2B, and 2C). These results indicate that AKHR1 mutants develop an obese phenotype. The same result was obtained with AKHR2 and AKHR1/AKHR2 transheterozygous mutant flies (unpublished data), as well as with flies lacking the AKH-producing cells of the neuroendocrine system due to targeted ablation by the cell-directed activity of the proapoptotic gene reaper (AKHR-2D mutant in Figure 2A). Conversely, chronic overexpression of AKH provided by a fat body–targeted AKHR transgene of otherwise wild-type flies largely depletes lipid storage droplets and organismal fat stores (Figure 2A and 2B). However, the obese phenotype of AKHR mutants is unresponsive to AKH (Figure 2A and 2B), indicating that AKHR is the only receptor transmitting the lipolytic signal induced by AKH in vivo. Collectively, these data demonstrate that AKH-
dependent AKHR signaling is critical for the chronic lipid-storage homeostasis in ad libitum–fed flies.

Studies on various insect species helped elucidate several components and mediators of the lipolytic AKH/AKHR signal transduction pathway (for review, see [22]). However, the identity of the TAG lipase(s) executing the AKH-induced fat mobilization program remained elusive. Besides the Drosophila homolog of the TG lipase from the tobacco hornworm Manduca sexta [32], the recently identified Brummer lipase, a homolog of the mammalian ATGL, is a candidate member of the AKH/AKHR pathway. This is based on the striking similarity between the phenotypes of AKHR and bmm mutants. Ad libitum–fed flies lacking either AKHR or bmm activity, store excessive fat (Figures 2A and 3A; and [21]), predominantly as TAG (Figure S3). Both mutants show incomplete storage-fat mobilization (Figure 3A and [21]) and starvation resistance (Figure 3C and [21]) in response to food deprivation. Starvation resistance of these mutants might be caused by their increased metabolically accessible fat stores (Figure 3) and/or changes in their energy expenditure due to locomotor activity reduction as described for flies with impaired AKH signaling [25,27]. Despite the phenotypic similarities of their mutants, however, AKHR and bmm are members of two different fat-mobilization systems in vivo. Several lines of evidence support this conclusion. On one hand, AKH overexpression reduces the excessive TAG storage of bmm mutants (Figure 2C), while on the other, bmm-induced fat mobilization can be executed in AKHR mutants (Figure S2). Thus, AKH/AKHR signaling is not a prerequisite for Brummer activity. Moreover, genetic epistasis experiments support this idea that AKHR and bmm belong to different control systems of lipocatabolism in vivo. Double-mutant analysis reveals that the obesity of AKHR and bmm single mutants is additive. Accordingly, AKHR bmm double-mutant flies store about four times as much body fat as control flies and accumulate excessive lipid droplets in their fat body cells (Figure 3A and 3B).

Thin layer chromatography (TLC) analysis was used to compare the storage-fat composition of AKHR and bmm single mutants with AKHR bmm double-mutant and control flies (Figure S3). Excessive body fat accumulation in AKHR bmm double-mutants is on the one hand due to TAG, which is increased compared to AKHR and bmm single-mutant flies. Additionally, an uncharacterized class of TAG (TAGX; for details see Protocol S1) appears exclusively in AKHR bmm double mutants (Figure S3 and unpublished data). In contrast to TAG, changes in diacylglycerol (DAG) content do not substantially contribute to the differences in body fat content in any of the analyzed genotypes (Figure S3). Taken together a quantitative increase and a qualitative change in the TAG composition account for the extreme obesity in AKHR bmm double-mutant flies.

To address the in vivo response of AKHR bmm double
mutants to induced energy-storage mobilization, flies were starved and their survival curve monitored. AKHR bmm double mutants die rapidly after food deprivation (Figure 3C). In contrast to the starvation-resistant obese AKHR and bmm single mutants, the double mutants are not capable of mobilizing even part of their excessive fat stores (Figure 3A). AKHR bmm double mutants do not, however, suffer from a general block of energy-storage mobilization because they can access and deplete their carbohydrate stores (Figure S4). These data demonstrate that energy homeostasis in AKHR bmm double-mutant flies is imbalanced by a severe and specific lipometabolism defect, which cannot be compensated in vivo.

The nature of Brummer as a TAG lipase and AKHR as a transmitter of lipolytic AKH signaling suggests that the extreme storage-fat accumulation and starvation sensitivity of ad libitum–fed AKHR bmm double mutants is due to severe lipolysis dysfunction. To address this possibility in vitro, lipolysis rate measurements on fly fat body cell lysates and lysate fractions of control flies were performed. Results, summarized in Figure S5A, show that the cytosolic fraction of fat body cells contains the majority of basal and starvation-induced lipolytic activity against TAG, similar to the activity distribution in mammalian adipose tissue [37]. Little basal and induced total TAG cleavage activity localizes to the lipid droplet fraction, whereas the pellet fraction including cellular membranes shows low basal, non-inducible TAG lipolysis. Lipolysis activity against DAG is similarly distributed between fat body cell fractions (Figure S5B). However, in accordance with the function of DAG as major transport lipid in Drosophila [38], DAG lipolysis in fat body cells is not induced in response to starvation (Figure S5B).

Based on the lysate fraction analysis of control flies, cytosolic fat body cell extracts were used to assess the basal and starvation-induced lipolytic activity of mutant and control flies on TAG, DAG, and cholesterol oleate substrates. Whereas DAG and cholesterol oleate cleavage activity of fat body cells is comparable between all genotypes and physiological conditions tested (Figure S5C and S5D), TAG lipolysis varies widely (Figure 4). Compared to control flies, basal TAG lipolysis of AKHR bmm double mutants is reduced by 80% and induced TAG cleavage is completely blocked, consistent with the flies’ extreme obesity and their inability to mobilize storage fat. The impairment of basal lipolysis in the double mutants is largely due to the absence of bmm function, because it is also detectable in bmm single-mutant cells, whereas basal lipolysis in AKHR mutants is not reduced. Interestingly, bmm mutants mount a starvation-induced TAG lipolysis response after short-term (6 h), but not after extended (12 h), food deprivation. Conversely, AKHR mutant cells lack an early lipolysis response, but exhibit strong TAG cleavage activity after extended food deprivation. These data suggest that induced storage-fat mobilization in fly adipocytes relies on at least two lipolytic phases: an early, AKH/AKHR-dependent phase and a later, Brummer-dependent phase. Accordingly, we speculate that the obesity of bmm and AKHR...
mutant flies is caused by different mechanisms: chronically low basal lipolysis in bmm mutants and, in AKHR mutants, lack of induced lipolysis during short-term starvation periods that is characteristic of organisms with discontinuous feeding behavior. We acknowledge, however, that in vitro lipolysis assays on artificially emulsified substrates allow only a limited representation of the lipocatabolism in vivo, because lipid droplet–associated proteins modulate the lipolytic response in the insect fat body [28,29] and mammalian tissue [9,15,16]. Moreover, excessive fat accumulation in AKHR mutants may be in part due to increased lipogenesis because AKH signaling has been demonstrated to repress this process in various insects [39–41].

The finding of the dual lipolytic control in the fly raises the question of whether the two systems involved act independently of each other or whether one system responds to the impairment of the other. Modulation of transcription is an evolutionarily conserved regulatory mechanism of lipases from the ATGL/Brummer family. ATGL is transcriptionally up-regulated in fasting mice [7], as is bmm transcription in starving flies ([21] and Figure 5A). Moreover bmm over-expression depletes lipid stores in the fat body of transgenic flies ([21] and Figure S2). Accordingly, we analyzed bmm transcription in response to modulation of AKH/AKHR signaling to assess a potential regulatory interaction between the two lipolytic systems. Compared to the moderate starvation-induced up-regulation of bmm in control flies, the gene is hyperstimulated in flies with impaired AKH signaling.

Figure 4. Impaired Basal and Blocked Starvation-Induced TAG Lipolysis in Fat Body Cells Lacking Both AKHR and brummer Gene Function

Fat body cells of control flies (AKHR<sup>rev</sup> bmm<sup>rev</sup>) exhibit basal TAG lipolysis, which is doubled by starvation-induced lipolysis after 6 h or 12 h of food deprivation. bmm mutant cells have reduced basal lipolysis and lack induced lipolysis after 12 h starvation. AKHR mutant cells lack early (6 h) induced lipolysis, but show strong starvation-induced lipolysis after 12 h food deprivation. AKHR<sup>rev</sup> bmm<sup>rev</sup> double mutants have reduced basal lipolysis and lack starvation-induced lipolysis altogether.

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As early as 6 h after food deprivation, bmm transcription is up-regulated by a factor of 2.5–3 in flies lacking the AKH-producing neuroendocrine cells (AKH-ZD). By contrast, bmm transcription in lean flies chronically expressing AKH in the fat body (B) is strongly reduced.

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Materials and Methods

Fly techniques. Fly strains used in this study are summarized in Table 1. Flies were propagated as described [21]. Flies of the genotype w+; P[w+mDeltatubulin=EP]/ARKHRGE16070 (previously called w++; P[w+mDeltatubulin=EP]/ARKHRGE16070) were obtained from GenExel, (http://genexel.com). The EP transposon construct [42] integration in this fly stock was

Table 1. Names, Genotypes, and References of Fly Stocks

| Name (Stock Number) | Genotype | Reference/Source |
|---------------------|----------|------------------|
| bmm+ (SGF540)      | w++; bmm[rev] | [21] |
| bmm- (SGF529)      | w++; bmm[1]/TM3 Sb float. | [21] |
| FB-Gal4 (RFK153)   | y[+] w[+] ; P[w+mW.hs] = Gav8FB P[w+mUAS-GFP 1010T2] #2; +/+ | [31] |
| FB-JO (RFK246)     | y[+] rev[P[w+mW.hs] = Gav8FB P[w+mUAS-GFP 1010T2]] #2; +/+ | [31] |
| UAS-bmm (SGF533)   | w[+]; /+; P[w+m-c] bmm[Scer(UAS) = UAS-bmm] #2d | [21] |
| CG1188B (RFK518)   | y[+] w[+] ; P[y+m-w] = Mae-UAS,6,11|CG1188B(A1332) | CyO P[Aab8] salm[A405.1M2] float | This study |
| AKHR[R61A] (RFK613) | y* float w[+]; rev[P[w+m-c] = EP]/AKHR(G6244)[/CyO float | This study |
| AKHR[R61A] (RFK640) | y* float w[+]; rev[P[w+m-c] = EP]/AKHR(G6244)[/CyO float | This study |
| AKHR[R639] (RFK639) | y* float w[+]; AKHR[1]/CyO float | This study |
| AKHR[R638] (RFK638) | y* float w[+]; AKHR[2]/CyO float | This study |
| UAS-AKHR (RFK592)  | w[+]; P[w+m-c] AKHR[Scer(UAS) = UAS-AKHR] #32A / TM3 Sb[1] U1 float | This study |
| AKHR[R725] (RFK778) | w[+]; AKHR[1]/CyO float; bmm[1]/TM3Sb float | This study |
| AKHR[R778] (RFK778) | y* float w[+]; rev[P[w+m-c] = EP]/AKHR(G6244)[A; bmm[rev] | This study |
| FB-Gal4 AKHR[R653] | w[+]; AKHR[1] P[w+mW.hs] = Gav8FB P[w+mUAS-GFP 1010T2] #2 / CyO float | This study |
| AKHR[R675] (SGF768) | w[+]; AKHR[1]/CyO-fz-lacZ float; P[w+m-c] bmm[Scer(UAS)=UAS-bmm] #2d / TM3-fz-lacZ Sb[1] y[+] float. | This study |
| UAS-AKHR (RFK656)  | y[+] w[+]; UAS-AKHR[+/-] | [25] |
| AKHR[R773] (RFK773) | y* float w[+]; UAS-AKHR[+/-] | This study |
| UAS-reaper (RFK778) | w[+]; P[w+m-m] = UAS-reaper / TM3; P[w+m-c] = ActGFP/;JM2, Ser[1] float. | Unpublished |
| akhp-Gal4 (RFK694) | w; akhp-Gal4, UAS-mCD8 GFP; akhp-Gal4; SMS-TM6 Tb | [26] |

The results presented here provide in vivo evidence that the fly contains two induced lipolytic systems. One system confers AKH/AKHR-dependent lipolysis, a signaling pathway, which assures rapid fat mobilization by cAMP signaling and PKA activity. Drosophila’s second lipolytic system involves the Brummer lipase, which is responsible for most of the basal and part of the induced lipolysis in fly fat body cells, likely via transcriptional regulation. Currently, it is unknown whether Brummer activity is post-translationally modulated by a β/δ hydroxlate domain-containing protein like the regulation of its mammalian homolog ATGL by CGI-58. Homology searches between mammalian and Drosophila genomes identify the CGI-58-related fly gene CG11882 and the putative Hsl homolog CG11055, providing additional support for the evolutionary conservation of fat-mobilization systems. However, differences in lipid transport physiology (i.e., DAG transport in Drosophila, and FFA in mammals) suggest a different substrate specificity or tissue distribution of fly Hsl compared to its mammalian relative.

Future studies will not only unravel the crosstalk between the two Drosophila lipocatobolic systems, but also disclose the identity of additional genes involved in this process, such as the upstream regulators of bmm. Our study substantiates the emerging picture of the evolutionary conservation between insect and mammalian fat-storage regulation and emphasizes the value of Drosophila as a powerful model system for the study of human lipometabolic disorders.

Figure 5. Antagonistic Transcriptional Regulation of brummer Lipase in Response to AKH/AKHR Lipolytic Signaling (A) Moderate transcriptional up-regulation of bmm in control flies (AKHR+) after 6 h food deprivation, but starvation-induced hypostimulation of bmm transcription in obese AKHR mutants (AKHR-) and flies lacking the AKH-producing neuroendocrine cells (AKH-ZD). By contrast, bmm transcription in lean flies chronically expressing AKH in the fat body (B) is strongly reduced.
localized by sequencing to chromosome 2L between positions 6716140 and 6716139 (FlyBase release 4.2.1) corresponding to positions 82/83 in the 5′ untranslated leader region of \( \text{AKHR} \) (Note: \( \text{AKHR} \) contains 289 base-pair [bp] and \( \text{AKHR} \) 18-bp residual P element sequences). Because \( \text{AKHR} \) and \( \text{AKHR} \) behaved indistinguishably in the assays tested, \( \text{AKHR} \) was used in all experiments labeled with \( \text{AKHR} \) and \( \text{AKHR} \). With the exception of Figure 1C (\( \text{AKHR} \)) and Figure 3C (\( \text{AKHR} \)).

The fly strain \( \text{CG11188}^{1332} \) was recovered as an autosomal integration line in a \( X \)-chromosomal gain-of-function screen using the \( \text{P}[\text{Mac-UGAS.6.11}] \) transposable element [43]. The \( \text{A1332} \) integration site was described as described [43] and localized at chromosome 2L between positions 67177080 (FlyBase database 4.2.1) corresponding to positions 82883 in the 5′ untranslated leader region of \( \text{CG11188-RA} \).

Transgenic fly strains allowing conditional expression of \( \text{AKHR}(\text{UAS-AKHR}) \), \( \text{AKHR} \) cDNA \( \text{GH19447} \) was cloned into vector pUAST (see below). Transgenic fly stocks were established by \( \text{P} \) element-mediated germline transformation as described [31].

Found at doi:10.1371/journal.pbio.0050137.sg001 (453 KB EPS).

**Lipolysis assays.** Abdominal adipocytes from 100–200 immature adult male flies (0–6 h of age) and subsequently fed or starved for 6 h or 12 h were manually released as described [21] and snap frozen in a tube. A total of 1.5 ml of 10% sucrose (w/v) was then layered on top of(MouseEvent to coast to rest. The most buoyant white layer of the gradient containing the \( \text{LD} \) was recovered by suction with a syringe (1 ml). About 2.5 ml of the 65% sucrose cushion were recovered and used as cytosol. LD were recovered from the surface layer after a washing cycle with 10 ml of buffer A (20,000×g, 10 min) and their volume adjusted to 1 ml with buffer A. The pellet obtained by the gradient centrifugation and containing total fly-adipocyte membranes was suspended in 100 μl of buffer A. Cytosolic extracts were used for assaying lipolysis of the different genotypes because these extracts account for the majority of the lipolytic activity recovered with total fly-adipocyte lysates (Figure S5A and S5B).

**Supporting Information**

**Figure S1.** Molecular Organization of the \( \text{AKHR} \) Gene Locus and \( \text{AKHR} \)-Dependent Storage-Fat Mobilization

(A) Genomic organization (exons in white, coding exons in grey) of the \( \text{CG11188} \) and the \( \text{AKHR} \) gene locus on chromosome 2L at cytogenetic position 27A1. Localization of \( \text{P} \) element-integration \( \text{A1332} \) in the fly stock \( \text{CG11188}^{1332} \). (B) Reduced fat-storage reduction upon fat body–targeted \( \text{AKHR} \) expression via the \( \text{A1332} \) P element or an independent \( \text{UAS-AKHR} \) transgene compared to controls. “Inducer +” refers to the presence of the fat-body–specific \( \text{FB-Gal4} \) chromosome; “Inducer −” refers to the presence of the genetically matched control chromosome \( \text{FBJO} \) (for details, see complete fly stock genotypes in Table 1). Found at doi:10.1371/journal.pbio.0050137.sg001 (453 KB EPS).

**Figure S2.** \( \text{AKHR} \) Function Is Not Essential for brummer-Induced Fat-Storage Mobilization

Organismal fat storage is reduced upon induction of a transgene causing \( \text{bmm} \) overexpression in the fat body of control (\( \text{bmm} \) induced \( \text{bmm} \) uninduced) and sublethal as well as \( \text{AKHR} \) (Figure S7). 

Supporting Information

Figure S1. Molecular Organization of the AKHR Gene Locus and AKHR-Dependent Storage-Fat Mobilization

(A) Genomic organization (exons in white, coding exons in grey) of the CG11188 and the AKHR gene locus on chromosome 2L at cytogenetic position 27A1. Localization of P element-integration A1332 in the fly stock CG111881332. (B) Reduced fat-storage reduction upon fat body–targeted AKHR expression via the A1332 P element or an independent UAS-AKHR transgene compared to controls. “Inducer +” refers to the presence of the fat-body–specific FB-Gal4 chromosome; “Inducer −” refers to the presence of the genetically matched control chromosome FBJO (for details, see complete fly stock genotypes in Table 1). Found at doi:10.1371/journal.pbio.0050137.sg001 (453 KB EPS).

Figure S2. AKHR Function Is Not Essential for brummer-Induced Fat-Storage Mobilization

Organismal fat storage is reduced upon induction of a transgene causing bmm overexpression in the fat body of control (bmm induced bmm uninduced) and sublethal as well as AKHR (bmm induced vs. AKHR bmm uninduced). “Induced” refers to the presence of the fat-body–specific FB-Gal4 chromosome, “uninduced” to the presence of the genetically matched control chromosome FBJO (for details, see complete fly stock genotypes in Table 1). Found at doi:10.1371/journal.pbio.0050137.sg002 (363 KB EPS).

**Lipolysis assays.** Abdominal adipocytes from 100–200 immature adult male flies (0–6 h of age) and subsequently fed or starved for 6 h or 12 h were manually released as described [21] and snap frozen in a tube. A total of 1.5 ml of 10% sucrose (w/v) was then layered on top of the gradient containing the LD was recovered by suction with a syringe (1 ml). About 2.5 ml of the 65% sucrose cushion were recovered and used as cytosol. LD were recovered from the surface layer after a washing cycle with 10 ml of buffer A (20,000×g, 10 min) and their volume adjusted to 1 ml with buffer A. The pellet obtained by the gradient centrifugation and containing total fly-adipocyte membranes was suspended in 100 μl of buffer A. Cytosolic extracts were used for assaying lipolysis of the different genotypes because these extracts account for the majority of the lipolytic activity recovered with total fly-adipocyte lysates (Figure S5A and S5B).

Tissue homogenization of abdominal fat bodies using a Leica TCS SP2 LSM (Leica, http://www.leica-microsystems.com) with 561-nm excitation/600–657-nm emission wavelength.

Statistical analysis. Mathematical significance of differences between datasets was analyzed using the unpaired \( t \) test and expressed as \( p \)-values.
Figure S3. Organismal Lipid Composition of AKHR and brummer Single Mutants Compared to Extremely Obese AKHR brummer Double-Mutant Flies

Body fat accumulation in AKHR and brummer single mutants (AKHRbr and brmbr) compared to controls (AKHRbr*brmbr*) is due to triacylglycerol (TAG) increase. Increased TAG content and an uncharacterized glyceride species (TAGX) add up to the extreme fat accumulation in AKHRbr*brmbr*. Note that DAG does not substantially contribute to the observed genotype-specific changes in the total glyceride content, although there is a significant increase (in brmbr mutants) and decrease (in AKHRbr* brmbr double mutants) of DAG compared to the control.

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Figure S4. AKHR and brummer Functions Are Not Essential for Starvation-Induced Glycerone Mobilization

Complete depletion of glycogen stores during starvation of AKHR and brmbr single mutants (AKHRbr and brmbr), as well as of AKHRbr* brmbr double mutants (AKHRbr*brmbr*), is similar to the genetically matched control (AKHRbr*brmbr*). Note the reduced glycogen content in ad lithium-fed AKHRbr brmbr double-mutant flies.

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Figure S5. Lipolytic Activity Analysis of Fat Body Cells from Fed or 6-h Starved Flies

TAG (A) and DAG (B) lipolysis activity of fat body cell lysate fractions from immature adult control flies (genotype: AKHRbr*brmbr*) in a histochemical assay. Note that DAG does not substantially contribute to the observed genotype-specific changes in the total glyceride content, although there is a significant increase (in brmbr mutants) and decrease (in AKHRbr* brmbr double mutants) of DAG compared to the control.

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Author contributions.
The study was designed by RPK. AA performed the Q-RT-PCR experiments. SG, JH, SF, TH, and RPK performed all other experiments except the in vitro lipolysis and lipid class identification studies done by GM who also wrote the corresponding part of Materials and Methods and Protocol S1. SG, JH, SF, TH, and RPK wrote the main text and AN.

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