Rab32 Is Important for Autophagy and Lipid Storage in Drosophila

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Abstract

Lipids are essential components of all organisms. Within cells, lipids are mainly stored in a specific type of organelle, called the lipid droplet. The molecular mechanisms governing the dynamics of lipid droplets have been little explored. The protein composition of lipid droplets has been analyzed in numerous proteomic studies, and a large number of lipid droplet-associated proteins have been identified, including Rab small GTPases. Rab proteins are known to participate in many intracellular membranous events; however, their exact role in lipid droplets is largely unexplored. Here we systematically investigate the roles of Drosophila Rab family proteins in lipid storage in the larval adipose tissue, fat body. Rab32 and several other Rabs were found to affect the size of lipid droplets as well as lipid levels. Further studies showed that Rab32 and Rab32 GEF/Claret may be involved in autophagy, consequently affecting lipid storage. Loss-of-function mutants of several components in the autophagy pathway result in similar effects on lipid storage. These results highlight the potential functions of Rabs in regulating lipid metabolism.

Introduction

Lipids, proteins and carbohydrates are the three major building components of all living organisms. Lipids provide energy for daily usage and also function as signaling molecules in the regulation of important biological processes [1]. To maintain proper physiological conditions, the metabolism and homeostasis of lipids must be precisely regulated. Defects in lipid metabolism can lead to health-threatening problems in humans, for example, obesity and insulin resistance [2,3,4].

In most animals, storage lipids are usually accumulated in adipose tissues. Within cells, neutral lipids, mainly triacylglycerol (TAG) and cholesterol ester (CE), are stored in a specific type of organelle, called the lipid droplet [5]. Under nutrient-rich situations, excess fatty acids can be converted to TAG through lipogenesis and stored in lipid droplets. Under some nutrient-limiting conditions such as starvation, lipids can be released from lipid droplets by lipolysis for cell usage. Maintaining the homeostasis of lipid droplets is therefore important for normal lipid metabolism and lipid-related diseases.

Lipid droplets contain a lipid core and a monolayer of protein-coated phospholipid membrane [6]. The size and the content of lipid droplets are largely regulated by the balance of lipogenesis and lipolysis, which is mediated by many lipases. PAT (Perilipin/APRP/TIP47) domain proteins, the best known lipid droplet-surface proteins, can interact with lipases [7]. PAT proteins regulate the lipid droplet surface access of lipase to modulate the lipolysis process [8,9]. Many fundamental aspects of the dynamics of lipid droplets, including their biogenesis, the transport of lipids in and out of lipid droplets, and intracellular trafficking of lipid droplets, are not well characterized.

Identifying the proteins involved in these processes will lead to a better understanding of the dynamics of lipid droplets. Lipid droplets from different types of cells/tissues in several organisms have been purified and many proteomic studies have been conducted to identify proteins associated with them [10,11,12]. These proteins are likely localized on the surface of lipid droplets and function directly in lipid droplet dynamics. Many members of the Rab small GTPase family have been associated with lipid droplets in proteomic studies [13,14]. Rab family proteins are known to function in intracellular membrane trafficking. They participate in many biological processes, including endocytosis and exocytosis, cytokinesis, melanosome formation, autophagosome formation, lysosome biogenesis, and signaling transduction [15]. The identification of Rabs in lipid droplet proteomic studies suggests a potential role for Rabs in regulating the dynamics of lipid droplets and/or lipid storage. Indeed, the recruitment of Rab18 to lipid droplets is regulated by the metabolic state of lipid droplets, implying that Rab18 may mobilize lipids stored in lipid droplets [16]. Although a start has been made in exploring the roles of Rab18 in lipid droplets, the functions of the majority of Rab proteins identified in proteomic studies in lipid storage are still unclear. In addition, it is possible that other Rabs which have not yet been identified in proteomic studies may also have roles in lipid storage and metabolism.
Rab proteins are evolutionarily conserved in many organisms. *Drosophila* has 31 Rabs, 23 of which have mouse and human orthologs [17]. Similar to mammals, lipids in *Drosophila* larvae are mainly stored in adipose tissue fat bodies. *Drosophila* lipid droplets are coated with the PAT domain proteins, PLIN1 and PLIN2. *plan2* mutants are lean, showing lower levels of TAG and small lipid droplets, while *plan1* mutants are adult-onset obese [18,19]. *Drosophila* has been used extensively as a model organism in lipid metabolism studies [20,21,22,23,24,25]. For example, a whole genome RNAi screen of S2 cells showed that about 1.5% of all the genes tested function in lipid droplet formation and utilization [26]. Another RNAi screen in adult flies identified about 500 obesity genes. The Hedgehog signaling pathway was shown to have a fat body-specific role in *Drosophila* and to function as a switch between brown and white adipose tissues in mammals, suggesting that fat storage mechanisms are conserved between *Drosophila* and mammals [27].

In this study, we have systemically investigated the potential roles of Rab proteins in the regulation of lipid storage in *Drosophila*. As a small GTPase, Rab protein can switch between its GDP-binding inactive form and GTP-binding active form. Guanine nucleotide exchange factor (GEF) switches GTPase from its inactive to its active form, while GTPase activating protein (GAP) inactivates GTPase. With the help of structural and functional analysis, specific amino acid changes can be made that keep Rab GTPase in its GDP-binding form (dominant negative/DN) or GTP-binding form (constitutive active/CA). Expression of the DN or CA form can therefore mimic loss-of-function or gain-of-function effects. To examine the effect on lipid droplets, we manipulated the activity of Rabs in the fat body using the Gal4-UAS system and a transgene collection of DN and CA forms of all 31 *Drosophila* Rabs [17]. Lipid droplet size changes were found in many DN- or CA-Rab-expressing larvae, suggesting that these Rabs may regulate the dynamics of lipid droplets. In particular, we analyzed the molecular function of Rab32 and Rab32 GEF/Claret in lipid storage in detail. We show that Rab32 may affect lipid storage through its effects on autophagy.

**Results**

**Systematic identification of Rabs that affect the size of lipid droplets**

To systematically investigate the potential functions of Rabs in regulating lipid storage, we performed a functional screen by manipulating Rab activity in a tissue-specific manner using the UAS-Gal4 system [28]. The fat body-specific *ppl-Gal4* was used to drive the expression of individual CAS-DN- or CAS-Rabs [29]. We then used Nile red dye to stain the lipid droplets in the fat body of wandering stage third instar larvae. We found that expression of DN-Rabs 1, 5, 14, 21, 23, 27, 32, 40, X4, and X6 resulted in small lipid droplets, while expression of DN-Rabs 7, 10, 39, and X3 led to large lipid droplets (Fig. 1A and 1B). When CA-Rabs were expressed in the fat body, CA-Rabs 21, 35, 39, and X3 expression reduced the size of lipid droplets and CA-Rabs 1, 4, 6, 10, 11, 14, 23, and X4 expression increased lipid droplet size (Fig. 1C and 1D). Thus, the DN- and CA-forms of Rabs 1, 14, 23, 39, X3, and X4, had opposite effects on the size of lipid droplets. In addition, Rabs 4, 5, 6, 7, 11, 27, 32, 35, 40, and X6, only affected lipid droplet size when either the DN- or CA-form was expressed, but not when both were expressed (Table 1). Intriguingly, both DN- and CA-forms of Rab10 and Rab21 exhibited similar effects on lipid droplet size. Since RNAi stocks for most Rabs are available, we next investigated whether knockdown these Rabs lead to similar effects as DN-Rabs. Consistently, RNAi of Rab1, 5, 21, 40, X4, and X6 reduced lipid droplet size (Fig. S1). RNAi of Rab10, 23, 27 did not affect the size of lipid droplets, although the knockdown efficiency remained to be evaluated. In addition, except Rab23, most Rabs tested are strongly expressed in the larval fat body (Fig. S2). Taken together, these results support the involvement of many Rabs in lipid droplet size control.

In addition, we compared our genetic results with results from previous proteomic studies, in which a total of 18 Rabs were identified (Table 1) [14]. Four of these 18 Rabs have no orthologs in *Drosophila* (Table 1). In our screen, a set of 18 Rab proteins was found to affect the size of lipid droplets, of which Rabs X3, X4, and X6 have no counterparts in mammals. Importantly, we noticed that Rabs 1, 5, 6, 7, 10, 11, 14, 21, 35, and 39 were found in both previous proteomic studies and our functional screen (Table 1). The significant overlap between these results suggests that these Rabs may function directly on lipid droplets to regulate the dynamics of lipid droplets. Interestingly, although Rabs 2, 8, 18, and 19 were identified in proteomic studies, neither their DN- nor CA-forms altered lipid droplet size in our study (Table 1). These Rab proteins may play roles in aspects of lipid droplets other than their size. Alternatively, they may regulate the size of lipid droplets under unusual conditions, such as starvation. Rabs 4, 23, 27, 32, and 40 were found in our screen, though they had not been identified in proteomic reports (Table 1), implying that these Rabs may affect the size of lipid droplets indirectly.

**Rab32/lightoid (ltd) and Rab32 GEF/claret (ca) affect the size of lipid droplets**

We next validated the screen results using mutant phenotypic analysis and then further investigated the functional mechanisms of Rabs in lipid droplet dynamics and lipid metabolism. We focused particularly on Rab32 for the following reasons. First, there are many more mutant alleles available for Rab32 than for other Rabs. Second, Rab32 is highly expressed in the fat body, which is consistent with a potential role in regulating lipid metabolism (FlyAtlas: http://flyatlas.org, and Fig. S2). Lastly, Rab32, also known as lightoid (*ltd*), acts in a well-known eye pigment granule biosynthesis pathway and many components of this pathway have previously been identified [30,31].

We found that the *ltd* loss-of-function mutants, *ltd* and *ltd* 2, had smaller lipid droplets than controls (Fig. 2A and 2B). This is consistent with DN-Rab32 expression results (Fig. 1A). Moreover, the *orange* mutant allele *rub* has been shown to rescue the small lipid droplet phenotype of *ltd* (Fig. S2). Claret has been reported as a GEF for Ltd and is essential for the activation of Ltd. As was the case for *ltd* mutants, *ca* mutants also had a small lipid droplet phenotype (Fig. 2A and 2B). In addition, *ltd*/*ca* double mutants exhibited the same small lipid droplet phenotype as both single mutants, consistent with a previous finding that they function together in the same pathway (Fig. 2A and 2B). Therefore, we conclude that both *Rab32/ltd* and *Rab32 GEF/ca* are required for maintaining normal lipid droplet size. These results also validated the results of our functional screen.

**ltd, ca, and rb are required for normal lipid storage**

Eye pigment granules in *Drosophila* are specialized types of lysosome-related organelles. The genetic pathway of eye pigment granule biosynthesis has been well studied in *Drosophila*. In addition to *ltd* and *ca*, at least nine more genes, including *garnet* (*g*), *carnation* (*car*), *ruby* (*rb*), *carmine* (*cm*), *purploid* (*pd*), *deep orange* (*do*), *orange* (*or*), *light* (*lt*) and *pink* (*pk*), are also required in this process [31]. We examined larval fat bodies in mutants of these nine genes and found that only *rb* mutants have small lipid droplets (Fig. 3A and 3B), indicating
that the regulation of lipid droplet size may share some components with, but may not be identical to, the eye pigment granule biogenesis pathway. Furthermore, \( \textit{shf}/\textit{lid} \) double mutants have the same small lipid droplet phenotype as \( \textit{rb} \) single mutant, suggesting that \( \textit{lid} \) and \( \textit{rb} \) act similarly in regulating lipid storage (Fig. 3B).

Changes in lipid droplet size in \( \textit{lid} \), \( \textit{ca} \), and \( \textit{rb} \) mutants may reflect changes in lipid levels. We measured larval TAG levels and
found that all three mutants had lower TAG levels compared to the wild type (Fig. 3C). For example, the TAG level in the ltd mutant larvae was only ~70% that of the wild type. Moreover, ltd and bmm double mutants had a similar lipid content to that of the single mutants. In addition, the levels of TAG and glucose are significantly reduced in ltd mutant adults compared to the wild type (Fig. 3D). These results suggest that ltd, ca, and rb are necessary for lipid storage.

Another piece of evidence suggesting that ltd, ca, and rb function in lipid storage comes from starvation tests. Under starved conditions, animals can mobilize stored lipids from the lipid droplet by lipidolysis for energy consumption. Animals with elevated TAG levels may be resistant to starvation, while animals with decreased TAG levels may be sensitive to starvation. We found that ltd, ca, and rb mutants were more sensitive to starvation than controls. For instance, nearly all the mutant animals were dead after a 48-hour period of starvation, while around 50% of the control animals were still alive (Fig. 3E). These results further confirm that lipid storage is impaired in these mutants and that lipid levels are decreased.

ltd and ca genetically interact with plin2 and bmm

What is the mechanism by which Rab32 affects lipid storage? Both increased lipidolysis and reduced lipogenesis may lead to the

Table 1. Comparison of Rabs identified in this study with previous proteomic studies [13,14].

| Proteomic studies (mammalian cells) | This study (Drosophila) |
|-----------------------------------|------------------------|
| Rab1 | Rab1 |
| Rab2 | No phenotype |
| Not Found | Rab4 |
| Rab5 | Rab5 |
| Rab6 | Rab6 |
| Rab7 | Rab7 |
| Rab8 | No phenotype |
| Rab9 | Rab10 |
| Rab10 | Rab11 |
| Rab11 | Rab14 |
| Rab14 | Rab18 |
| Rab18 | No phenotype |
| Rab19 | No phenotype |
| Rab21 | Rab21 |
| Not Found | Rab23 |
| Rab24 | Not available |
| Not Found | Rab27 |
| Not Found | Rab32 |
| Not Found | Rab33 |
| Not Found | Rab34 |
| Not Found | Rab35 |
| Not Found | Rab39 |
| Rab39 | Rab40 |
| Not Found | Rab41 |
| Not available* | RabX3 |
| Not available | RabX4 |
| Not available | RabX6 |

*Not available means the homolog can’t be found.

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Reduced lipid storage phenotype in Rab32/ltd and its GEF/ca mutants. Using a Gal4-SREBP>UAS-GFP fluorescent reporter system which has been used as an indicator of lipogenesis in Drosophila [32], we found that lipogenesis is not reduced in ca mutants (Fig. 4A).

The lean phenotype in ltd, ca, or rb mutants resembles that of plin2 mutants. PLIN2 is a PAT domain protein localized on the surface of lipid droplets and is involved in lipidolysis. plin2 mutants show a reduction in TAG level and small lipid droplets, likely due to increased lipolysis (Fig. 4B) [19,33]. We tested the genetic interaction between plin2 and ltd or ca. Lipid droplets in plin2;ca double mutants were the same size as those of the ca single mutant. plin2;ltd double mutants yielded similar results, suggesting that plin2 and ltd or ca may affect the same process (Fig. 4B). Moreover, fat body-specific expression of plin2 using either the UAS-plin2-EYFP transgene line or the plin2 EP line (EY07901) significantly rescued the ca mutant phenotype (Fig. 4B). These results indicate that Rab32 GEF/ca genetically interacts with plin2 and Rab32 and may ultimately affect lipidolysis.

brummer (bmm), the Drosophila homolog of the mammalian adipocyte triglyceride lipase (ATGL) gene, is an important fat storage regulator in Drosophila. bmm mutants have reduced lipolysis, accumulations of TAG, and enlarged lipid droplets (Fig. 4C) [33]. Since Rab32/ltd likely acts on lipolysis, we tested its genetic interaction with bmm. We generated ltd/bmm double mutants and found that mutation of bmm could suppress the ltd mutant phenotype in a dose-dependent manner. Removal of one copy of bmm in ltd mutants significantly suppressed the small lipid droplet phenotype. Lipid droplet size in ltd/bmm double mutants was similar to that in bmm mutants (Fig. 4C). This dose-dependent suppression of ltd by bmm further supports that Rab32/ltd may modulate lipolysis.

Rab32 is localized in the lysosome and/or autophagosome

We next examined the subcellular localization of Rab32 in the fat body. Rab32 exhibited a ring-like localization pattern when we used ppl-Gal4 to drive UAS-Rab32-EYFP expression (Fig. 5A). In addition, ppl-Gal4-driven UAS-CA-Rab32-EYFP showed a similar localization pattern, while the ring-like localization pattern was lost in ppl-Gal4-driven UAS-DN-Rab32-EYFP. The DN-Rab32-EYFP
signal was diffuse throughout the cytosol. These results indicate that Rab32 is localized on the surface of some vesicles/organelles and that its location may be critical for its normal function. Are these vesicles/organelles lipid droplets? We used PLIN1-mCherry to label lipid droplets [25], and found that Rab32-EYFP did not co-localize with PLIN1-mCherry at all (Fig. 5A), indicating that it is not located in lipid droplets. These findings are consistent with the fact that Rab32 was not found in previous proteomic studies and suggest that Rab32 affects lipid storage in organelles other than lipid droplets.

Since Rab32 is known to function in the biogenesis of the eye pigment granule [30], which is a lysosome-related organelle, we wondered whether Rab32 is localized to the lysosomes or lysosome-related organelles. We found that Rab32-EYFP co-localizes perfectly with lysotracker, suggesting that Rab32 is localized in the lysosome or lysosome-related organelles in fat body cells (Fig. 5B).

**References**

[25] PLIN1-mCherry

[30] Eye pigment granule
During insect metamorphosis, the fat body, salivary gland, and midgut undergo programmed autophagy. Programmed autophagy is induced by ecdysone at the L3 stage. At the early L3 stage, puncta of Atg5 and Atg8 (autophagy markers) are found in the fat body, indicating the formation of the autophagosome. At the late L3 stage, autophagosomes fuse with lysosomes and can be labeled by both lysosomal markers and autophagosome markers [34]. To determine whether the ring-like Rab32-positive vesicles observed here were autophagosomes, we co-expressed RFP-Atg8a with Rab32-EYFP in the fat body. We found that the RFP-Atg8a-labeled autophagosomes were coated by Rab32-EYFP (Fig. 5B). These results indicate that Rab32 is localized in autophagosomes and suggest that Rab32 may have a potential regulatory function in autophagy.

Autophagy is impaired in ltd and ca mutants and defective autophagy leads to small lipid droplets

To address whether Rab32 activity is required for autophagy in larval fat body, we labeled autophagosomes with GFP-huLC3. huLC3 is the human homolog of Atg8 and has been widely used as an autophagosome marker in autophagy activity assays [34]. GFP-huLC3-positive structures were also labeled by lysotracker (Fig. 6A and 6B). We observed many GFP-huLC3-positive and lysotracker-positive autophagosomes in Rab32 GEF ca heterozygous animals, indicating that autophagy was normal. However, in ltd or ca homozygous mutant animals, the number of GFP-huLC3-positive autophagosomes was dramatically reduced, indicating that the autophagy process was impaired in these mutants (Fig. 6A and 6B). These data suggest that Rab32 activity is required for the autophagy process of fat body at the late L3 stage. We further examined whether Rab32 regulates autophagy in other tissues. Salivary gland is an ideal in vivo system for studies of autophagic cell death [35]. Normally, the cortical tGPH (tubulin-GFP-Pleckstrin-Homology) signal is lost at 13.5 hr after pupae formation, the time of salivary gland cell death (Fig. 6C). However, in ltd mutants, the cortical signal persists in the salivary gland, indicating autophagic cell death defects (Fig. 6C). Together, these results suggest that autophagy is impaired in ltd and ca mutants.

Several previous studies provide supporting evidence for a connection between autophagy and lipid storage as well as lipolysis. For example, knock-out of either Atg5 or Atg7 leads to reduced lipid accumulation and impaired adipocyte differentiation in mice [36,37]. We wondered whether down-regulation of autophagy in Drosophila could also lead to a similar effect. We analyzed Atg7 loss-of-function mutants and found that the size of
lipid droplets was smaller in the mutants than in controls (Fig. 6D and 6E). Additionally, Atg7;ca double mutants did not exhibit an enhanced phenotype, indicating that Atg7 and ca likely act in the same genetic pathway (Fig. 6D and 6E). To further confirm this result, we used ppl-Gal4 to drive UAS-Atg1 or Atg6 RNAi to specifically knock-down these genes in fat body cells. Knock-down of Atg1 or Atg6 led to small lipid droplets, suggesting an essential role of autophagy in lipid storage in fat body cells (Fig. 6D and 6E). Therefore, we concluded that Rab32 may regulate lipid storage by affecting autophagy.

**Discussion**

Lipid droplets are the main storage sites of neutral lipids in all cells, however, the dynamics of lipid droplets are poorly characterized. Here we systematically investigated the functions of all of the 31 Drosophila Rabs in the dynamics of lipid droplets and lipid storage by expressing their DN- and CA- forms. Eighteen Rabs were identified, 10 of which, including Rab1, had been found in previous proteomic studies. Rab1 is important for ER to Golgi transport by tethering the COPII-coated vesicles to Golgi through its effector p115 [38]. Interestingly, it was reported that COPI and COPII involved pathway delivers ATGL to lipid droplets to mediate lipolysis [39]. Five Rabs are not present in previous proteomic lists. These Rabs may not act on lipid droplets directly and instead may act on other organelles to influence lipid storage. Rab32 is an example of one of these Rabs.

Rab32/ltd and its GEF ca have well known functions in the biogenesis of a specific type of lysosome-related structure, called the eye pigment granule [30]. Many mutants have been found which have defective eye pigments. Proteins encoded by these genes include enzymes required for eye pigment biogenesis, ABC transporters responsible for the trafficking of pigment precursors, and the so-called “granule group” [31]. Four granule group genes, g, car, or, and rb, encode homologs of different AP-3 subunits which are believed to be involved in protein trafficking into lysosomes. Among them, only rb is required for lipid storage (Fig. 3A), suggesting that AP-3 subunits may have different roles in the regulation of lipid metabolism.

The regulation of lipid storage involves both the biosynthesis and the usage of lipids. Lipids are mainly stored in the lipid droplet, a monolayer-membrane-bound organelle, which is different structurally from lysosomes and lysosome-related organelles. Our studies of Rab32 reveal that the lysosomal pathway and the regulation of lipid storage may converge at points such as lipolysis. One explanation is that Rab32 may function in these different processes in a similar way. Alternatively, Rab32 could affect the lysosome and lysosome-related processes, subsequently influencing lipid storage.

Our results support the second possibility. First, we found that Rab32 is localized in autophagosomes, but not lipid droplets. Its location appears to be important for Rab32 function, since the DN form of Rab32 is mainly present in the cytosol. Second, it is known that autophagy affects lipid storage [40,41]. The autophagosome is

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Figure 6. Autophagy is impaired in ca mutants and defective autophagy leads to small lipid droplets. (A) Autophagy marker EGFP-huLC3-positive puncta are greatly reduced in ltd and ca homozygous mutants compared to the ca heterozygous control. Scale bar: 20 μm. (B) Quantification of EGFP-huLC3-marked autophagosomes in ca heterozygous and ltd and ca homozygous mutants. The error bars represent the standard deviation. **: P < 0.001. (C) tGPH reporter in control and ltd mutant salivary glands at 13.5 hr after pupae formation. tGPH cortical signal persists in ltd mutants. Scale bar: 50 μm. (D) Nile red staining of lipid droplets in wandering stage third instar larval fat body cells. Autophagy mutants or Atg1 and Atg6 RNAi animals have small lipid droplets. Scale bar: 20 μm. (E) Quantification of lipid droplet size in different genetic backgrounds. The error bars represent the standard deviation. *: P < 0.001.
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a special lysosome-related organelle. Lipid storage is reduced in the adipocytes of mice autophagy mutants [36,37]. Third, levels of the autophagy activity marker GFP-huLC3 are reduced in the adipocytes of mice autophagy mutants [36,37]. In addition, hepatocyte-specific knockout ATG7 results in elevated hepatic lipids [41]. Interestingly, in contrast, knockout of ATG5 or ATG7 in the pre-adipocyte cell line 3T3-L1 leads to decreased TAG accumulation, affecting adipocyte differentiation [36,37]. In vivo, adipocyte-specific knockout ATG7 mice are lean and have greatly reduced white adipocyte mass, but increased brown adipocyte mass. Mutant white adipocytes exhibit features resembling brown adipocytes, such as an increased rate of fatty acid β-oxidation, suggesting that autophagy may affect adipocyte differentiation [37]. Therefore, autophagy may affect lipid metabolism in a tissue-specific manner.

Our results also support the involvement of autophagy in lipid metabolism. During the wandering third instar larvae to pupae transition, animals do not feed and are in a state resembling starvation or nutrient-deprivation. Programmed autophagy of fat bodies and other tissues is important for providing energy and other nutrients for development. Mutations in or tissue-specific knockdown of autophagy components lead to reduced lipid storage (Fig. 6C and 6D). These results suggest that in autophagy mutants, more lipids may be mobilized from lipid droplets to compensate for the shortage of energy. It is conceivable that by affecting autophagy, Rab32 likely regulates lipid storage through lipolysis. The genetic interactions between Rab32/lim and lipolysis-related genes, bnm1 and pln2, further support this hypothesis. This study has highlighted the potential functions of Rab32 in regulating lipid metabolism. Further studies will elucidate the intermingled relationship between autophagy and lipid metabolism during development.

Materials and Methods

Fly strains
Drosophila stocks were maintained in standard corn meal food, unless specified. Canton-S (CS), w1118 or yw were treated as controls. bnm1 mutants were kindly provided by Dr. Ronald P. Kühlheim [33]. The P[Gal4-dSREBP] transgene was kindly provided by Dr. Robert B. Rawson. The UAS-RFP-Aglia transgene was kindly provided by Dr. Ernst Hafen [44]. UAS-plin1-mCherry and UAS-plin2-EYFP transgene lines were generated using a standard protocol. All other strains were obtained from the Bloomington Stock Center.

Fat body dissection and imaging
Wandering stage third instar larvae were dissected in 1xPBS, fixed with 4% paraformaldehyde for 75 min, washed twice with 1xPBS, and then stained with Nile red (0.5 μg/ml) or Bodipy 493/503 (1 μg/ml) for 60 min. After washing twice with 1xPBS, the samples were observed under a confocal microscope. The sizes of lipid droplets were measured using NIS-Elements BR 3.0 software (Nikon). The five largest lipid droplets in every fat body cell were measured and their average size was counted as the lipid droplet size for each cell. A total of 30 cells from at least 3 images were measured for each genotype. The lipogenic reporter Gal-4>SREBP>UAS-GFP fluorescence images were taken using a confocal microscope. Mean fluorescence intensity from 50 fat body cells were compared between mutants and control. Lysotracker (Molecular Probes) staining was performed according to the manufacturer’s instructions. Briefly, wandering stage third instar larvae were dissected in 1xPBS and incubated with 10 μM Lysotracker red DND-99 before observing immediately under a confocal microscope. Salivary gland cortical tGPH signal at 13.5 hr after pupae formation was captured as previously described [35]. For quantification purpose, images were taken using equivalent exposure conditions for controls and mutants. A two-tailed Student’s t-test was used to determine the significance of differences.

Supporting Information

Figure S1 RNAi validation of Rab5 genes that affect the size of lipid droplets. (A) Bodipy staining of lipid droplets in wandering stage third instar larval fat body cells. Scale bar: 10 μm. (B) Quantification of the effects of Rab RNAi. ***: P<0.001. (TIF)

Figure S2 Rab32 is expressed and functions in the fat body. (A) The fat body expression of ca, cb, and several Rab genes analyzed by RT-PCR. (B) Bodipy staining of lipid droplets in wandering stage third instar larval fat body cells. The fat body specific expression of Rab32 by ppl-Gal4 driver can rescue the lid lipid droplet phenotype. Scale bar: 10 μm. (C) Quantification of the rescuing effect. **: P<0.001. (TIF)
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References

1. Licorish M, Cantley LC (1994) Lipid second messengers. Cell 77: 329–334.
2. Horton JD, Goldstein JL, Brown MS (2002) SREBP: transcriptional mediators of lipid homeostasis. Cold Spring Harb Symp Quant Biol 67: 491–498.
3. Shi Y, Burn P (2004) Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. Nat Rev Drug Discov 3: 695–710.
4. Szendroedi J, Roden M (2009) Ectopic lipids and organ function. Curr Opin Lipidol 20: 59–56.
5. Fiere RV, Jr., Walther TC (2009) Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139: 855–860.
6. Martin S, Parton RG (2006) Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 7: 373–378.
7. Kimmel AR, Brassemi DL, McAndrews-Hill M, Sztalryd C, Londo C (2010) Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular, lipid storage droplet proteins. J Lipid Res 51: 468–471.
8. Duncan RE, Ahmadian M, Javorksi K, Sarkadi-Nagy E, Sul HS (2007) Regulation of lipolysis in adipocytes. Annu Rev Nutr 27: 79–101.
9. Granneman JG, Moore HP, Krishnamoorthy R, Rasheed M (2009) PERILIPIN lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abih5) and adipose triglyceride lipase (Atg5). J Biol Chem 284: 34358–34364.
10. Bella M, Riedel D, Jansch L, Dieterich G, Wehland J, et al. (2006) Regulation of lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abih5) and adipose triglyceride lipase (Atg5). J Biol Chem 284: 34358–34364.
11. Fujimoto Y, Ibae H, Sakai J, Makita M, Noda J, et al. (2004) Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. Biochim Biophys Acta 1644: 47–59.
12. Liu P, Ying Y, Zhao Y, Mundy DI, Zhu M, et al. (2004) Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. J Biol Chem 279: 3767–3770.
13. Liu P, Bartz R, Zehnder JK, Ying YS, Zhu M, et al. (2007) Rab-regulated interaction of early endosomes with lipid droplets. Biochim Biophys Acta 1773: 784–793.
14. Zehnder JK, Huang Y, Peng G, Pa J, Anderson RG, et al. (2009) A role for lipid droplets in intermembrane lipid traffic. Proteomics 9: 914–921.
15. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.
16. Martin S, Driessen K, Dixson S, Zerial M, Parton RG (2005) Regulated localization of Rab11 to lipid droplets: effects of lipolytic stimulation and inhibition of lipid droplet catabolism. J Biol Chem 280: 42525–42535.
17. Zhang J, Schulze KL, Hiesinger PR, Suyama K, Wang S, et al. (2007) Thirty-one flavors of Drosophila rab proteins. Genetics 176: 1307–1322.
18. Bella M, Bulankina AV, Hsiao HH, Urlaub H, Jackle H, et al. (2003) Coatomer-dependent protein delivery to lipid droplets. Biochim Biophys Acta 1773: 784–793.
19. Zehnder JK, Huang Y, Peng G, Pa J, Anderson RG, et al. (2009) A role for lipid droplets in intermembrane lipid traffic. Proteomics 9: 914–921.
20. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.
21. Martin S, Driessen K, Dixson S, Zerial M, Parton RG (2005) Regulated localization of Rab11 to lipid droplets: effects of lipolytic stimulation and inhibition of lipid droplet catabolism. J Biol Chem 280: 42525–42535.
22. Zhang J, Schulze KL, Hiesinger PR, Suyama K, Wang S, et al. (2007) Thirty-one flavors of Drosophila rab proteins. Genetics 176: 1307–1322.
23. Bella M, Bulankina AV, Hsiao HH, Urlaub H, Jackle H, et al. (2003) Coatomer-dependent protein delivery to lipid droplets. Biochim Biophys Acta 1773: 784–793.
24. Schlegel A, Stainier DY (2007) Lessons from “lower” organisms: what worms, flies, and zebrafish can teach us about human energy metabolism. PLoS Genet 3: e199.

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Conceived and designed the experiments: XH CW. Performed the experiments: CW ZL. Analyzed the data: CW ZL XH. Wrote the paper: XH CW.

25. Tian Y, Bi J, Shui G, Liu Z, Xiang Y, et al. (2011) Tissue-autonomous function of Drosophila seipin in preventing ectopic lipid droplet formation. PLoS Genet 7: e1001364.
26. Guo Y, Walther TC, Rao M, Sturman N, Goshima G, et al. (2008) Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. Nature 453: 657–661.
27. Popišilka J, Schramek D, Schindler H, Cronin SJ, Nehme NT, et al. (2010) Drosophila genome-wide obesity screen reveals hedgehog as a determinant of brown versus white adipose cell fate. Cell 146: 140–160.
28. Duffy JB (2002) GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34: 1–15.
29. Colombani J, Rasini S, Pantalacci S, Radimerski T, Montague J, et al. (2003) A nutrient sensor mechanism controls Drosophila growth. Cell 114: 739–749.
30. Ma J, Pleasen H, Trimaren JE, Edelman-Novemsky I, Ren M (2008) Lighloid and Claret: a rab GTase and its putative guanine nucleotide exchange factor in biogenesis of Drosophila eye pigment granules. Proc Natl Acad Sci U S A 101: 11652–11657.
31. Lloyd Y, Ramaswami M, Kramer H (1998) Not just pretty eyes: Drosophila eye-colour mutations and lysosomal delivery. Trends Cell Biol 8: 257–259.
32. Kunte AS, Matthews KA, Rawson RB (2006) Fatty acid autotrophy in Drosophila larvae lacking SREBP. Cell Metab 3: 439–448.
33. Grouwe S, Middler A, Felti S, Trumagel N, Petry S, et al. (2003) Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metab 1: 923–930.
34. Singh R, Xiang Y, Wang Y, Baikati K, Cuervo AM, et al. (2009) Autophagy regulates adipose mass and differentiation in mice. J Biol Chem 284: 7842–7854.
35. Singh R, Kaushik S, Felti S, Trumagel N, Petry S, et al. (2003) Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metab 1: 923–930.
36. Singh R, Kaushik S, Felti S, Trumagel N, Petry S, et al. (2003) Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metab 1: 923–930.