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RESEARCH ARTICLE

Drosophila Lipin interacts with insulin and TOR signaling pathways in the control of growth and lipid metabolism

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ABSTRACT

Lipin proteins have key functions in lipid metabolism, acting as both phosphatidate phosphatases (PAPs) and nuclear regulators of gene expression. We show that the insulin and TORC1 pathways independently control functions of *Drosophila* Lipin (dLipin). Reduced signaling through the insulin receptor strongly enhanced defects caused by dLipin deficiency in fat body development, whereas reduced signaling through TORC1 led to translocation of dLipin into the nucleus. Reduced expression of dLipin resulted in decreased signaling through the insulin-receptor-controlled PI3K–Akt pathway and increased hemolymph sugar levels. Consistent with this, downregulation of *dLipin* in fat body cell clones caused a strong growth defect. The PAP but not the nuclear activity of dLipin was required for normal insulin pathway activity. Reduction of other enzymes of the glycerol-3 phosphate pathway affected insulin pathway activity in a similar manner, suggesting an effect that is mediated by one or more metabolites associated with the pathway. Taken together, our data show that dLipin is subject to intricate control by the insulin and TORC1 pathways, and that the cellular status of dLipin impacts how fat body cells respond to signals relayed through the PI3K–Akt pathway.

KEY WORDS: TOR signaling, Insulin signaling, Lipin

INTRODUCTION

Normal growth and the maintenance of a healthy body weight require a balance between food intake, energy expenditure and organismal energy stores. Two signaling pathways, the insulin pathway and the target of rapamycin (TOR) complex 1 (TORC1) pathway, play a crucial role in this balancing process. Insulin or, in *Drosophila*, insulin-like peptides called Dilps are released into the circulatory system upon food consumption and stimulate cellular glucose uptake while promoting storage of surplus energy in the form of triacylglycerol (TAG or neutral fat) (DiAngelo and Birnbaum, 2009; Rulifson et al., 2002; Saltiel and Kahn, 2001). Nutrients, in particular amino acids, activate the TORC1 pathway, which stimulates protein synthesis leading to cellular and organismal growth (Wullschleger et al., 2006). The two pathways are interconnected to allow crosstalk, but the extent and biological significance of crosstalk seems to be highly dependent on the physiological context and could be different in different animal groups. For instance, tuberous sclerosis protein TSC2, which together with TSC1 inhibits TORC1 signaling, can be phosphorylated by Akt, the central kinase of the insulin pathway, in both mammals and *Drosophila melanogaster* (Manning, 2004; Potter et al., 2002). However, phosphorylation of TSC2 by Akt (Akt1 in *Drosophila*) is not required for normal growth and development in *Drosophila* (Dong and Pan, 2004), whereas in mammalian cells Akt phosphorylation of TSC2 is required for normal TORC1 activity and the resulting activation of ribosomal protein kinase S6K1 (Manning et al., 2002).

Studies in mice have identified one of the three mammalian lipin paralogs, lipin 1, as a major downstream effector mediating effects of insulin and TORC1 signaling on lipid metabolism (Csaki and Reue, 2010; Harris and Finck, 2011; Peterson et al., 2011). In both *Drosophila* and mice, proteins of the lipin family function as key regulators of TAG storage and fat tissue development (Reue, 2009; Ugrankar et al., 2011). Lipins execute their biological functions through two different biochemical activities, a phosphatidate phosphatase (PAP) activity that converts phosphatidic acid into diacylglycerol (DAG) and a transcriptional co-regulator activity, mediated by an LxxIL motif located in close proximity to the catalytic motif of the protein (Finck et al., 2006). The PAP activity of lipin constitutes an essential step in the glycerol-3 phosphate pathway that leads to the production of TAG, which is stored in specialized cells in the form of fat droplets (adipose tissue in mammals and fat body in insects). In addition, the product of the PAP activity of lipin, DAG, is a precursor for the synthesis of membrane phospholipids. As a transcriptional co-regulator, mammalian lipin 1 directly regulates the gene encoding nuclear receptor PPARα, which regulates mitochondrial fatty acid β-oxidation (Finck et al., 2006), and the yeast lipin homolog has been shown to regulate genes required for membrane phospholipid synthesis (Santos-Rosa et al., 2005).

In cultured adipocytes, insulin stimulates phosphorylation of lipin 1 in a rapamycin-sensitive manner, suggesting that phosphorylation is mediated by mammalian (m)TORC1 (Harris et al., 2007; Huffman et al., 2002; Péterfy et al., 2010). Phosphorylation by mTOR blocks nuclear entry of lipin 1 and, thus, access to target genes. Interestingly, non-phosphorylated lipin 1 that has migrated into the nucleus affects nuclear protein levels, but not mRNA levels, of the transcription factor SREBP1, which is a key regulator of genes that are involved in fatty acid and cholesterol synthesis. This effect requires the catalytic activity of lipin 1, suggesting that not all nuclear effects of the protein result from a direct regulation of gene transcription (Peterson et al., 2011). The lowering of nuclear SREBP protein abundance by lipin 1 counteracts the effects of Akt on lipid metabolism, which activates lipogenesis in a TORC1-dependent manner through activation of SREBP (Porstmann et al., 2008).

Lipins are not only subject to control by insulin and TORC1 signaling, they also have an effect on the sensitivity of tissues to insulin. Lipin-1-deficient mice exhibit insulin resistance and elevated insulin levels, whereas overexpression in adipose tissue increases their sensitivity to insulin (Phan and Reue, 2005; Reue et al., 2000). Similarly, in humans, lipin 1 levels in adipose tissue are

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inversely correlated with glucose and insulin levels as well as insulin resistance (Suviolahti et al., 2006; Yao-Borengasser et al., 2006). Although these data indicate that adipose tissue expression of lipin 1 is an important determinant of sensitivity to insulin, the underlying mechanism remains poorly understood.

Here, we present evidence that the only *Drosophila* lipin homolog, dLipin, controls the sensitivity of the larval fat body to stimulation of the insulin, phosphoinositide 3-kinase (PI3K), Akt pathway in a cell-autonomous manner. *dLipin* mutant larvae have increased hemolymph sugar levels, and larval fat body cells that are deficient of dLipin exhibit a severe growth defect. Loss-of-function and rescue experiments show that the PAP activity of dLipin and an intact glycerol-3 phosphate pathway are required for normal insulin pathway activity in fat body cells. Similar to the control of lipin 1 in mammalian cells, the insulin–PI3K pathway controls functions of dLipin in fat tissue development and fat storage, and the TORC1 pathway controls nuclear translocation of dLipin. However, in an apparent contrast to regulation of lipin 1 in mammals, our data suggest that the two pathways exert at least part of their effects on dLipin independently of one another.

RESULTS

dLipin is required in a cell-autonomous manner for fat storage and cell growth

We have previously found that animals that lack dLipin are characterized by severely reduced fat body mass and fat droplet size (Ugrankar et al., 2011). Interestingly, individual fat body cells were greatly enlarged in the mutant, whereas the number of fat body cells was reduced. The size increase might indicate that dLipin normally limits cell growth in a cell-autonomous manner. Alternatively, the size increase might be caused by a non-cell autonomous mechanism that compensates for insufficient numbers of fat cells by increasing individual cell size and, thus, fat storage capacity. To address these possibilities, we generated fat body cell clones lacking dLipin. Knockdown of *dLipin* in individual cells was accomplished by using RNA interference (RNAi) with the FLP–GAL4 system (Blair, 2003; Neufeld et al., 1998). Antibody staining showed that dLipin protein was strongly reduced in the knockdown cells (Fig. 1A). Low levels of dLipin expression were associated with the reduction of lipid droplets, indicating that dLipin has an essential cell-autonomous function in TAG synthesis. dLipin-deficient cells were significantly smaller in size than surrounding cells that expressed dLipin normally (Fig. 1C). The nuclei of these cells were smaller as well, and the nucleocytoplasmic ratio was significantly higher than in control cells (Fig. 1D). A similar increased nucleocytoplasmic ratio was found after ubiquitous knockdown of *dLipin* using a tubulin-GAL4 driver. Fat body cells were rounded after ubiquitous RNAi knockdown, similar to cells in *dLipin* mutants (Ugrankar et al., 2011), and were characterized by highly variable size. Some cells were very small, but contained comparatively large nuclei (Fig. 1B). Because the fat body is an endoreplicating tissue, these data suggest that a lack of dLipin affects the number of endoreplication cycles and, to an even larger degree, cytoplasmic growth. Thus, lack of dLipin does not enhance, but limits, the growth of individual cells, supporting the hypothesis that the hypertrophic fat body cells observed in *dLipin* mutants are the result of a secondary compensatory mechanism.

Lack of dLipin decreases insulin pathway activity in fat body cells

We were intrigued by the growth defect of fat body cells that lacked dLipin, which suggested that these cells had decreased sensitivity to growth factor stimulation. Cell growth in *Drosophila* is controlled by the insulin receptor (InR)–PI3K–Akt pathway (Saucedo and Edgar, 2002). To test the hypothesis that dLipin has an effect on sensitivity to insulin, we took advantage of an *in vivo* reporter of insulin–PI3K signaling, *tGPH* (Britton et al., 2002). *tGPH* encodes the pleckstrin homology (PH) domain of the *Drosophila* Steppke (Grp1) protein fused to GFP and is controlled by the *tubulin* promoter. The Steppke PH domain is specifically recruited to the cell membrane by binding to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), the second messenger that is generated by PI3K (Britton et al., 2002). In flies expressing this gene, green fluorescence at the cell membrane strongly depends on the activity of the insulin signaling pathway (Britton et al., 2002). As expected, fat body cells of feeding third-instar larvae expressing *tGPH* showed a strong association of PH–GFP with the cell membrane (Fig. 2). In contrast, in *dLipin* mutants or after fat-body-specific knockdown of *dLipin* with RNAi, the association of PH–GFP with the cell membrane was strongly reduced, indicating that the production of $PI(3,4,5)P_3$ is severely compromised in fat bodies that lack dLipin (Fig. 2A). Cells of the larval salivary glands, which were unaffected by the knockdown of *dLipin*, showed unchanged cell membrane localization of PH–GFP (Fig. 2A). In addition to reduced $PI(3,4,5)P_3$ levels, phosphorylation at residue Ser505 of the protein kinase Akt, the central target of the InR–PI3K pathway, was diminished, which is indicative of reduced InR–PI3K signaling (Kockel et al., 2010) (Fig. 2C). Finally, hemolymph sugars (combined trehalose and glucose) were increased by 38%, consistent with reduced sensitivity of the fat body to insulin (Fig. 2E). Combined, these data support the conclusion that a lack of dLipin impairs the sensitivity to insulin of the fat body, the major metabolic tissue of the fly larva.

Our results so far suggest that impaired sensitivity to insulin of cells lacking dLipin is caused by a cell-autonomous defect in signaling through the second messenger $PI(3,4,5)P_3$, which could result from an attenuation of PI3K activity or increased activity of the lipid phosphatase PTEN, which antagonizes $PI(3,4,5)P_3$ accumulation. Alternatively, decreased insulin pathway activity might be caused by a scarcity of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) in the cell membrane, the substrate of PI3K. To distinguish between these possibilities, we used a $PI(4,5)P_2$-specific reporter, PLCδPH–GFP (Pinal et al., 2006). PLCδPH-GFP showed strong association with the cell membrane, indicating that dLipin does not have a major impact on $PI(4,5)P_2$ levels in the plasma membrane (Fig. 2B). Thus, lack of dLipin does not affect the supply of substrate for PI3K, suggesting that the activity of PI3K or PTEN might be affected. This conclusion is further supported by the observation that expression of a constitutively active form of the catalytic subunit of PI3K (Dp110CAAX) could restore some of the active Akt lost after RNAi knockdown of *dLipin* (Fig. 2C). At the same time, knockdown of *dLipin* counteracted the increased cell growth induced by Dp110CAAX (Fig. 2D). Thus, lack of dLipin affects signaling through the second messenger $PI(3,4,5)P_3$.

In summary, a lack of dLipin leads to impaired cell growth, reduced levels of $PI(3,4,5)P_3$ and elevated levels of circulating sugars. Taken together, these data strongly suggest that dLipin has an essential role in maintaining the sensitivity to insulin of the larval fat body.

The PAP activity of dLipin and normal TAG synthesis are required for the responsiveness of fat body cells to insulin

We wondered whether the PAP activity of dLipin or its transcriptional co-regulator activity, or both, are required for

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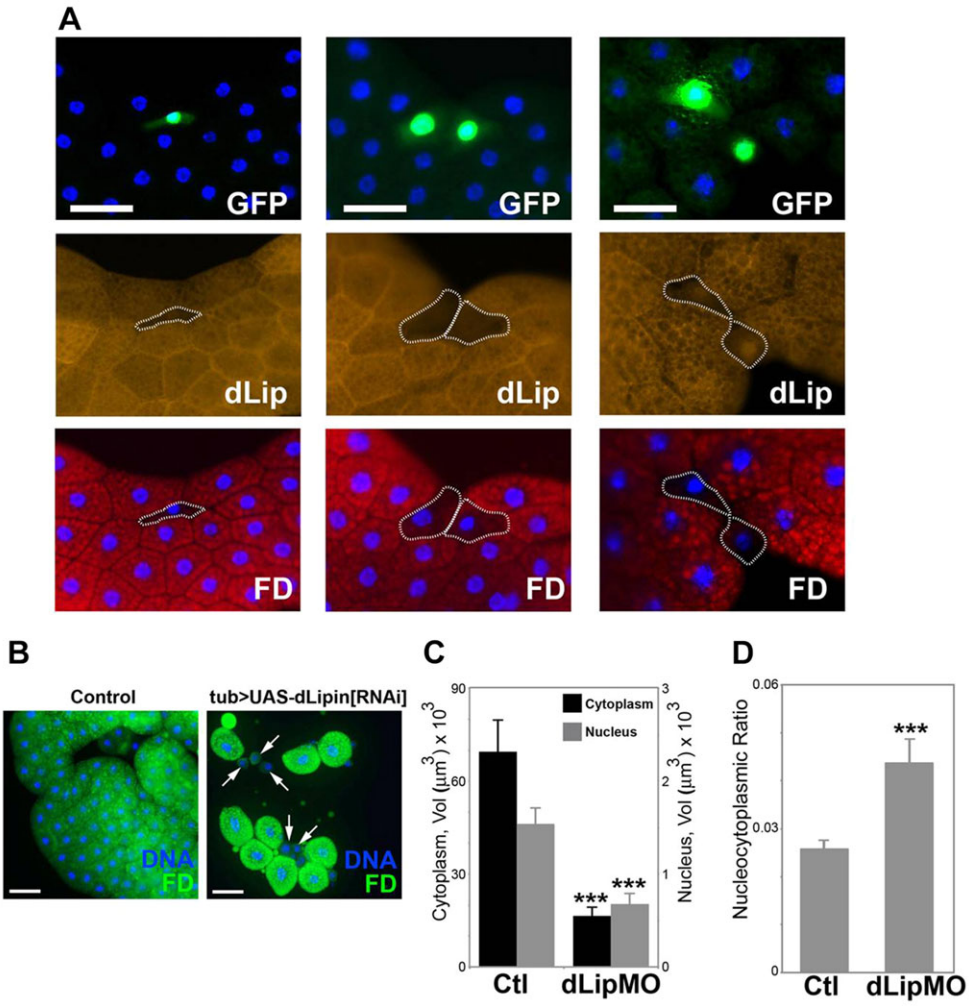

Fig. 1. dLipin is required in a cell-autonomous manner in the fat body for normal fat droplet formation and cell growth. (A) Fat body cells in mosaic animals that are deficient of dLipin protein contain few fat droplets (FD) and are reduced in size. Fat body cells that had been subjected to RNAi against dLipin (dLipin[RNAi]) are marked by nuclear GFP (green); DNA is stained with DAPI (blue). Fat droplets were stained with LipidTOX Deep Red, and dLipin protein was detected using an affinity-purified antibody against dLipin (dLip). Scale bars: 50 µm. (B) After ubiquitous knockdown of *dLipin* using a *tubulin*-GAL4 driver, cells were rounded and of highly variable size; some very small cells contained large nuclei but very little cytoplasm (arrows); fat droplets were stained with Bodipy, and DNA with DAPI. Control fat body is from a larva of the same cross carrying UAS-dLipin[RNAi], but not the tubulin driver. Scale bars: 100 µm. (C,D) Cell size was decreased and the nucleocytoplasmic ratio increased in mosaic cells that lacked dLipin (dLipMO) compared to control cells (Ctl). Student's unpaired *t*-test, ***P<0.001; error bars indicate s.e.m.

sensitivity to insulin of fat body cells. To address this question, we constructed UAS transgenes that express dLipin protein lacking PAP activity or the ability to enter the cell nucleus where dLipin participates in gene regulation. dLipinΔPAP contains an amino acid substitution (D812E) in the catalytic motif that leads to complete loss of PAP activity (Finck et al., 2006). dLipinΔNLS lacks the nuclear localization signal (NLS; amino acids 276–281) of dLipin. UAS-dLipinΔPAP, UAS-dLipinΔNLS, and a control wild-type transgene (UAS-dLipinWT) were expressed at similar levels when activated by GAL4 (Fig. S1). We noticed that expression of dLipinΔNLS causes dominant-negative effects (S.S., data to be reported elsewhere), suggesting that not only is dLipinΔNLS unable to enter the nucleus, but that it is blocking nuclear entry of endogenous wild-type dLipin. To test this prediction, we expressed dLipinΔNLS in animals with reduced TOR activity, which normally show robust translocation of dLipin into the nucleus (Fig. 7; discussed further below). Notably, expression of dLipinΔNLS led to an apparently complete exclusion of dLipin from the cell nucleus (Fig. S2).

Expression of dLipinWT or dLipinΔNLS in *dLipin* mutants (*dLipin*^{e00680}/*Df(2R)Exel7095*) rescued defects in fat body cell morphology and lipid droplet formation (Fig. 3A). However, dLipinΔPAP did not rescue (Fig. 3A) and, in contrast to dLipinWT and dLipinΔNLS, significantly enhanced lethality in *dLipin* mutant animals (data not shown). Similarly, dLipinΔPAP was not able to restore PIP_3 levels in the cell membrane of fat body cells that lacked dLipin, whereas PIP_3 levels at the membrane showed a clear increase when dLipinWT or dLipinΔNLS were expressed (Fig. 3B). These data suggest that decreased insulin pathway activity is caused by a loss of the PAP activity provided by dLipin and not by loss of nuclear functions of dLipin in gene expression. Thus, it seems to be the role of dLipin in the glycerol-3 phosphate pathway and, therefore, TAG production that is required for normal sensitivity to insulin of fat body cells. To test this prediction, we asked whether the reduced activity of other enzymes of the glycerol-3 phosphate pathway had similar effects on PIP_3 levels in the cell membranes of fat body cells. Indeed, RNAi knockdown in the fat body of GPAT4 (encoded by CG3209) or AGPAT3 (encoded by CG4729), the enzymes that catalyze the two reactions immediately preceding dephosphorylation of phosphatidic acid by dLipin, reduced cell membrane association of PH–GFP to a similar extent as that of knockdown of *dLipin* (Fig. 3C; compare Fig. 2C). Taken together, these data suggest that dLipin influences the sensitivity to insulin of fat body cells through the effect it has on the glycerol-3 phosphate pathway and resulting changes in the concentrations of metabolites, such as TAGs or fatty acids.

Fat body defects are strongly enhanced and viability strongly decreased in larvae lacking both dLipin and active insulin receptor

We next asked whether a simultaneous reduction of dLipin and InR activity exacerbates the defects observed after interference with the

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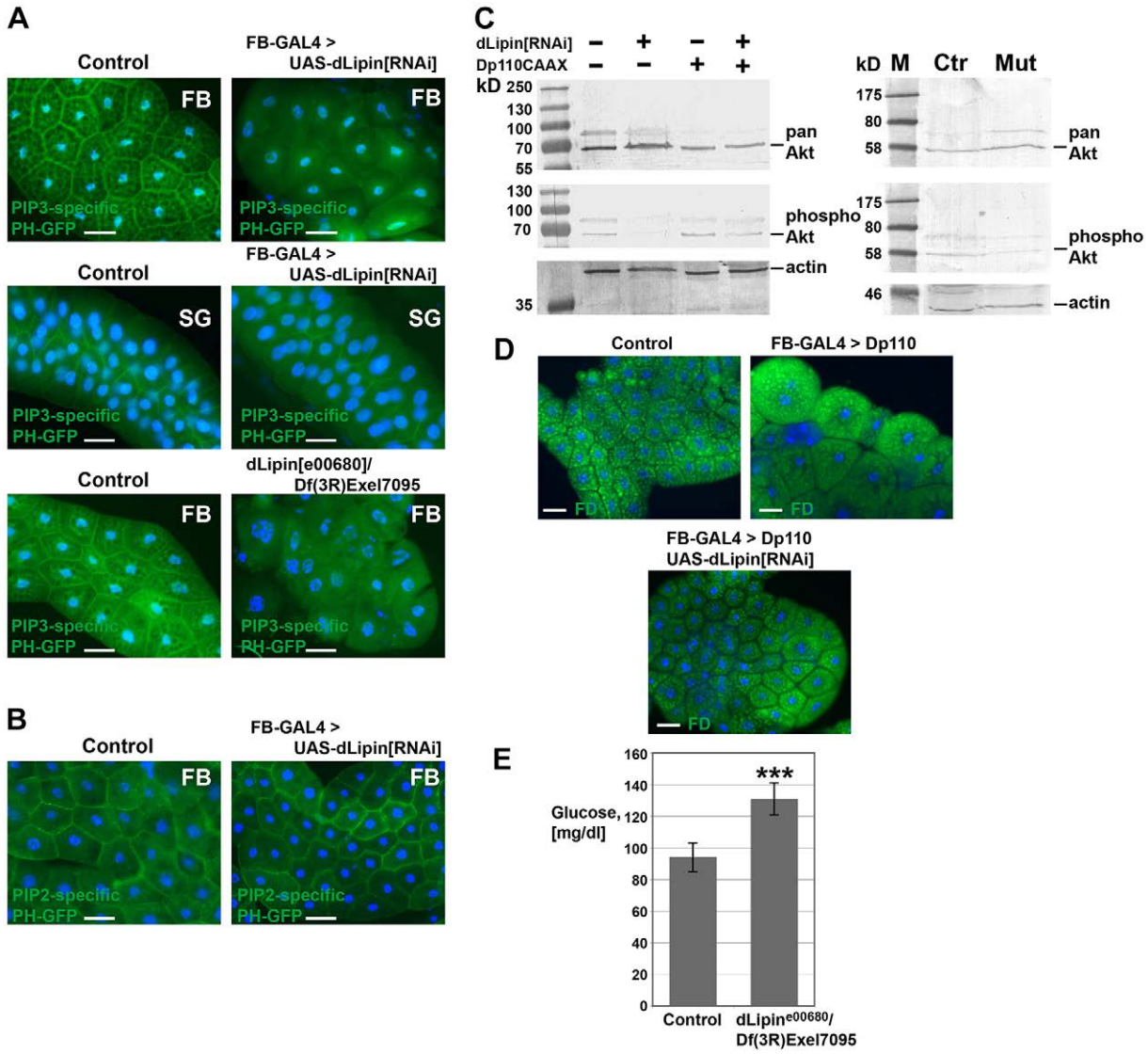

Fig. 2. PI3K signaling is attenuated in fat body cells that lack dLipin. (A) The second messenger PIP_3 was reduced in cell membranes of fat body cells that lacked dLipin. *dLipin* was knocked down specifically in the fat body (FB) using the GAL4 driver Cg-GAL4 (FB-GAL4) in animals expressing the PIP_3 reporter *tGPH*. Membrane association of PH–GFP was strongly reduced in fat bodies lacking dLipin, but not in the fat body of animals carrying FB-GAL4 or UAS-dLipin[RNAi] alone (control). The same lack of membrane association of PH–GFP was seen in the fat body of *dLipin* mutants [$dLipin^{e00680}/Df(2R)Exel7095$]. Membrane association was unaffected in the larval salivary glands (SG), indicating that knockdown of *dLipin* in the fat body does not have a systemic effect on insulin signaling. (B) Knockdown of *dLipin* in the fat body of animals that expressed the PIP_2 reporter PLCδPH–GFP did not lead to a change in PH–GFP association with the cell membrane. (C) Western blot analysis of Akt activity in fat bodies with reduced dLipin. Phospho-Akt was reduced, whereas total Akt protein (pan-Akt) was unchanged after RNAi knockdown of *dLipin* (dLipin[RNAi]). Expression of a constitutively active form of Dp110 (Dp110CAAX) counteracted the reduction of phospho-Akt after *dLipin* knockdown. A similar reduction in phospho-Akt was seen in the fat body of *dLipin* mutants [Mut, $dLipin^{e00680}/Df(2R)Exel7095$]. Detection of actin served as a control for loading and transfer. Ctr, control. (D) Expression of Dp110CAAX (Dp110) in the fat body led to an increase in cell size that was counteracted by knockdown of *dLipin*. Fat droplets (FD) are stained with Bodipy (green). The control fat body was from a larva carrying Dp110 without the other transgenes. (E) Titers of circulating sugars were elevated in animals lacking dLipin. Hemolymph samples were obtained from feeding third-instar larvae heterozygous for $dLipin^{e00680}$ or *Df(2R)Exel7095* (control) or transheterozygous $dLipin^{e00680}/Df(2R)Exel7095$ larvae. Trehalose was enzymatically converted to glucose, and total glucose was measured using a colorimetric assay. Student's unpaired *t*-test, ***$P<0.01$; error bars indicate s.d. Scale bars: 50 µm.

activity of either of the proteins alone. Expression of a dominant-negative form of InR (InR-DN) in the fat body did not interfere with the normal development of the tissue. Fat body cells contained large lipid droplets, indicating that there was no major effect on fat storage. However, the average size of the fat body cells was reduced by about 60% (Fig. 4B). RNAi knockdown of *dLipin* alone in the fat body had mild effects in most animals, resulting mainly in reduced fat droplet size (Fig. 4B; and see Ugrankar et al., 2011). The viability of the animals was unaffected by interference with either InR or dLipin. In stark contrast, when the activity of both InR and dLipin was reduced, most animals died during larval development and showed a severe underdevelopment of the larval fat body (Fig. 4A). None of the animals reached the adult stage. The fat body cells and nuclei were greatly enlarged, cells were rounded and contained very small lipid droplets (Fig. 4B). The severity of this phenotype closely resembled the severity of the phenotype of *dLipin* mutants, which are characterized by a similarly underdeveloped fat body, increased cell and nucleus size, decreased fat droplet size, and larval lethality (Fig. 4B) (Ugrankar et al., 2011).

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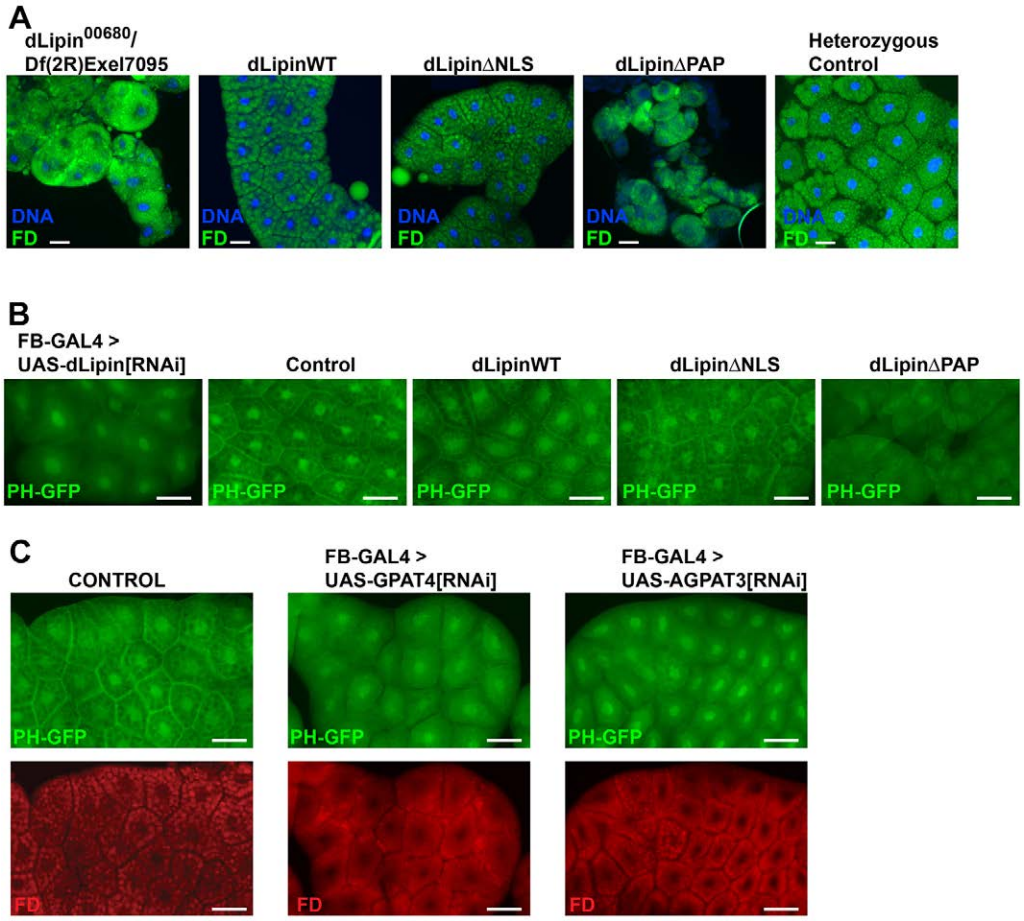

Fig. 3. PI3K signaling depends on the PAP activity of dLipin and an intact glycerol-3 phosphate pathway. (A) Expression of wild-type dLipin (dLipinWT) and dLipinΔNLS, but not dLipinΔPAP, rescued defects in fat body cell morphology and lipid droplet size in *dLipin* mutants [$dLipin^{e00680}/Df(2R)Exel7095$]. The r^4-GAL4 driver was used to activate expression of UAS transgenes in the fat body. Control fat body was from a heterozygous larva from the same cross that produced mutant larvae. Fat droplets (FD) are stained with Bodipy (green), and DNA with DAPI (blue). (B) Loss of PIP_3 at the cell membrane after knockdown of *dLipin* in the fat body was restored by expression of dLipinWT and dLipinΔNLS, but not by expression of dLipinΔPAP. The control fat body was from a larva carrying UAS-dLipin [RNAi] without the FB-GAL4 driver. PIP_3 is marked by PH–GFP (green). (C) RNAi knockdown in the fat body of GPAT4 and AGPAT3 leads to loss of PIP_3 at the cell membrane and a reduction of fat droplet size. PIP_3 is marked by PH–GFP (green), and fat droplets (FD) by LipidTOX (red). Control animals carried FB-GAL4 and tGPH only. Scale bars: 50 µm.

To determine whether InR-DN reduces the expression of dLipin in the fat body cells, we stained fat bodies expressing InR-DN with an antibody against dLipin. The dLipin protein level and distribution were similar to those in control cells (Fig. 4C). Western blot analysis confirmed that InR-DN did not substantially affect dLipin protein levels (Fig. 4D). Together with data showing that insulin affects phosphorylation of dLipin (Bridon et al., 2012), these results suggest that signaling through InR controls dLipin function through a post-translational mechanism, as it does in mammalian species, where lipin 1 undergoes phosphorylation in response to insulin (Csaki and Reue, 2010).

In contrast to the moderate effect of InR-DN on fat body development and survival, disruption of PI3K signaling by overexpression of p60 (also known as Pi3K21B), the regulatory subunit of PI3K, led to a severe reduction of fat body mass and cell size (Fig. 7A), and resulted in strong larval and pupal lethality. p60 is known to exert strong dominant-negative effects when overexpressed (Weinkove et al., 1999). In fat bodies of animals overexpressing p60, dLipin protein was strongly reduced (Fig. 4D). Thus, taken together, the data obtained with InR-DN and p60 suggest that signaling through InR–PI3K is required for both dLipin activity and expression.

dLipin and TORC1 interact in the control of growth and fat body mass

Cellular and organismal growth is controlled by a complex interplay between growth factor signaling through PI3K–Akt and the nutrient-sensitive TOR kinase (Oldham et al., 2000; Zhang et al., 2000). We therefore asked whether we could detect a genetic interaction between *dLipin* and *Drosophila TOR*. Animals heterozygous for the hypomorphic TOR^{k17004} allele, which causes a severe growth defect in homozygous animals, develop normally (Zhang et al., 2000; Fig. 5A). Likewise, larvae in which *dLipin* had been reduced ubiquitously through expression of double-stranded *dLipin* RNA from a heat-inducible transgene showed apparently normal larval development and looked very similar to TOR^{k17004} heterozygous larvae. However, when larvae were subjected to the same knockdown of *dLipin* in a TOR^{k17004} heterozygous background, we observed a reduction in fat body mass, leading to the transparent appearance that is typical for *dLipin* mutant larvae (Ugrankar et al., 2011; Fig. 5A). Dissection revealed that the fat body in these larvae was underdeveloped and TAG levels were significantly reduced compared to levels in *dLipin* knockdown or TOR^{k17004} heterozygous larvae alone (Fig. 5B). Although fat body mass was reduced, individual fat body cells lacking both TOR and dLipin resembled cells that lacked only dLipin (Fig. 5C). Pupae that developed from *dLipin* knockdown larvae exhibited lethality that was significantly enhanced by simultaneous reduction of TOR (Fig. 5D), probably caused by the enhanced depletion of energy stores in these animals. The observed enhancement of *dLipin* loss-of-function phenotypes in a *TOR* mutant heterozygous background indicates that the level of active TOR influences the ability of dLipin to execute functions in fat body development and fat storage.

TOR is a member of two different protein complexes, TORC1 and TORC2. Consistent with the demonstrated role of TORC1 in fat metabolism (Wullschleger et al., 2006), we found that RNAi

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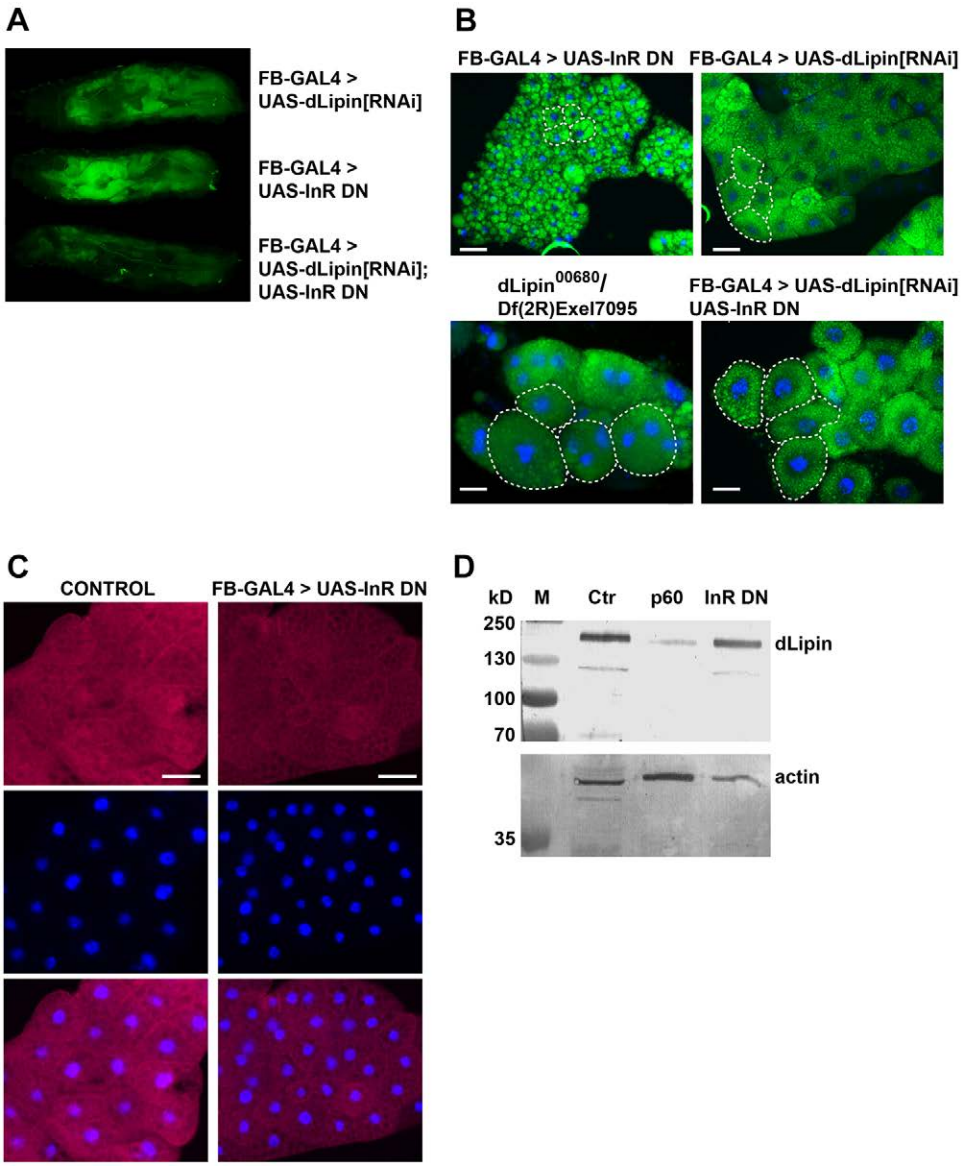

Fig. 4. Reduced insulin signaling strongly enhances the *dLipin* loss-of-function phenotype. Expression of dominant-negative InR (InR-DN) and RNAi knockdown of *dLipin* through expression of UAS-dLipin[RNAi] were driven, either separately or in combination, by the fat body driver Cg-GAL4 (FB-GAL4). (A) Larvae in which activity of both InR and dLipin had been reduced exhibited a severe underdevelopment of the larval fat body. Larvae that expressed InR-DN or the dLipin[RNAi] construct alone exhibited strong fluorescence emitted by the fat body marker *Dcg-GFP* (Suh et al., 2007). In contrast, combined expression strongly reduced fluorescence, visualizing the observed underdevelopment of the larval fat body. (B) InR-DN caused a strong reduction in fat body cell size to about 40% of the size of control cells and the formation of large lipid droplets (control cells, which are not shown here, carried either GAL4 driver or InR responder alone). In striking contrast, when InR-DN was expressed and *dLipin* was knocked down at the same time, both the fat body cells and cell nuclei were greatly enlarged, and cells were rounded. The cytoplasm harbored many very small fat droplets. As previously observed (Ugrankar et al., 2011), knockdown of *dLipin* alone did not have a major effect on cell size or shape, but led to a similar reduction of fat droplet size. The severe fat body phenotype observed after reduction of the activity of both InR and dLipin mimics the phenotype of *dLipin* mutants [$dLipin^{e00680}/Df(2R)Exel7095$] whose fat body cells are about twice as big as normal fat body cells, rounded and characterized by the presence of small fat droplets (Ugrankar et al., 2011). Fat droplets were stained with Bodipy (green) and DNA with DAPI. Scale bars: 50 µm. Dashed lines outline individual cells. (C) Staining of the fat body of w^{1118} control larvae and larvae expressing InR-DN (FB-GAL4>UAS-InR-DN) with an antibody against dLipin. Reduction of insulin signaling by InR-DN does not substantially affect the amount or distribution of dLipin protein. Scale bars: 50 µm. (D) Western blot analysis of dLipin in the fat body after expression of InR-DN or p60. Compared to w^{1118} control animals (Ctr), the dLipin protein level was not substantially changed after FB-GAL4-driven expression of InR-DN, whereas it was greatly reduced after expression of the regulatory PI3K subunit p60. Actin was used as a loading control. M, markers.

knockdown of a specific component of TORC1, raptor, but not rictor, which is specific for TORC2, resulted in phenotypic interactions with dLipin. Although we failed to detect an interaction with TORC2, we cannot exclude that a more rigorous pursuit of this possibility could reveal such an interaction. However, for the purpose of this study, we restricted our further experimental work to TORC1. Simultaneous RNAi knockdown in the fat body of *dLipin* and *raptor* had a strong negative effect on larval growth and fat body cell size. Larvae with fat-body-specific knockdown of either *raptor* or *dLipin* were able to pupariate, whereas double-knockdown larvae remained small, persisted in the food for several days and died without being able to pupariate (Fig. 6A). Fat body

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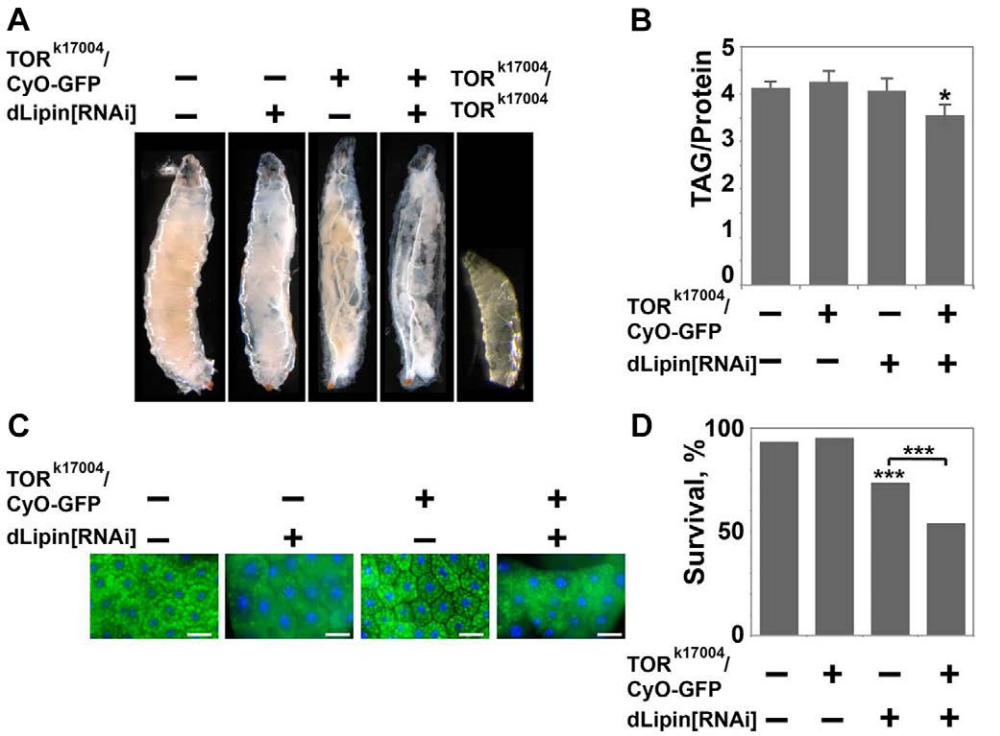

Fig. 5. *dLipin* and *TOR* interact in the control of fat storage. *dLipin* was downregulated by using RNAi in wild-type *TOR* larvae or larvae heterozygous for the TOR^{k17004} allele. (A) Knockdown of *dLipin* in a TOR^{k17004} heterozygous background (TOR^{k17004}/CyO-GFP) led to impaired fat body development, apparent by the increased transparency of the larvae. In contrast to TOR^{k17004} heterozygotes, larvae homozygous for TOR^{k17004} showed a severe growth defect. (B) Measurement of triacylglycerides (TAGs) showed that the impaired fat body development seen in A was accompanied by a significant reduction of fat stores. Student's unpaired *t*-test, $*P<0.05$; error bars indicate s.e.m. (C) Knockdown of *dLipin* by RNAi in a TOR^{k17004} heterozygous background did not change the cellular phenotype caused by *dLipin* knockdown, which includes reduced lipid droplet size. Scale bars: 50 µm. (D) Reduced activity of *TOR* in a TOR^{k17004} heterozygous background was sufficient to significantly enhance pupal lethality caused by *dLipin* knockdown. Fisher's exact test, $***P<0.001$.

cells of double-knockdown animals were extremely small (Fig. 6B), but contained comparatively large nuclei, leading to a more than threefold increase of the nucleocytoplasmic ratio of these cells compared to the control cells (Fig. 6F). Cytoplasmic growth was affected approximately fivefold by the simultaneous knockdown of *dLipin*, whereas nuclear growth did not seem to be significantly affected. This suggests that knockdown of *dLipin* using the fat body driver does not significantly affect endoreplication and, thus, DNA content, which correlates well with measurement of the nucleus size (Maines et al., 2004; Sun and Deng, 2007). The larval growth defect of double-knockdown animals and the inability to pupariate could be rescued by expression of wild-type dLipin (dLipinWT) or dLipin deficient in nuclear translocation (dLipinΔNLS), but not by dLipin lacking PAP activity (dLipinΔPAP) (Fig. 6A). Similarly, the enhanced growth retardation of fat body cells was rescued by dLipinWT and dLipinΔNLS, but not dLipinΔPAP (Fig. 6C). The observation that *dLipin* knockdown enhanced only the cytoplasmic growth defect of *raptor* knockdown cells, but not the nuclear growth defect, suggests that lack of dLipin does not affect cell growth by further reducing TORC1 activity. Because lack of dLipin does reduce PI3K signaling (Fig. 2), this result is consistent with a model in which InR–PI3K can control cell growth independently of TORC1.

TORC1, but not insulin signaling, controls nuclear translocation of dLipin

To determine how TORC1 controls dLipin activity, we examined the effects of reduced TOR and raptor expression on dLipin protein expression and distribution. Both *in situ* antibody staining of fat body cells and western blots indicated that the cells contained less dLipin protein after fat-body-specific RNAi-knockdown of *TOR*. Furthermore, dLipin was enriched in the cell nuclei (Fig. 7A,B). A similar reduction in protein levels and nuclear translocation were observed after RNAi knockdown of *raptor* (Fig. 7A). These data indicate that TORC1 controls the nuclear translocation of dLipin and suggest that dLipin has, primarily, gene regulatory functions in cells that exhibit low TORC1 activity. Because TORC1 activity is controlled by nutrients, we asked whether a similar shift in intracellular localization could be observed in fat bodies of fasting larvae. Indeed, western blot analyses of cytoplasmic and nuclear fractions of fat body homogenates from fasting and fed larvae indicated that the relative levels of dLipin in the nucleus increase when nutrients are scarce (Fig. 7C). In contrast to a reduction in TOR or raptor activity, a reduction of InR (Fig. 4C) or PI3K (Fig. 7A) activity did not result in nuclear translocation of dLipin. Taken together, these data show that TORC1 controls the expression and nuclear translocation of dLipin, thus controlling alternative functions of dLipin under different physiological conditions.

DISCUSSION

Our data indicate that the normal growth of fat body cells depends on sufficient levels of dLipin (Fig. 1). Interestingly, cytoplasmic growth seems to be more affected by a lack of dLipin than endoreplicative growth, as indicated by an increased nucleocytoplasmic ratio (Figs 1 and 6). How does dLipin affect growth? Fat body cells of *dLipin* mutants and cells in which *dLipin* was downregulated using RNAi exhibited a striking lack of the second messenger PIP_3 in the cell membrane, associated with reduced cellular levels of active Akt (Fig. 2). These data indicate that dLipin has an influence on signaling through the canonical InR–PI3K–Akt pathway. PIP_2 levels in the cell membrane were unchanged in dLipin-deficient fat bodies, indicating that the lack of PIP_3 was not caused by a scarcity of the substrate of PI3K. Because RNAi knockdown of *dLipin* was sufficient to prevent an increase in cell growth induced by overexpression of a constitutively active form of the catalytic subunit of PI3K, Dp110 (Fig. 2D), it seems that disruption of the InR–PI3K–Akt pathway occurs either at the level of PI3K or the PI3K antagonist PTEN.

The hemolymph of *dLipin* mutant larvae contained increased levels of sugars (Fig. 2E), a condition which could result from

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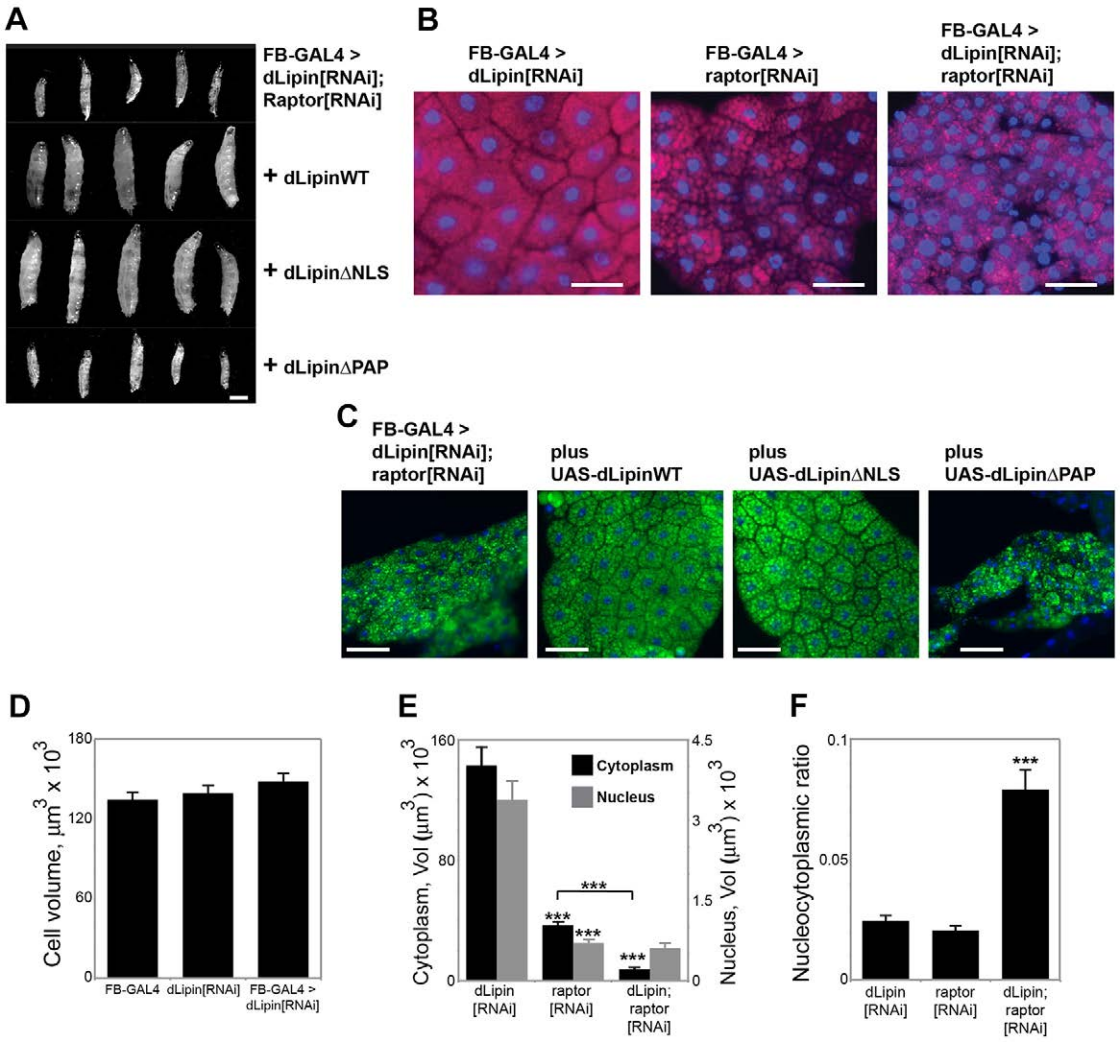

Fig. 6. Lack of *dLipin* strongly enhances growth defects caused by reduced activity of TORC1 in the fat body. (A) Larvae that lack both dLipin and raptor in the fat body (FB-GAL4>dLipin[RNAi]; raptor[RNAi]) exhibited a severe growth defect that could be rescued by expression of wild-type dLipin (dLipinWT) or dLipin without a nuclear translocation sequence (dLipinΔNLS), but not by dLipin lacking the PAP motif (dLipinΔPAP). Double-knockdown larvae of reduced size survived in the food for up to 7 days and did not pupariate. In contrast, rescued larvae grow to approximately normal size and pupariate normally. Scale bar: 1 mm. (B) *dLipin* knockdown strongly enhanced the growth defect of fat body cells that lacked raptor. Fat body cells were stained with LipidTOX (red) to visualize fat droplets and DAPI (blue) to visualize DNA and the cell nucleus. Double-knockdown cells contained very small fat droplets, suggesting that a lack of raptor enhances the *dLipin* phenotype. (C) Fat body expression of wild-type dLipin or dLipin lacking NLS, but not of dLipin lacking the PAP motif, rescues part of the growth defect of *dLipin raptor* double-knockdown cells. Fat droplets were stained with Bodipy (green). Scale bars in B,C: 50 µm. (D) As previously observed (Ugrankar et al., 2011), knockdown of *dLipin* by FB-GAL4 did not significantly affect cell size. (E) In contrast, knockdown of *raptor* using FB-GAL4 had a strong effect on cell size, and this effect was 2.8-fold enhanced by simultaneous knockdown of *dLipin*. (F) However, the nucleus size was not or only marginally reduced by the additional knockdown of *dLipin*, resulting in a significant increase of the nucleocytoplasmic ratio of double-knockdown cells. Error bars indicate s.e.m. and asterisks indicate results of Student's unpaired *t*-tests (***P<0.0001).

insulin resistance and/or decreased Dilp levels. Our data strongly suggest that insulin resistance, at least, contributes to increased sugar levels for two reasons. First, reduction of dLipin specifically in the fat body reduces insulin responses in this tissue, but not in other tissues (Fig. 2A). This suggests that insulin (Dilp) levels are unaffected. Second, our mosaic data show that a lack of dLipin affects cell growth, which is controlled by the InR–PI3K–Akt pathway (Saucedo and Edgar, 2002), in a cell-autonomous manner (Fig. 1A). Thus, individual cells that lack dLipin showed impaired growth in an otherwise normal physiological background (Fig. 1A), further supporting the notion that lack of dLipin affects insulin (Dilp) sensitivity, but not insulin signaling itself. Consistent with our observations in *Drosophila*, insulin resistance is one of the phenotypes exhibited by fatty lipid dystrophy (*fld*) mice that lack lipin 1 (Reue et al., 2000). Similar to mice, expression of lipin 1 in humans is positively correlated with the sensitivity of liver and adipose tissue to insulin (Suviolahti et al., 2006; Yao-Borengasser et al., 2006). However, mechanisms that mediate the effects of lipins on sensitivity to insulin are not well understood. Our data show that the PAP activity of dLipin is required for normal sensitivity to insulin and that reduction in the levels of GPAT4 or AGPAT3, two other enzymes of the glycerol-3 phosphate pathway, has effects on membrane-associated PIP_3 similar to those of reduction of dLipin levels (Fig. 3C). This suggests that the effect of dLipin on the sensitivity to insulin is mediated by intracellular changes in metabolites, e.g. TAGs or fatty acids, that are brought about by changes in flux through the glycerol-3 phosphate pathway.

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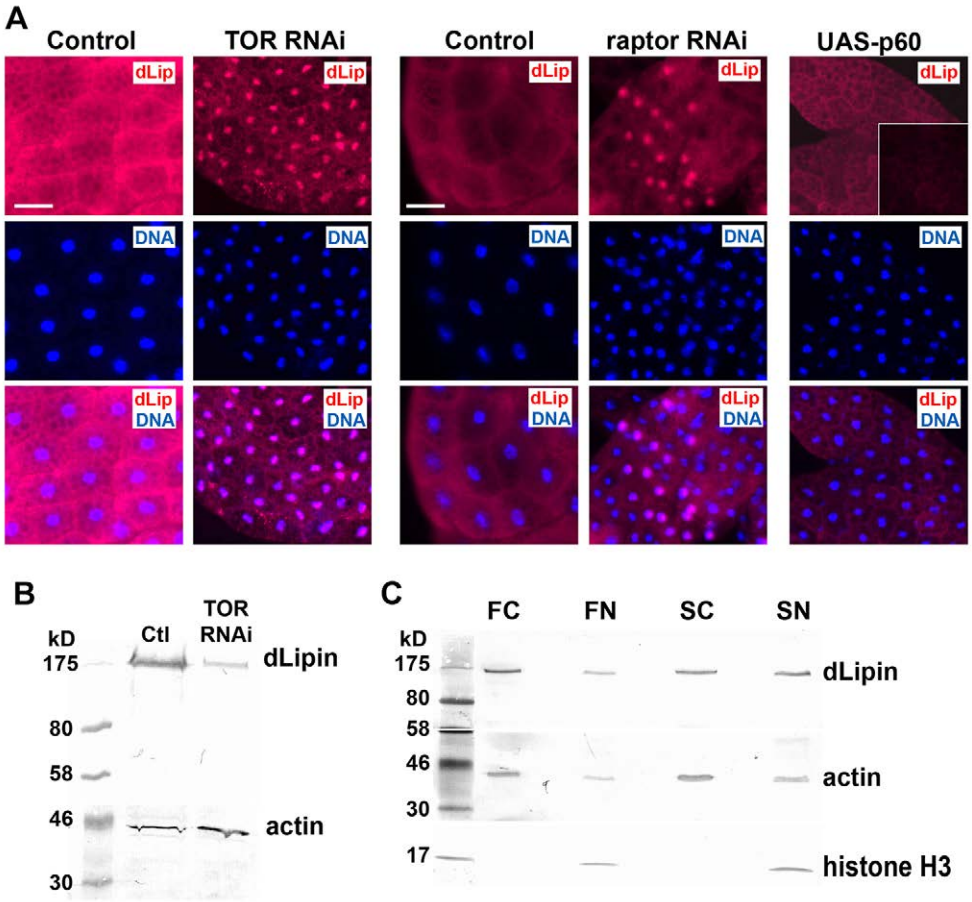

Fig. 7. dLipin preferentially localizes to the cell nucleus when TORC1 activity is low or animals are fasting. (A) Staining with an antibody against dLipin (dLip) indicates that dLipin predominantly resides in the cytoplasm in fat body cells of third-instar larvae that express TOR or raptor normally (control). Knockdown of *TOR* or *raptor* in the fat body by using RNAi (FB-GAL4>UAS-TOR[RNAi] or UAS-raptor[RNAi]) leads to a decrease of dLipin in the cytoplasm and an increase in the cell nuclei (DAPI, blue). In contrast, reduction of PI3K activity (UAS-p60) did not lead to nuclear translocation of dLipin. Staining in the p60 background is shown at increased brightness to make intracellular distribution of dLipin visible. The inset shows the unaltered, weak staining intensity, which supports data obtained by western blot analysis, indicating that expression of p60 strongly reduces dLipin protein (Fig. 4D). Scale bars: 50 µm. (B) Western blot analysis confirms that the total amount of dLipin in the fat body was reduced after *TOR* knockdown (TOR RNAi). (C) Similar to *TOR* or *raptor* knockdown, starvation caused a change from cytoplasmic to nuclear localization of dLipin. The fat body of larvae that had been fed normally or starved for 4 h was dissected, homogenized and fractionated into cytoplasm and nuclei. Fractions were dissolved in SDS sample buffer and subjected to western blot analysis. The blot shown represents an example of four replicas that all showed similar results. FC: fed, cytoplasm; FN: fed, nucleus; SC: starved, cytoplasm; SN: starved, nucleus. Detection of actin in B, C served as a loading control, and detection of histone H3 in C as a control for cell fractionation. Ctl, control.

Our data show that reduced activity of InR in dLipin-deficient fat bodies leads to a phenotype that strongly resembles the severe fat body phenotype of *dLipin* loss-of-function mutants (Fig. 4B). This observation strongly suggests that reduced signaling through InR further reduces the activity of dLipin. Because reduced activity of InR has no substantial impact on dLipin protein levels (Fig. 4D), a likely explanation for this effect is that the InR pathway controls the activity of dLipin through post-translational modification. This interpretation is supported by data showing that phosphorylation of *dLipin* in *Drosophila* S2 cells responds to insulin stimulation (Bridon et al., 2012), and it is consistent with a substantial body of evidence showing that mammalian lipin 1 is regulated through phosphorylation in response to insulin signaling (Harris et al., 2007; Huffman et al., 2002). This suggests that functions of the insulin signaling pathway in the regulation of lipins are evolutionarily conserved.

In contrast to reduced signaling through the InR–PI3K pathway, reduced signaling through TORC1 led to translocation of dLipin into the nucleus (Fig. 7A). A similar translocation into the cell nucleus has been observed for lipin 1 after loss of TORC1 in mammalian cells (Peterson et al., 2011). Consistent with the role of TORC1 as a nutrient sensor, we observed nuclear enrichment of dLipin during starvation (Fig. 7C), and we have previously shown that the presence of dLipin is crucial for survival during starvation (Ugrankar et al., 2011). Taken together, these data suggest that both dLipin and lipin 1 have essential nuclear, gene regulatory functions during starvation. What are the genes controlled by nuclear lipins, and how do they control gene expression? In the mouse, it has been shown that lipin 1 can directly activate the gene encoding nuclear receptor PPARα and that overexpression of lipin 1 leads to the activation of genes involved in fatty acid transport and β-oxidation, TCA cycle and oxidative phosphorylation, including many target genes of PPARα. At the same time, expression of genes involved in fatty acid and TAG synthesis is diminished (Finck et al., 2006). This suggests that lipins directly regulate genes to promote the utilization of fat stores during starvation, although gene expression studies are necessary at physiological protein levels that distinguish between the effects of nuclear and cytoplasmic lipin to confirm this

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hypothesis. Chromatin immunoprecipitation experiments with both yeast and mammalian cells have shown that lipins associate with regulatory regions of target genes, suggesting that nuclear lipins act as transcriptional co-regulators (Finck et al., 2006; Santos-Rosa et al., 2005). Interestingly, however, lipin 1 that has translocated into the nucleus can also influence gene expression through an unknown PAP-dependent mechanism that controls nuclear levels of the transcription factor SREBP, which positively controls the expression of genes required for sterol and fatty acid synthesis (Peterson et al., 2011). This suggests that nuclear lipins use alternate mechanisms to bring about changes in gene expression. It will be interesting to further investigate these mechanisms, taking advantage of the large size and the polytene chromosomes of fat body cells in *Drosophila*.

Interestingly, we observed robust nuclear translocation of dLipin after reducing TORC1 activity (Fig. 7A), but we did not see nuclear translocation of dLipin when signaling through the insulin pathway was reduced, neither after moderate (InR-DN) or severe reduction (p60) (Figs 4C and 7A). This suggests that the InR–PI3K pathway can control functions of dLipin independently of TORC1 in *Drosophila*. Two observations further support this proposition. First, reduction of dLipin affects cytoplasmic and endoreplicative growth differently when enhancing growth defects associated with diminished TORC1 activity, leading to an increase in the nucleocytoplasmic ratio (Fig. 6B,F). We did not see such an increase after reduction of TORC1 alone, suggesting that enhancement of the growth defect is an additive effect that is caused by reduced PI3K–Akt signaling and not by further reduction of TORC1 activity. Second, reduction of TORC1 in the fat body leads to a systemic growth defect (Colombani et al., 2003), whereas lack of dLipin in the fat body does not affect organismal growth (Ugrankar et al., 2011), and reduction of dLipin did not affect the growth of animals that lack TOR (Fig. 5A; and data not shown).

Our data do not indicate that InR–PI3K signaling has an effect on the intracellular distribution of dLipin, whereas insulin stimulates cytoplasmic retention of lipin 1 in mammalian cells in a rapamycin-sensitive manner (Harris et al., 2007; Péterfy et al., 2010). This suggests that the effect is mediated by TORC1, which can also regulate lipin 1 in certain cells in a rapamycin-insensitive manner (Peterson et al., 2011). However, it is noteworthy that lipin 1 contains at least 19 serine and threonine phosphorylation sites (Harris et al., 2007; Peterson et al., 2011), and that some of these sites appear to be recognized by kinases other than TOR (Grimsey et al., 2008). In view of these findings, and considering that not all insulin-stimulated phosphorylation events on lipin 1 are sensitive to rapamycin (Harris et al., 2007), it cannot be excluded that one or more other insulin-sensitive kinases contribute to the regulation of lipin 1 and other lipins. Data on the insulin and TORC1 regulation of lipin 1 were obtained with cultured cell lines. Our whole-animal data suggest that, indeed, an additional pathway might exist through which insulin regulates functions of lipins independently of TORC1. It is important to note that genetic studies in *Drosophila* have provided a number of examples that indicate that the insulin and TORC1 pathways act independently of one another when studied in the context of specific tissues during normal development. For instance, activity of the ribosomal protein kinase S6K, which is a major target of TORC1 in both flies and mammals, is unaffected by mutations of insulin pathway components in *Drosophila* (Oldham et al., 2000). Furthermore, insulin and TORC1 independently control different aspects of hormone production by the *Drosophila* ring gland (Colombani et al., 2005; Layalle et al., 2008). It will be interesting to see whether whole-animal studies in mammalian systems will reveal a similar, at least partial, independence of insulin and TORC1 signaling in the control of lipins. Specifically, future work will have to address in detail the functional importance of the many phosphorylation sites found in both mammalian and fly lipins, and identify all kinases involved, to determine the extent to which regulation is conserved between fly and mammalian lipins.

MATERIALS AND METHODS

Fly stocks

Flies carrying the hypomorphic *dLipin*^{e00680} allele (Ugrankar et al., 2011) were from the Exelixis insertion collection at Harvard Medical School (Thibault et al., 2004). Tub>CD2>Gal4 and hsflp; UAS-GFP/CyO flies were provided by Gerard Campbell (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA), and PLCδPH–GFP flies (Pinal et al., 2006) by Franck Pichaud (MRC Laboratory for Molecular Biology, University College London, London, UK). *Dcg-GFP* flies (Suh et al., 2007) were obtained from Jonathan M. Graff (Department of Developmental Biology, UT Southwestern Medical Center, Dallas, TX), and r^{4}-*gal4* flies (Suh et al., 2007) from Jae Park (Department of Biochemistry & Cellular & Molecular Biology, The University of Tennessee, Knoxville, TN). The UAS-dLipin[RNAi] stock (number 36007) and stocks for RNAi knockdown of AGPAT3 (number 48593) and GPAT4 (number 10281) were obtained from the Vienna *Drosophila* Resource Center. Flies carrying Df(2R)Exel7095 (number 7860), Cg-GAL4 (number 7011), *TOR*^{k17004} (number 11218), tGPH (number 8164); transgenes for RNAi knockdown of raptor (number 41912), TOR (number 33951), or for misexpression of p60 (number 25899); or carrying Dp110CAAX (number 25908), and InR-DN (number 8252) were obtained from the Bloomington *Drosophila* Stock Center.

Generation of genetic mosaics in the fat body

RNAi knockdown of *dLipin* in fat body cell clones was achieved by 'flip out' activation of GAL4 using the FLP-FRT system (Arsham and Neufeld, 2009; Britton et al., 2002). Flies carrying UAS-dLipin[RNAi] and Tub>CD2>Gal4 were crossed with flies carrying hsflp; UAS-GFP. The progeny were kept at 25°C, a temperature at which leaky expression of heat-shock inducible FLP leads to activation of GAL4 expression in the few cells that are marked by GAL4-induced activation of GFP (Britton et al., 2002). Fat bodies with GFP-positive cells were stained with an antibody against dLipin and LipidTOX Deep Red (Life Technologies).

dLipin knockdown in *TOR*^{k17004} heterozygous background

To reduce *dLipin* in a *TOR*^{k17004} heterozygous background, we used a heat-inducible dLipin[RNAi] transgene, hs-dsLipin3. Animals of the genotype *TOR*^{k17004}/CyO-GFP; hs-dsLipin3/hs-dsLipin3, and control animals of the genotypes *TOR*^{k17004}/CyO-GFP, and hs-dsLipin3/hs-dsLipin3 were subjected to daily 30-min heat shocks at 37°C. Wandering third-instar larvae were collected for analysis of fat body development and triglyceride quantification.

Plasmid construction and transgenics

For construction of hs-dsLipin3, two PCR products derived from *dLipin* cDNA GH19076 were sequentially cloned into *Bam*HI–*Xba*I and *Eco*RI/*Xba*I-cut transformation vector pCaSpeR-hs-act (Thummel et al., 1988) (primer pair 1, 5′-GCAGCGCGATGGCGGGATCCAGTGCTCGCCC-3′, 5′-GTCCAAATCTAGACGTGGATTGCTAGTGGGGG-3′; primer pair 2, 5′-GCAGCGCGATGGCGAATTCCAGTGCTCGCCC-3′, 5′-CGCTCA-TGGCCTCATTCTAGACCTGCTCCGG-3′). For construction of UAS-dLipinΔPAP and UAS-dLipinΔNLS, mutations were introduced into *dLipin* cDNA GH19076 using the Change-IT Multiple Mutation Site-Directed Mutagenesis kit (Affymetrix). A C-to-G nucleotide exchange leading to an amino acid exchange (D812E) in the catalytic motif of dLipin (dLipinΔPAP) was introduced using primers of the sequence 5′-GGTGG-TGATCTCGGAGATTGACGGCACCATCA-3′ and 5′-GCCATTCAGC-CGTACGACTAGGTTAGGC-3′. Deletion of an 18-bp sequence encoding the NLS of dLipin (dLipinΔNLS lacking amino acids 276–281) was

introduced using primers of the sequences 5′-GGTGTCCAAGAGCAAA-ACCTCGCAAATGAAGAAGA-3′ and 5′-GCCATTCAGCCGTACGAC-TAGGTTAGGC-3′. Successful mutagenesis was confirmed by sequencing. Mutated and wild-type cDNAs (dLipinWT) were isolated by *Xho*I and *Eco*ICRI digest and cloned into the *Xba*I and *Xho*I sites of transformation vector pUASTattB (Bischof et al., 2007). Injection of transforming plasmids into embryos of w^{1118} and phiC31 recipient strains was performed at BestGene (Chino Hills, CA).

Antibody staining of fat bodies, and cell size measurements

Fat body tissue was stained with an affinity-purified antibody directed against dLipin as described previously (Ugrankar et al., 2011). The antibody against dLipin was used at a dilution of 1:400. Secondary antibodies used were Rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) and Alexa-Fluor-647-conjugated goat anti-rabbit IgG (Molecular Probes), both used at a 1:1000 dilution. Cell and nuclear volumes were derived from measurements of cell and nuclear areas performed with AxioVision (Zeiss) or ImageJ (Schneider et al., 2012) software. Cell and nuclear radii were calculated using the formula $r=\sqrt{A/\pi}$ and converted to volumes using the formula $V=4/3\pi r^3$ (Oldham et al., 2000). Cytoplasmic volumes were obtained by subtraction of the nuclear volume from the total cell volume.

Lipid staining and triglyceride assay

Fat droplets were stained with either Bodipy 493/503 or LipidTOX Deep Red (Life Technologies), as noted in the figure legends. Fat bodies were fixed before staining for 30 min in 4% formaldehyde and incubated for 30–60 min in the dark with 1 µg/ml Bodipy or in a 1:400 dilution of LipidTOX in PBS, pH 7.4. Tissue was mounted in Slowfade Gold antifade reagent with DAPI (Life Technologies). Fat droplets were visualized by fluorescence microscopy using a Carl Zeiss Axio Imager.M1 microscope controlled by AxioVision software using GFP (Bodipy) and Cy5 (LipidTOX Deep Red) filters.

Triglycerides were measured using the Infinity triglycerides assay (Thermo Scientific), as described previously (Ugrankar et al., 2011).

Hemolymph sugar measurement

The sugar content of hemolymph from feeding third-instar larvae was measured using the Glucose Colorimetric Assay kit from Cayman Chemical (catalog number 10009582; Ann Arbor, MI). A volume of 0.5 µl hemolymph was collected from five larvae using a micropipette and transferred into 19 µl PBS, pH 7.4. After centrifugation at 13,000 ***g*** for 10 min, 10 µl of supernatant was added to 100 µl of sodium phosphate assay buffer provided by the kit. Samples and standards were incubated at 37°C overnight with 0.05 units/ml trehalase (Sigma-Aldrich) to release glucose from trehalose. Samples and standards were then measured according to the instructions of the manufacturer, and glucose concentrations determined. Analyses were performed in biological triplicates for *dLipin* mutant larvae ($dLipin^{e00680}/Df(2R)Exel7095$) and heterozygous control larvae.

Starvation and western blot analyses

Pre-wandering third-instar larvae of the strain w^{1118} were transferred to either standard food (fed larvae) or cotton plugs soaked in PBS (starved larvae). After 4 h, fat bodies were dissected out and homogenized in 10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM benzamide, 0.5 mM PMSF and 0.6% NP-40. Cytoplasmic and nuclear fractions were separated, essentially as described previously by Petersen et al. (1999), and taken up in SDS sample buffer for SDS-PAGE. Western blotting of the separated proteins was performed using standard procedures. Antibodies against dLipin (Ugrankar et al., 2011) were used at a dilution of 1:1500 and antibodies against actin (Sigma-Aldrich) at 1:500. Antibodies against histone H3 (1:1000; Cell Signaling Technology) or Pipsqueak (Schwendemann and Lehmann, 2002; 1:1500) were used to ascertain the quality of the nuclear and cytoplasmic preparations.

For the western blots shown in Figs 4 and 7, antibodies against dLipin were used at a dilution of 1:1000. Pan-Akt and phospho-Akt rabbit monoclonal antibodies (Cell Signaling Technology; Fig. 2) were used at a dilution of 1:1000.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.S. co-designed experiments, carried out most of the experimental work, made images and analyzed the data. R.U. contributed to the analysis of the TOR–dLipin interaction and carried out the TAG measurements. S.E.G. contributed to the analysis of the dLipin–Dp110 interaction. M.P. carried out the western blot analysis of dLipin distribution under starvation conditions. M.L. obtained funding for and directed the project, designed experiments, carried out the survival studies, created the figures and wrote the manuscript.

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Supplementary information

Supplementary information available online at
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