dMyc expression in the fat body affects DILP2 release and increases the expression of the fat desaturase Desat1 resulting in organismal growth

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A B S T R A C T

Drosophila dMyc (dMyc) is known for its role in cell-autonomous regulation of growth. Here we address its role in the fat body (FB), a metabolic tissue that functions as a sensor of circulating nutrients to control the release of Drosophila Insulin-like peptides (Dilps) from the brain influencing growth and development. Our results show that expression of dMyc in the FB affects development and animal size. Expression of dMyc, but not of CycD/cdk4 or Rheb, in the FB diminishes the ability to retain Drosophila Insulin-like peptide-2 (DILP2) in the brain during starvation, suggesting that expression of dMyc mimics the signal that remotely controls the release of Dilps into the hemolymph. dMyc also affects glucose metabolism and increases the transcription of Glucose-transporter-1 mRNA, and of Hexokinase and Pyruvate-Kinase mRNAs, key regulators of glycolysis. These animals are able to counteract the increased levels of circulating trehalose induced by a high sugar diet leading to the conclusion that dMyc activity in the FB promotes glucose disposal. dMyc expression induces cell autonomous accumulation of triglycerides, which correlates with increased levels of Fatty Acid Synthase and Acetyl CoA Carboxylase mRNAs, enzymes responsible for lipid synthesis. We also found the expression of Stearoyl-CoA desaturase, Desat1 mRNA significantly higher in FB overexpressing dMyc. Desat1 is an enzyme that is necessary for monosaturation and production of fatty acids, and its reduction affects dMyc’s ability to induce fat storage and resistance to animal survival.

In conclusion, here we present novel evidences for dMyc function in the Drosophila FB in controlling systemic growth. We discovered that dMyc expression triggers cell autonomous mechanisms that control glucose and lipid metabolism to favor the storage of nutrients (lipids and sugars). In addition, the regulation of Desat1 controls the synthesis of triglycerides in FB and this may affect the humoral signal that controls DILP2 release in the brain.

Introduction

Myc activity controls fundamental processes including animal development, cell proliferation, and tissue growth (Bellosta and Gallant, 2010; Dang, 2012). In Drosophila mutations in the sole dmyc gene reduce body size (hence the name diminutive) (Johnston et al., 1999) and analysis of dmyc’s target genes reveals a high prevalence for genes regulating ribosome biogenesis and protein synthesis (Grewal et al., 2005; Hulf et al., 2005; Orian et al., 2003). Myc activity lies downstream of numerous growth factors and nutrient signaling pathways including the SWH (Salvador–Warts–Hippo) pathway, to control growth in epithelial cells (Huang et al., 2005; Neto-Silva et al., 2010; Ziosi et al., 2010), and of the insulin/TOR signaling pathway, that regulates dMyc protein stability through the ubiquitin degradation pathway (Parisi et al., 2011; Zhang et al., 2006). Myc function is known to control metabolic pathways upstream of glycolysis and glutaminolysis (Osthus et al., 2000; Wise et al., 2008), particularly in cancer cells where c-Myc activity couples glucose and glutamine metabolism with growth and biomass (Dang, 2012; Yuneva et al., 2012). Much less is known about the role of Myc in the regulation of lipid metabolism. A few studies pinpoint at the ability of adipocytes and liver cells to increase c-myc mRNA in response to caloric restriction, suggesting that Myc may be necessary to maintain the basal metabolic rate of these cells during low nutrient conditions.

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(Horikawa et al., 1986; Kim et al., 1991). Previous studies demonstrated that animals expressing c-myc in the liver showed increased blood glucose disposal and resistance to streptozotocin-induced diabetes. These animals favor glycolysis and lipogenesis (Valera et al., 1995), suggesting a function for Myc in the regulation of carbohydrate metabolism in non-cancerous conditions (Riu et al., 1996).

In Drosophila, the fat body (herein referred as FB) is a metabolic tissue with similar physiological functions as the mammalian adipose tissue and liver. The FB acts as storage for sugars and fats, which are mobilized to sustain basal cellular energy supplies when nutrients are scarce (Liu et al., 2009), and regulates systemic growth in response to nutrients and hormonal signals (Tennen and Thummel, 2011). The FB functions as a sensor for amino-acids in the hemolymph (Britton and Edgar, 1998; Davis and Shearn, 1977), and when their concentration is abundant, releases humoral factors to remotely control the metamorphosis to proceed (Delanoue et al., 2010), while the opposite instar by ecdysone signaling to reduce growth allowing the onset of growth and confers resistance to starvation. Animals expressing dMyc demonstrate that expression of dMyc in the FB induces systemic growth, fat storage and resistance to starvation, placing this enzyme as a novel component in dMyc's function in controlling lipid metabolism and systemic growth.

Materials and methods

Fly husbandry

cg-GAL4 (Bloomington stock 7011), UAS-CS1 RNAi (Bloomington stock 40836), UAS-dMyc RNAi (VDRC stock 2947 and Bloomington stock 25784), UAS-Pyruvate Kinase RNAi (VDRC stock 49533), UAS-Hexokinase-C RNAi (VDRC stock 35337) and UAS-Desat1 RNAi (VDRC stock 33338). UAS-HA-dMyc (Bellosta et al., 2005), yw hs-Flp122;; Act5C > FRT-CD2-FRT > Gal4; UAS-GFP/TM6b (Saucedo et al., 2003), hemolentin-GAL4 (Goto et al., 2001), pumpless-GAL4 (Zinke et al., 1999). Animals were raised at low density, at 25 °C on a standard corn meal containing 6 g/L agar, 75 g/L corn flour, 100 g/L white sugar, 50 g/L fresh yeast, and 10 g/L inactivated yeast powder, along with nipagin and propionic acid (Acros Organic).

Nile-red and oil-red O tissue staining

For Nile-Red: Larvae were bisedected in PBS. Fat bodies were isolated from carcasses, fixed for 20 min in 4% paraformaldehyde (Electron Microscopy Science) in PBS, washed in 0.3% PBS triton X-100 and incubated for 30 min in a 0.01 mM Nile Red Solution (SIGMA), then photographed under a Zeiss LSM510 confocal microscope. Oil Red O staining of larval tissues was performed as described previously (Gutierrez et al., 2007).

Quantitative real time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNAs were quantified using a UV spectrophotometer and RNA integrity was confirmed with ethidium bromide staining. The purified RNA (1 μg) was used as template for cDNA synthesis using SuperScript II (Invitrogen). The SYBR Green PCR Kit (Qiagen) was used, and products were quantified using the ABI Prism 7300 system. The relative level for each gene was calculated using the 2-DDCt method (Hult et al., 2005) and reported as arbitrary units compared to actin5C as a control. At least three separate experiments were performed in duplicate. Primers were designed using Primer3 (Rozen and Skalletsy, 2000). Primers list in available in the Supplementary materials.

Starvation assay

At least 30 staged late L2 larvae from each genotype for each replica were collected and transferred in single well plastic plates containing paper soaked with PBS; survival rate was determined every 12 h. These experiments were repeated three times. Statistical analysis was performed using a z-test (Supplementary Appendix A).

Larval development

At least 30 staged L1 larvae (24 h AED) of each genotype were collected in grape agar and transferred into regular corn meal food. The developmental stage was scored every 12 h and identified according to the mouth hook/spiracle morphology (Britton and Edgar, 1998). To quantify growth differences, age-matched larvae were collected from the culture medium, washed and killed by microwave pulse. Larval volume was measured using Photoshop software and the formula \(4/3\pi r^2(L-\pi \text{length} l/\text{width})\) was used.

Glucose and triglyceride measurement

Trehalose and TAG levels were measured in both fed and starved larvae. 72 h larvae were either kept in normal fly food or starved for 24 h in petri dishes containing filter paper moistened with PBS. Five to ten larvae were homogenized on ice in 200 μl of PBS for glucose assays, or in 0.1% PBS Tween for trehalose assays. Homogenates were centrifuged for 3 min at 5000 rpm and the supernatants were incubated at 70 °C for 5 min to inactivate endogenous enzymes. For glucose assays, trehalose was converted to glucose by adding Trehalase (Sigma) at a final concentration of 0.025 U/ml and incubating for 15 min at 37 °C. The samples were mixed with 500 μl of Glucose...
Reagent HK (Sigma) for 15 min at room temperature and absorbance measured at 340 nm according to the manufacturer's protocol. Glucose measurements from undigested samples were subtracted to give the amount of glucose obtained from trehalose. For hemolymph assays, hemolymph was pooled from 5 larvae and 1 μl collected, diluted 1:20 in PBS, and heat inactivated at 70 °C for 5 min. 2 μl of diluted hemolymph was used for each glucose assay. Triglyceride measurements were performed by incubating 20 μl of sample with 400 μl of Free Glyceral Reagent (Sigma) for 15 min at RT, followed by 100 μl Triglyceride Reagent (Sigma) for 15 min, and absorbance read at 540 nm according to the manufacturer's protocol. Glucose and triglyceride concentrations were normalized against total protein concentration in each sample as measured by a BCA assay (Pierce). Assays were repeated a minimum of three times. Triglycerides were also measured in fat bodies collected from ten larvae per genotype.

Western blot

Tissues from staged third instar larvae were collected in lysing buffer (50 mM Heps pH 7.4, 150 mM NaCl, 1% Triton, 1 mM EDTA), with phosphatase and protease inhibitors (Roche). Protein concentrations were quantified using Bradford reagent. Equal amounts were resolved on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Western blotting was performed using mouse monoclonal anti dMyc antibody (Prober and Edgar, 2002), rat monoclonal anti epitope tag HA antibody (Roche) and mouse monoclonal anti-actin antibody (Millipore). Enhanced chemiluminescence (ECL-GE Healthcare) was used for detection. Quantification of dMyc proteins was performed from photographs using ImageJ. The intensity of each band (pixels) was compared to the mean pixels corresponding to the same band in control or RNAi animals. 

Anti-DILP2 and anti dFOXO immunofluorescence

Brains from larva at 96 h AEL were dissected in PBS, fixed in PBS/4% formaldehyde for 20 min, and washed in PBS-0.3% Triton X-100 (PBT). Tissues were blocked in PBT/5% BSA. Primary rat-anti-DILP2 was used overnight at 4 °C (Geminard et al., 2009) followed by Alexa488 anti rat-secondary antibodies (Invitrogen). Tissues were mounted in Vectashield with DAPI (Vectorlabs) and fluorescence images were acquired using a Zeiss LSM 510 confocal microscope. The quantification of the mean pixels corresponding to each band was compared to the intensity of the same band in control from the same blot.
to the intensity of fluorescence in the IPCs was determined using the histogram tool in ImageJ, using similar parameters as described previously (Geminard et al., 2009). Data are expressed in arbitrary units, and represent the average fluorescence intensity in the IPCs and relative standard deviations were calculated using four independent experiments, each including at least ten animals of each genotype and conditions. dFOXO staining FBs from larva at 96 h AEL or starved for 24 h in PBS, were dissected in PBS, fixed in PBS/4% formaldehyde for 20 min, and washed in PBS-0.3% Triton X-100 (PBT). Tissues were blocked in PBT/5% BSA. Primary rat-anti-dFOXO was used overnight at 4 °C followed by Alexa-488 anti rat-secondary antibodies (Invitrogen). Tissues were mounted in Vectashield with DAPI (Vectorlabs) and fluorescence images were acquired using a Zeiss LSM 510 confocal microscope. The anti dFOXO antibody was developed during this study using the region CASMETSRYEKRRGRAKKRVEALR from the Drosophila peptide sequence as an antigen. The peptide was linked to a protein carrier and injected into rabbit. The serum was affinity purified using the peptide used as antigen and AP anti-dFOXO used 1:200 (Prosci-inc, CA).

### Results

DMyC expression in the fat body affects animal size

To understand whether Myc activity in adipocytes regulates metabolic pathways that affect growth and survival, we modulated DMyC expression in the larval fat body (FB), using the UAS-Gal4 binary system (Brand et al., 1994). The cg (collagen type IV) promoter (Asha et al., 2003) fused with Gal4, was used to drive expression of UAS-dMyc or UAS-dMyc-RNAi in the FB. DMyC protein expression was analyzed in FB from yw1118; Gal4; UAS-HA-dMyc (hereinafter called cg-dMyc), yw1118; Gal4; UAS-dMyc-RNAi (hereinafter called cg-dMyc-RNAi), and yw1118; Gal4 (hereinafter called cg-) third instar larvae, using anti dMyc antibodies (Prober and Edgar, 2002). These data showed that in the FB anti dMyc antibodies recognize two major bands of 100 and 130 KDa bands that were significantly reduced in extracts from cg-dMyc RNAi FBs (Fig. 1A, left panel). However, since dMyc was expressed with an HA tag, we were able to use anti-HA antibody to detect one band at 100 KDa that was present only in the extracts of FB from cg-HA-dMyc RNAi FBs (Fig. 1A, right panel). Analysis of organismal size and developmental delay revealed that cg-dMyc animals develop with a similar rate as cg larvae (Fig. 1C and Table 1), but they reach pupation at a significantly bigger size than control animals. Similar results were obtained using two independent insertions of UAS-dMyc (t-test P-values < 0.05 for both lines). In contrast, RNAi-mediated reduction of DMyC in the FB resulted in animals that reached pupation with a delay of 48 h at their third instar transition (Fig. 1C and Table 1); these animals were smaller throughout development (P < 0.0001 in a t-test when compared to cg- larvae) and eclosed as small adults (Fig. 1B–D and E). Two independent chromosomal insertions were used for these experiments, and the dMyc-RNAi line on Chromosome III resulted in larval lethality, a defect that was rescued by coexpression of UAS-dMyc (Table 1, and Supplementary Fig. 1). Phenotypic analysis of the adult animals showed that cg-dMyc flies were 17% heavier than control cg- animals (Fig. 1D,E), on the contrary, RNAi-mediated reduction of dMyc resulted in flies that eclosed with and with significantly reduced body size and weight (Fig. 1D,E and Table 1) and two days of delay (Supplementary Fig. 1B). To assess whether the differences in body weight measured in cg-dMyc and cg-dMyc-RNAi adults animals reflected changes in the size of the peripheral organs, we measured the cell size and number in the wing and the eye of adult animals. In the wing blade each cell is accessorized with a single bristle called trichome. We measured the number of trichomes in a defined area of the wing blade in animals of the three different genotypes. This analysis revealed that flies expressing dMyc in the FB had an increased size and number of the cells

### Table 1

The indicated UAS-transgenes were expressed in the fat body using the cg-Gal4 promoter. Two different lines were used for UAS-dMyc and UAS-dMyc-RNAi with different chromosomal insertions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pupal length (mm)</th>
<th>Days of development</th>
<th>Adults at eclosion (%)</th>
<th>Area wing (mm²)</th>
<th>Size of thricomes (µm²)</th>
<th>Total number of thricomes/wing</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg-Gal4/+</td>
<td>3.05 ± 0.07 (63)</td>
<td>10.72 ± 0.31 (443)</td>
<td>99.8 (443)</td>
<td>196 ± 0.06 (16)</td>
<td>133 ± 9.5 (16)</td>
<td>14713 ± 1094 (16)</td>
</tr>
<tr>
<td>cg-Gal4/UAS-dMyc (1)-/+</td>
<td>3.24 ± 0.07 (52)</td>
<td>11.27 ± 0.07 (287)</td>
<td>100.0 (287)</td>
<td>2.24 ± 0.6 (16)</td>
<td>149 ± 6.1 (16)</td>
<td>15060 ± 1094 (16)</td>
</tr>
<tr>
<td>cg-Gal4/UAS-dMyc (2)-/+</td>
<td>3.18 ± 0.09 (46)</td>
<td>11.50 ± 0.22 (356)</td>
<td>99.92 (356)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>cg-Gal4/UAS-dMyc-RNAi (1)-/+</td>
<td>2.80 ± 0.14 (48)</td>
<td>12.90 ± 0.37 (304)</td>
<td>105.48 (304)</td>
<td>1.40 ± 0.10 (16)</td>
<td>113 ± 6.1 (16)</td>
<td>12404 ± 0980 (16)</td>
</tr>
<tr>
<td>cg-Gal4/UAS-dMyc-RNAi (2)-/+</td>
<td>2.35 ± 0.17 (20)</td>
<td>PL</td>
<td>PL</td>
<td>PL</td>
<td>PL</td>
<td>PL</td>
</tr>
</tbody>
</table>

* From egg-deposition until adults eclosion.

Data were calculated as % of the total animals of the expected genotype.

See materials and methods for calculation. PL: pupal lethal. nd: not determined.

***P-value from Student t-test is P < 0.001 compared to cg-Gal4. In parenthesis is the total number of animals in the experiments.
in their wings (Fig. 1E, \( P < 0.0001 \) in a \( t \)-test) indicating that expression of \( d\text{Myc} \) in the FB increases the size of the wings, thus peripheral organs. On the contrary, \( cg-d\text{Myc-RNAi} \) animals displayed an opposite phenotype with wings that were smaller due to a reduced number and size of their cells (Fig. 1E, \( P < 0.0001 \) in a \( t \)-test). Similar conclusions were obtained when the analysis was performed in the ommatidia that constitute the compound eye (Fig. 1E). \( d\text{Myc} \) effect on systemic growth was specific for its activity in the fat cells since we obtained similar results using \( pum	ext{pless-Gal4} \) (Zinke et al., 1999) that also drives the expression in the FB (Supplementary Fig. 2). Since the \( cg \) promoter is active in the FB and in the hemocytes (Asha et al., 2003), we used the \( hemolectin-Gal4 \) (Goto et al., 2003) and \( Serpent-hemolectins-Gal4 \) (Bruckner et al., 2004) to show that expression of \( d\text{Myc} \) in hemocytes was not associated with any alteration in developmental timing or body size (Supplementary Fig. 3). In order to understand whether the systemic growth induced by \( d\text{Myc} \) was associated with its role in increasing the cell size of the FB, we expressed the growth inducers cyclin D/Cdk4 (Prober and Edgar, 2002) and Rheb (Patel et al., 2003; Saucedo et al., 2003; Stocker et al., 2003) and cell size and organinal size were measured. This analysis showed that even though expression of \( d\text{Myc, Cdc4/Cdk4} \) or Rheb induced cell-autonomous growth (Supplementary Fig. 4), only expression of \( d\text{Myc} \) was able to induce growth at the organinal level (Fig. 1E). Taken together these data suggest that increasing \( d\text{Myc} \) activity in the FB specifically induces systemic growth.

d\text{Myc} expression in the FB affects DILP2 release from the insulin producing cells

During feeding, the FB remotely controls DILP2 release from the brain by secreting factors in the hemolymph to induce the release of DILP2 from the insulin producing cells (IPCs) (Geminard et al., 2009). Given the systemic effect on growth by \( d\text{Myc} \) expression in the FB we analyzed if misexpression of \( d\text{Myc} \) in the FB affects DILP2 protein localization within the IPCs. Animals were grown in corn medium then starved in PBS, and DILP2 protein in the IPCs was quantified by immunofluorescence. This analysis showed that while DILP2 protein accumulates in the IPCs of \( cg \)-control larvae after starvation (\( P < 0.001 \), in a \( t \)-test), in \( cg-d\text{Myc} \) animals this regulation was absent and the intensity of fluorescence in the IPCs was similar in animals in feeding or starving conditions (\( P = 0.957 \)) (Fig. 2A, Supplementary Fig. 5). On the contrary, animals with reduced \( d\text{Myc} \) levels in the FB showed an accumulation of DILP2 in the IPCs when kept in feeding conditions (Fig. 2A, \( P = 0.004 \), in a \( t \)-test and Supplementary Fig. 5). The lack of DILP2 protein accumulation after starvation in \( cg-d\text{Myc} \) animals was attributable mainly to changes in DILP2 protein since \( d\text{ilp2} \) mRNA levels did not change in these animals (Supplementary Fig. 6A-D). To analyze if DILP2 activity was solely responsible for mediating the systemic growth induced by \( d\text{Myc} \), we expressed \( d\text{Myc} \) in the FB of \( d\text{ilp2,3,5} \) mutant animals. \( d\text{ilp2,3,5} \) homozygous mutant flies are viable and hatch as small adults with a delay of about 14 days (Gronke et al., 2010). These experiments showed that reduction of \( d\text{ilp2,3,5} \) expression blocked the effect of \( d\text{Myc} \) on systemic growth (Fig. 2B, \( P = 0.114 \)) suggesting that \( d\text{Myc} \) activity in the FB requires functional dilps signaling to induce systemic growth. The use of a triple mutant background was necessary to avoid compensatory effects between the different dilps (Broughton et al., 2008; Ikeya et al., 2002). \( d\text{ilp2,3} \) and 5-mRNAs levels in the IPCs did not vary among the three different genotypes (Supplementary Fig. 6A,B), however we measured a significant decrease in the of \( d\text{ilp2,3} \) and 5-mRNAs levels in \( cg \)-control animals upon starvation, which was not visible in brains from \( cg-d\text{Myc} \) larvae (Supplementary Fig. 6C,D).

DILPs stimulate the uptake of circulating sugars from the hemolymph (Rulifson et al., 2002; Tatar et al., 2003), and their levels in the IPCs are inversely proportional to the amount of circulating trehalose (larval sugar) (Geminard et al., 2009). Trehalose is a dimer of two molecules of glucose and is transported as a monomer through the hemolymph to the tissues where it is uptaken by a class of glucose transporters (Escher and Rasmuson-Lestander, 1999). During starvation, the concentration of trehalose decreases over time, as a direct result of DILP2 retention in the IPCs (Dus et al., 2011; Luo et al., 2012) hence we decided to measure the level of trehalose in the hemolymph as an indirect readout for DILP2 activity. This analysis showed that starvation the levels of circulating trehalose in \( cg-d\text{Myc} \) animals decreased more rapidly than in \( cg \)-control larvae (Fig. 2C, and Supplementary Fig. 7), indirectly supporting the hypothesis that \( cg-d\text{Myc} \) larvae have higher concentration of circulating DILP2 than \( cg \)-control animals.

d\text{Myc} expression in the FB affects sugar storage, glycolytic enzymes and counteracts increased glycemia

We then analyzed whether the expression of \( d\text{Myc} \) in the FB affects the storage and mobilization of trehalose in the whole animal. \( cg-d\text{Myc} \) larvae raised on regular food, displayed a significant increase in trehalose concentration in the whole body, whereas \( cg-d\text{Myc-RNAi} \) animals, of same developmental stage, showed the opposite effect (Fig. 3A). After 24 h of starvation, trehalose levels were reduced with a similar ratio in all three genotypes indicating that \( d\text{Myc} \) in the FB did not influence sugar mobilization. Analysis of enzymes responsible for glucose metabolism, showed an increase in the level of the glucose transporter Glut1-mRNA, and of the glycolysis enzymes Hexokinase-C and...
Hexokinase-A-mRNAs and of Pyruvate Kinase-mRNAs (Fig. 3B). In addition, we found that expression of dMyc correlated with the increased level of dilp6-mRNA. Dilp6 is expressed in the FB and produced during the non-feeding stage (Okamoto et al., 2009; Slaidina et al., 2009; Zhang et al., 2009). In adult fly, dilp6 transcription is regulated by dFOXO and correlates with a decrease in DILP2 release, resulting in reduced insulin signaling (Bai et al., 2012).

Transgenic mice expressing c-myc in the liver maintain lower glycaemia after treatment with streptozotocin, a drug that causes a predisposition to diabetes (Riu et al., 1996). We analyzed whether a similar mechanism was also present in flies. Feeding larvae a high-sugar diet (HSD) increases sugars in the hemolymph (Musselman et al., 2011), and while cg-animals fed a HSD exhibited a rise in circulating trehalose (Fig. 3C, P < 0.05), the cg-dMyc counterparts maintained an invariant trehalosemia (Fig. 3C, P = 0.159). These data support the idea that dMyc in the FB is able to reduce the level of sugars in the hemolymph via a remote control of dilps and through a tissue autonomous regulation of glucose uptake in FB. In vertebrates, increase in glucose metabolism is associated to glutamine uptake to promote cell survival (Welling et al., 2010), in addition, c-myc induces the consumption of glutamine to favor growth in cancer cells (Wise et al., 2008). In cg-dMyc FBs, we found increased expression of Glutaminase (CG42708) and of Glutamine synthetase1 (GS1) mRNAs, suggesting that dMyc may control glutamine metabolism in Drosophila FB.

dMyc expression in the FB affects storage of TAG and survival in low nutrient conditions

Morphological analysis of FBs, using the lipid-specific dye Nile Red, revealed that fat cells from cg-dMyc animals contained cytoplasmic lipid droplets smaller in size than in cg-controls (Fig. 4A B and E). This effect was more exacerbated in clones expressing dMyc using the actin-promoter (Supplementary Fig. 8A). On the contrary, the lipid droplets from cg-dMyc-RNAi FB cells were abnormally enlarged (Fig. 4C and E, and Supplementary 8B) resembling vesicles from starved animals (Fig. 4D). Analysis of the cell size and triglyceride (TAG) content showed that fat cells from cg-dMyc animals were larger (Fig. 4F) and contained more TAG per protein compared to cg-control (Fig. 4G). The opposite effect was observed in FB from cg-dMyc-RNAi animals. Increased levels of TAG result from increased synthesis or reduced degradation of fatty acids. Thus we analyzed if the expression of specific enzymes involved in fatty acid metabolism were affected by dMyc expression. This analysis revealed that FBs from cg-dMyc animals have increased levels of Perilipin2-mRNA, the orthologue of the vertebrate lipid storage droplet gene-2 (LSD-2) (Bickel et al., 2009), a protein that functions to regulate lipid storage and favors lipogenesis (Gronke et al., 2003; Okumura, 2011; Tansey et al., 2001). In addition, we found increased expression of CG11198 and CG3524 (Palanker et al., 2009), encoding for putative orthologues of the vertebrate Acetyl-Co Carboxylyase (ACC) and Fatty Acid Synthase (FAS) respectively. dMyc also upregulates the expression of CG5887-Desat1 mRNA (Wicker-Thomas et al., 1997), the orthologue of the vertebrate Stearoyl CoA Desaturase (SDC1) an enzyme that has been associated together with ACC and FAS with the control of de-novo synthesis of lipids in the liver (Patton and Ntambi, 2009). On the contrary, the expression of CG21077-mRNA (Palanker et al., 2009), encoding for a putative orthologue of a carnitine transporter, a key regulator of beta-fatty acid oxidation was reduced in cg-dMyc FB (Fig. 4H), indicating that the fat cells in these animals have increased fatty synthesis and reduced oxidation.

Lipids stored principally in the mid-gut and gastric caeca, are cleared from those tissues during starvation and accumulate in the oenocytes, a cluster of cells with functions similar to the vertebrate liver (Gutierrez et al., 2007). To analyze whether dMyc expression in the FB affects lipid mobilization, third instar larvae were starved, and lipid content was visualized in feeding and after starvation using Oil Red O (Palanker et al., 2009). In feeding conditions lipids were present more abundantly in the mid-gut and gastric caeca of cg-dMyc animals (Fig. 4I middle panel). These animals also showed the presence of lipid vesicles in their oenocytes, which during feeding are normally emptied of lipids (compare insets in the left and middle panel). After 24 h of starvation, lipids were equally cleared from the mid-gut and caeca from all three genotypes and accumulate in the oenocytes with no difference between the three genotypes (Fig. 4I, lower panels). Measurement of TAG from whole animals in feeding conditions confirmed that cg-dMyc larvae have a small but significant increase in the level of TAG compared to cg-controls (P < 0.05), while cg-dMyc-RNAi displayed a dramatic reduction in their lipid content (P < 0.001) (Fig. 4J). Upon starvation the TAG stores were reduced at a similar rate in all three genotypes, suggesting that expression of dMyc in FB does not affect lipid mobilization.

Animals store triglycerides and sugars to survive periods of scarce nutrient availability. We analyzed whether the differences in the content of sugars (Fig. 3A) and TAG (Fig. 4J) between the animals of the three genotypes could affect their ability to survive starvation. Animals were raised in normal food until they reached the mid-second larval instar, at which point they were transferred to PBS and their viability scored every 12 h. These experiments showed that whereas only 50% of cg- control survived after 80 h of starvation, the half-life of cg-dMyc animals during starvation...
reached 100 h ($P < 0.0001$, in a $z$-test analysis, see Appendix A for statistic analysis). Conversely, cg-dMyc-RNAi larvae reached their half-life at about 56 h of starvation (Fig. 4K) ($P < 0.0001$, in a $z$-test).

From these data, we conclude that expression of dMyc in FB locally and remotely increases lipid concentration and storage, and correlates with a better survival during nutrient starvation.

**Reduction of Desat1 in FB, but not of PyK and HK-C affects the ability of dMyc to induce systemic growth**

The increase of dMyc expression in the FB during starvation (Fig. 5A) is induced by the transcription factor FOXO that translocates to the nucleus (Fig. 5B) to directly induce $dmyc$-mRNA (Teleman et al., 2008). In order to assess which pathways were sustained by dMyc in the FB during starvation, we undertook a proteomic approach to compare the proteomes of FBs from animals with different dMyc levels in feeding and starving conditions. To confirm that a more sustained expression effectively correlates with an increase in protein abundance, we focused our attention on the genes that were previously identified by quantitative RT-PCR analysis (Figs. 3B and 4H). This analysis revealed that FBs expressing dMyc have significantly increased Desat1, Pyruvate Kinase, Hexokinase-C and Glutamine Synthetase1 protein levels (Table 2) and the expression of these enzymes was further increased upon starvation (Table 2, $P < 0.0001$, using a Student’s $t$ test). Next we used the cg-Gal4 driver in combination with UAS-RNAi lines to analyze if the silencing of these genes in the FB interferes with the ability of dMyc to induce organismal growth.

This analysis revealed that reduction of PyK or HK-C expression did not affect the ability of dMyc to induce organismal growth, as dMyc expression was able to significantly induce growth ($*** P < 0.001$) as measured in adults (Fig. 5C) and in pupae (Supplementary Fig. 9), even when PyK or HK-C expression was reduced. On the contrary, reduction of Desat1 completely blunted the ability of dMyc to influence body size (Fig. 5C). Reduction of GS1 in the FB with the available RNAi line resulted in pupal lethality, preventing us from further performing any genetic analysis (Supplementary Fig. 9). Efficiency of the RNAi line in FB was tested (Supplementary Fig. 10).

**Reduction of Desat1 in the FB affects accumulation of TAGs and dMyc resistance to starvation**

In mice, mutation of $scd1$ reduces the level of TAG in the adipose tissue resulting in leaner animals (Ntambi et al., 2002).
rate as cg-Desat1-RNAi animals (Fig. 6B). Suggesting that reduction of Desat1 compromises the ability of cg-dMyc larvae to survive on a starvation regimen. cg-Desat1 RNAi animals are smaller, and their level of TAG in the FB is reduced compared to cg-control larvae (Fig. 6A). We then analyzed if reduction of Desat1 affected the size of the fat cells. We use the UAS/Gal4 technique to induce flip-out clones expressing UAS-Desat1-RNAi under the actin promoter. Clones were marked by co-expression of UAS-GFP and scored in the FB. The size of Desat1-RNAi GFP-positive cells was compared to that of wt GFP-negative cells surrounding the clones (Fig. 6C). This analysis revealed that reduction of Desat1 decreased the cell-size of the fat cells to 74% (compared to 100% in control wt cells) (P < 0.0001, using a t-test). Expression of dMyc increased the size of the cells to 179%, but when dMyc was expressed in combination with Desat1 RNAi, its ability to induce growth was reduced to 139% (Fig. 6D). However, the ability of dMyc to induce growth in a Desat1 RNAi background (23±39%) was not significantly different to that measured in cg-control fat cells (79%), leading to the conclusion that depletion of Desat1 in the FB did not affect dMyc’s ability to promote cell growth.

Discussion

Here we show that modulation of dMyc activity in the fat body (FB) controls animal size, recapitulating the phenotype of ubiquitous dMyc expression (de la Cova et al., 2004; Johnston et al., 1999; Pierie et al., 2004). This function is specific for dMyc activity since other known growth inducers like CycD/Cdk4 or Rheb, do not produce the same effect (Fig. 1E). (Arsham and Neufeld, 2006; Wullschleger et al., 2006) Recent data showed that expression of Rheb in Drosophila S2 cells stimulates ribosomal biogenesis and cell size but did not promote glucose import (Hall et al., 2007). On the contrary, we found that in the FB dMyc up-regulates the expression of Glucose transporter-1 mRNA (Fig. 7), a condition that may explain the increased intracellular concentration of glucose (not shown). Stimulating protein synthesis alone is not sufficient to promote organismal size, indeed decreasing ribosomal biogenesis in the FB, using a mutation in the ribosomal-protein Rpl14 Minute (3) 66D (Saebø-Larsen et al., 1997) did not blunt dMyc’s ability to induce organismal growth (Supplementary Fig. 11), supporting the idea that specific metabolic signals in addition to protein synthesis are induced by dMyc in FB leading to increase in body size.

The FB functions as a sensor for the amino acid concentration in the hemolymph and in response to a diet rich in proteins, produces humoral factors that control the release of DILP2 from the brain (Geminard et al., 2009). Our data show that dMyc expression in the FB inhibits the retention of DILP2 in the IPCs during starvation (Fig. 2A), suggesting that an enhanced dMyc activity in the FB mimics a fed state or is sufficient to induce the secretion of factors responsible for DILP2 release from the IPCs. Accumulation of DILP2 in the IPCs leads to decreased trehalosemia (level of circulating trehalose in the hemolymph) (Geminard et al., 2009). The ability of cg-dMyc animals to decrease trehalosemia and to affect the amount of DILP2 released during starvation, together with the finding that DILP2 is required for the dMyc-dependent systemic increase in size, led us to conclude that dMyc control of systemic growth depends on the presence of circulating DILPs. However the nature of the humoral factors governing DILP2 release and the mechanism that triggers the secretion of these factors are not clear yet. To attempt at the identification of the pathways involved in their production, we analyzed by quantitative RT-PCR the expression levels of key enzymes of glycolysis, glutamine and lipid metabolic pathways upon dMyc induction in FB.

Table 2

Quantitation and identification of digest of proteins in FB from cg-dMyc and cg-control larvae.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>UniProt</th>
<th>Ratio fed</th>
<th>Ratio starved</th>
<th>P-value fed</th>
<th>P-value starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desat1</td>
<td>Q7K4Y0</td>
<td>1.18</td>
<td>2.64</td>
<td>0.0012</td>
<td>8.86E-43</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>KPYK</td>
<td>1.78</td>
<td>2.28</td>
<td>6.42E-07</td>
<td>2.72E-32</td>
</tr>
<tr>
<td>Hexokinase C</td>
<td>Q7Y7W9</td>
<td>1.41</td>
<td>1.98</td>
<td>0.003</td>
<td>3.82E-28</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>GLNA1</td>
<td>1.38</td>
<td>1.43</td>
<td>1.52E-24</td>
<td>2.02E-21</td>
</tr>
</tbody>
</table>

The data are expressed as ratio from FB of cg-dMyc/cg-control animals in feeding or after 24 h of starvation in PBS. P-values were calculated using ANOVA from at least four independent experiments.

We found that reduction of Desat1 in the FB (cg-Desat1 RNAi) results in smaller pupae (Supplementary Fig. 9) and adults with reduced body size (Fig. 5C). The larval FB of cg-Desat1 RNAi animals was composed of smaller cells (not shown) containing less TAG (Fig. 6A, P=4.5E-06; using a Student's t test). Expression of dMyc increased the levels of TAG/protein of about 20% in both cg- control and in cg-Desat1 RNAi; dMyc larval FB (Fig. 6A). However, the content of TAG/protein in cg-Desat1 RNAi; dMyc animals was lower than that of cg-control animals (Fig. 6A, P=0.003; using a Student's t test). We then analyzed whether reduction of Desat1 could affect the ability of cg-dMyc animals to resist starvation. This analysis showed that while cg-dMyc larvae reached their half-life after 100 h of starvation, cg-Desat1-RNAi; dMyc larvae reached the same point only after 48 h, with a similar conclusion that depletion of Desat1 compromises the ability of cg-dMyc larvae to survive on a starvation regimen. cg-Desat1 RNAi animals are smaller, and their level of TAG in the FB is reduced compared to cg-control larvae (Fig. 6A). We then analyzed if reduction of Desat1 affected the size of the fat cells. We use the UAS/Gal4 technique to induce flip-out clones expressing UAS-Desat1-RNAi under the actin promoter. Clones were marked by co-expression of UAS-GFP and scored in the FB. The size of Desat1-RNAi GFP-positive cells was compared to that of wt GFP-negative cells surrounding the clones (Fig. 6C). This analysis revealed that reduction of Desat1 decreased the cell-size of the fat cells to 74% (compared to 100% in control wt cells) (P < 0.0001, using a t-test). Expression of dMyc increased the size of the cells to 179%, but when dMyc was expressed in combination with Desat1 RNAi, its ability to induce growth was reduced to 139% (Fig. 6D). However, the ability of dMyc to induce growth in a Desat1 RNAi background (23±39%) was not significantly different to that measured in cg-control fat cells (79%), leading to the conclusion that depletion of Desat1 in the FB did not affect dMyc's ability to promote cell growth.
In the FB dMyc increases cell autonomously the level of Hexokinase-A, the putative homolog of human Hexokinase-IV or glucokinase, Hexokinase-C, and of Pyruvate Kinase-mRNAs (Fig. 3B) key enzymes that control glycolysis. In a mouse model for Type 2 diabetes, the expression of c-Myc in the liver induced the transcription of glycolytic enzymes and of the glucose transporter GLUT2, resulting in decreased glycemia and counteracting streptozotocin (STZ) induced diabetes (Riu et al., 1996). Similarly, we found that cg-dMyc larvae, grown on a high sugar diet (1 M sucrose), were able to reduce their level of trehalosemia (Fig. 3C). Our results could be explained by the ability of dMyc to increase Glut-1 mRNA that favored glucose uptake, and by dMyc’s ability to increase the rate of glucose consumption via glycolysis.

By quantitative PCR analysis we revealed that expression of dMyc in the FB upregulates Glutaminase and Glutamine Synthetase (GS1) mRNAs, key regulators in glutamine metabolism (Fig. 3B). In addition, we found an increase in the expression of the Drosophila orthologue of the vertebrate LAT1/SLC1A5 also called minidiscs (mnd) (Martin et al., 2000) and of SLC38/11, two members of the L-and N-type family of amino acid transporter-1 (Mackenzie and Erickson, 2004) (Supplementary Fig. 12). In vertebrates, LAT1/SLC1A5 controls the efflux of L-glutamine that precedes the import of essential amino acids to induce the activation of mTOR signaling (Nicklin et al., 2009), while in flies, mnd, which is highly expressed in the FB, was shown to function non autonomously to control organism size (Martin et al., 2000). With this data we could hypothesize that dMyc in the FB controls glutamine flux to favor the uptake of essential amino acids resulting in activation of glutamine metabolism. Glutamine flux is also controlled by Glutamine Synthetase1, and very recently GS1 was identified in vertebrates, as a direct target of FOXO-3 to maintain glutamine homeostasis and to promote autophagy leading to cell survival (van der Vos et al., 2012). Because in the FB, dFOXO is necessary to sustain dmyc-mRNA expression during starvation.
and FAS promoter to induce their expression, and to reduce protein (Mlx) and MondoA (Uyeda and Repa, 2006). Mlx and MondoA Carbohydrate Response-Element Binding Protein, Max-Like proteins.

In the vertebrate liver and adipocytes FAS and ACC are transcriptionally regulated in response to glucose concentration by the Carbohydrate Response-Element Binding Protein, Max-Like protein (Mlx) and MondoA (Uyeda and Repa, 2006). Mlx and MondoA form heterodimers that bind to the E-box sequences in the ACC and FAS promoter to induce their expression, and to reduce glucose uptake (Billin and Ayer, 2006; Billin et al., 2000). In the presence of high levels of glucose, the activity of Mlx/Mondo heterodimers decreases and their negative effect on glucose uptake is relieved, resulting in increased glucose concentration (Kaadige et al., 2009). In addition, Drosophila Mondo interacts genetically with dMyc (Billin and Ayer, 2006) raising the possibility that misexpression of dMyc may change the stoichiometry of Mondo/Mlx heterodimers to control ACC and FAS and glucose uptake in the FB.

In support to the data on the transcriptional targets induced by dMyc in the FB, our analysis of proteomes of fat bodies from animals with different dMyc levels revealed a significant increase of the glycolytic enzymes (PyK and HK-C), Glutamine Synthetase (GS1) and Desat1 particularly during starvation (Table 2). However, to our surprise, we found that only reducing Desat1 blunted the ability of dMyc to induce organismal growth (Fig. 5C). Desat1, or SC1 in vertebrates, is the rate limiting enzyme that catalyzes the biosynthesis of monounsaturated fatty acids, components of triglycerides and phospholipids (Mauvoisin and Mounier, 2011). In Drosophila Desat1 protein was increased in proteomes from FB from starved animals, while desat1 mutant animals were found defective in autophagy, a survival mechanism that is activated during starvation (Kohler et al., 2009). We found that reduction of Desat1 reduced the ability of fat cells to store TAG, and cg-Desat1-RNAi animals were less resistant to starvation. We found Desat1 activity necessary for dMyc’s ability to induce fat synthesis and for the animal to resist starvation (Fig. 6). One possible explanation is that reduction of Desat1 unable animals to accumulate TAG in their fat cells, thus their storage necessary to resist starvation. In our model, we place Desat1 function downstream of dMyc activity (Fig. 7), however we did not find dMyc binding sites in the promoter region of Desat1, suggesting that dMyc is acting indirectly to control desat1mRNA expression. In human, SC1/Desat1 plays a relevant role in carcinogenesis to control de-novo synthesis of lipids and its level is upregulated in different tumors (Igal, 2010; Scaglia et al., 2009). FAS and ACC expression are also increased in cancer cells, where their ability to promote de novo synthesis of lipids provides a growth advantage to the tumor cells (Menendez and Lupu, 2007). Myc’s ability to control FAS and ACC and SC1/Desat1 expression suggest a novel function for Myc to control lipid metabolism and survival that may be relevant in normal and pathological conditions.

Our studies are centered to uncover novel metabolic pathways induced by dMyc in the FB. dMyc expression in this organ is developmentally regulated by edcsyne, which reduces dmyc mRNA in late third instar to constrain growth (Delanoue et al., 2010). At this developmental time the systemic reduction of insulin signaling activates dFOXO, which results in dmyc upregulation (Fig. 5A) (Teleman et al., 2008). We think that opposite mechanisms may coexist in the FB to regulate dMyc expression. Activation of these pathways is regulated by the metabolic status of the animals and by their developmental stage. For example, at the end of larval development growth is reduced and dMyc expression needs to be shut down, however if the animal is in starvation and the nutrients are reduced, dMyc expression is induced to ensure the activation of specific metabolic processes necessary for the animal to survive.

In conclusion, our findings establish a novel link between dMyc function in the fat body and the non-cell-autonomous control of growth, metabolism, and survival of the organism. Our data identify two potential signals that are influenced by dMyc (Fig. 7). The first mimics the non-autonomous control of DILP2 release from the brain induced by humoral factors produced by the FB during feeding. The second identifies Desat1 as a novel component for dMyc activity, that may be relevant for its function in controlling fatty acid metabolism and de-novo synthesis of lipids as a mechanism of cell survival. These observations may have significant implications for our understanding not only of the systemic control of growth and metabolism but also create the need to explore Myc function at the physiological level in controlling lipid homeostasis and metabolism in diseases such as diabetes and cancer.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.04.008.

References


