ATF-2 Regulates Fat Metabolism in *Drosophila*

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ATF-2 is a member of the ATF/CREB family of transcription factors that is activated by stress-activated protein kinases such as p38. To analyze the physiological role of *Drosophila* ATF-2 (dATF-2), we generated dATF-2 knockdown flies using RNA interference. Reduced dATF-2 in the fat body, the fly equivalent of the mammalian liver and adipose tissue, decreased survival under starvation conditions. This was due to smaller triglyceride reserves of dATF-2 knockdown flies than control flies. Among multiple genes that control triglyceride levels, expression of the *Drosophila* PEPCK (dPEPCK) gene was strikingly reduced in dATF-2 knockdown flies. PEPCK is a key enzyme for both gluconeogenesis and glyceroneogenesis, which is a pathway required for triglyceride synthesis via glycerol-3-phosphate. Although the blood sugar level in dATF-2 knockdown flies was almost same as that in control flies, the activity of glyceroneogenesis was reduced in the fat bodies of dATF-2 knockdown flies. Thus, reduced glyceroneogenesis may at least partly contribute to decreased triglyceride stores in the dATF-2 knockdown flies. Furthermore we showed that dATF-2 positively regulated dPEPCK gene transcription via several CRE half-sites in the PEPCK promoter. Thus, dATF-2 is critical for regulation of fat metabolism.

INTRODUCTION

ATF-2 (activating transcription factor-2) is a member of the ATF/CREB (CRE-binding protein) transcription factor family that has a bZIP-type DNA-binding domain (Hai *et al.*, 1989; Maekawa *et al.*, 1989). ATF-2 can form either homodimers or heterodimers with c-Jun: the dimers subsequently bind to the cyclic AMP response element (CRE: 5'-TGACGTCA-3') and positively regulate transcription (Hai and Curran, 1991). Stress-activated protein kinases (SAPKs) such as p38 and JNK (c-Jun N-terminal protein kinase) phosphorylate ATF-2 at Thr-69 and Thr-71 close to the N-terminal transcriptional activation domain containing the zinc finger domain and thereby enhance its *trans*-activating capacity (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). Activation of ATF-2 by p38/JNK is thought to play a role in apoptosis (Gupta *et al.*, 1995). ATF-2 is also activated by insulin, epidermal growth factor, and serum via a two-step mechanism involving two distinct Ras

effector pathways (Ouwens *et al.*, 2002). A group of ATF-2 target genes have been identified that are involved in multiple biological phenomena. The target genes of c-Jun/ATF-2 heterodimers, which are implicated in growth control, include *c-jun* itself and *interferon-β* (van Dam *et al.*, 1993; Falvo *et al.*, 2000). The platelet-derived growth factor receptor α gene, which is critical for proliferation of cytotrophoblasts, is an ATF-2 target and its expression level is decreased in the placenta of *Atf-2* mutant mice (Maekawa *et al.*, 1999).

ATF-2 is ubiquitously expressed and is found in liver and white adipose tissue (WAT), which are critical organs for metabolic regulation. This expression profile of ATF-2 indicates the possibility that ATF-2 plays an important role in metabolic regulation. In fact, ATF-2 activates transcription of the phosphoenolpyruvate carboxykinase-cytosolic (PEPCK-C) gene in hepatoma cells by directly binding to the CRE in its promoter via the p38 pathway (Cheong *et al.*, 1998). It was also suggested that all-trans-retinoic acid activates the p38 pathway leading to phosphorylation and activation of ATF-2, thereby enhancing PEPCK gene transcription (Lee *et al.*, 2002). PEPCK catalyzes the first committed step in hepatic gluconeogenesis and is a rate-limiting enzyme of gluconeogenesis. Recently, PEPCK has been also shown to play a key role in lipid homeostasis via glyceroneogenesis in WAT (Reshef *et al.*, 2003). Glyceroneogenesis is defined as the *de novo* synthesis of glycerol-3-phosphate from pyruvate, lactate, or certain amino acids. However, it is difficult to examine in a whole animal system whether ATF-2 is critical for metabolic regulation via regulating PEPCK expression, since *Atf-2* null mutant mice die immediately after birth due to a respiration defect (Maekawa *et al.*, 1999). Furthermore, no tissue-specific *Atf-2* knockdown mice are available at present. Thus, the role of ATF-2 in metabolic regulation still remains elusive.

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Abbreviations used: ACC, acetyl coenzyme A carboxylase; AcCoAS, acetyl coenzyme A synthetase; ATF, activating transcription factor; BBF, box B-binding factor; CRE, cyclic AMP response element; CREB, CRE-binding protein; *da*, *daughterless against*; dATF-2, *Drosophila* ATF-2; dp38, *Drosophila* p38; dsRNA, double-stranded RNA; FAS, fatty acid synthetase; G4, Gal4; IR, inverted repeat; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MHC, myosin heavy chain; PEPCK, phosphoenolpyruvate carboxykinase; MKP, MAPK phosphatase; RNAi, RNA interference; SAPK, stress-activated protein kinase; WAT, white adipose tissue.

Because *Drosophila* has a low degree of gene redundancy and therefore fewer related genes than mammals, it is sometimes advantageous to analyze the *Drosophila* homologue of a mammalian gene of interest. Furthermore, in *Drosophila* the expression of double-stranded RNA (dsRNA) corresponding to a part of the gene can down-regulate the gene's mRNA by RNA interference (RNAi; Kennerdell and Carthew, 2000). Furthermore the *Drosophila* RNAi system enables the tissue-specific knockdown by using Gal4/UAS system (Brand and Perrimon, 1993). To elucidate the physiological role of ATF-2, we first identified the *Drosophila* ATF-2 homologue (dATF-2; Sano *et al.*, 2005). Like mammalian ATF-2, dATF-2 has the bZIP-type DNA-binding domain and the p38 phosphorylation sites in its C- and N-terminal regions, respectively. dATF-2 binds to the cAMP response element as a homodimer or a heterodimer with *Drosophila* Jun and activates transcription. *Drosophila* p38, but not *Drosophila* JNK, phosphorylates dATF-2, and enhances dATF-2-dependent transcription.

Here, we have demonstrated that reduction of dATF-2 function in the fat body by transgenic RNAi decreases survival under dietary restriction and triglyceride as stored energy. These phenotypes are correlated with the decreased levels of *dPEPCK* gene transcription in the fat body, which is positively regulated by dATF-2. Thus, dATF-2 plays a critical role in regulation of fat metabolism, especially triglyceride synthesis.

MATERIALS AND METHODS

Generation of dATF-2 Transgenic Flies

Transgenic dATF-2 RNAi flies were generated using the inducible RNAi method (Kennerdell and Carthew, 2000). A 566-base pair-long cDNA fragment (nucleotide position, 59–624 of the dATF-2 coding sequence) was amplified by PCR and inserted as an inverted repeat (IR) into a modified pUAST transformation vector, pUAST-R57 (Shigen, Mishima, Japan), which possesses an IR formation site consisting of paired KpnI-CpoI and XbaI-SfiI restriction sites. The IR was constructed in a head-to-head orientation using a combination of tag sequences on PCR primers and restriction sites on the vector. Transformation of *Drosophila* embryos was performed using *w*¹¹¹⁸ as a recipient strain. The primer sequences for RNAi construct were as follows: forward, 5'-AAGGCTACATGGCCGACCGCAAAATCCCCAGAGGGCGACA-3'; reverse, 5'-AAT CTAGAGGTACCGAAGGGATGAATGCACCACT-3'. In this study, we used flies carrying one copy of the RNAi construct combined with one copy of the Gal4 driver. For ubiquitous and tissue specific knockdowns, we used the following drivers: da-Gal4 (ubiquitous), c564-Gal4 (fat body), and MHC-Gal4 (muscle). da-Gal4 and c564-Gal4 were obtained from the Bloomington *Drosophila* stock center. MHC-Gal4 was a kind gift from G. Davis (University of California, San Francisco). In the experiments using the transgenic dATF-2 RNAi flies, we used control flies which carried only the GAL4 driver and have the same genetic background as the test flies.

For generation of transgenic flies harboring an extra copy of the dATF-2 gene, the Nael-SalI. A 13.6-kb genomic DNA fragment covering the full portion of the dATF-2 gene was prepared from *Drosophila* embryonic DNA. The digested DNA fragment was subcloned into the EcoRV-SalI site of the pBluescript KS(-) vector. The plasmid was digested with SalI and ligated with the NotI linker. After NotI digestion, the DNA fragment was inserted into the NotI site of the P-element vector, pCaSpeR-AUG-βGal. Transformation of *Drosophila* embryos was performed using *w*¹¹¹⁸ as a recipient strain. In the experiments using the transgenic dATF-2 flies, we used control flies that have the same genetic background as the transgenic dATF-2 flies.

Starvation Assay

The starvation experiments were performed with 7-d-old male flies. In an experiment, for each genotype batches of 20–25 flies were transferred to vial containing 1.3% agarose. Dead flies were counted every 8 h for survival rate calculations. Data are the average with SE from three independent experiments (at least 1 vial/experiment for each genotype; thus data are derived from a total of 4 to 12 vials for each genotype). The mean and SE of data from 4 to 12 vials was plotted.

Body Weight, Triglyceride, and Trehalose Measurements

Seven-day-old male flies were weighed in batches of 30. Data are the average with SE from at least six independent experiments. For triglyceride measure-

ments, the entire bodies of a group of three adult males or fat bodies from a group of six third instar larvae were homogenized in 0.1% Tween 20 on ice, heated at 70°C for 5 min to inactivate endogenous enzymes, and centrifuged. The triglycerides in the supernatants were measured using the Serum Triglyceride Determination Kit (TR0100; Sigma, St. Louis, MO). Total protein was measured in the same sample using Bio-Rad protein assay reagent (Richmond, CA). To measure the hemolymph trehalose level, 1 μl of hemolymph from a group of five third instar larvae was put into 9 μl of buffer (5 mM Tris-HCl, pH 6.6, 137 mM NaCl, 2.7 mM KCl), heated at 70°C for 5 min, and incubated with 1 μl of trehalase (Sigma) at 37°C for 12 h, and a commercial glucose (HK) assay kit (Sigma) was used to determine the trehalose level. All assays were done at least eight times.

Fat Body Analysis by Fluorescence Microscopy

Larval fat bodies were dissected from third instar larvae, fixed in 4% paraformaldehyde/PBS for 20 min at room temperature, and washed with 0.1% Triton X-100/PBS (PBTX). Tissues were then blocked in PBTX containing 5% BSA over night. After RNase A treatment (400 μg/ml in PBS at 37°C for 30 min), DNA was stained with 5 μg/ml propidium iodide for 30 min and washed at 4°C overnight. Membranes were stained with FITC-phalloidin (Molecular Probes, Eugene, OR) at 1 μg/ml for 10 min. After washing with PBS, fat bodies were mounted in 50% glycerol/PBS. For Nile red staining, third larval fat bodies were dissected and fixed as described above. Tissues were incubated in Nile red (Sigma) solution (0.1 M stock solution in DMSO diluted to 1:250,000). For immunostaining of dATF-2, dissected larval fat bodies were fixed and blocked as described above. Fat bodies were immunostained with anti dATF-2 polyclonal antibody (1:200) at 4°C overnight. After RNase treatment, nuclear was stained with 1 μM of TO-PRO-3 iodide (Molecular Probes) at room temperature for 10 min. All fluorescent images were acquired using confocal laser scanning microscopy (Zeiss, Thornwood, NY).

Quantitative RT-PCR

Total RNA was prepared from third instar larval fat bodies by a standard TRIzol (Invitrogen, Carlsbad, CA) procedure. Fat bodies were obtained from six male larvae. Quantitative RT-PCR was performed on the Applied Biosystems (Foster City, CA) ABI Prism 7000 Sequence Detection System (using the Quantitect Probe RT-PCR Kit [204443; Qiagen] or the SYBR Green One-Step qRT-PCR Kit [Invitrogen]). All assays were done in three independent experiments and were normalized to *rp49* levels, and errors were propagated in all calculations. The sequences of forward/reverse primers and TaqMan probes were as follows: dATF-2, forward 5'-CCCAGTAGCGCCAGTCTGA-3', reverse 5'-GTGGATAATGGCCTTCAATCG-3', probe 5'-CCACCTCACAGCTGCCCATCAAGG-3'; dPEPCK, forward 5'-CGCCAGCGACATGGATGCT-3', reverse 5'-GTACATGGTGGCACCCTTCA-3', probe 5'-GAGTGCAGCAGGATTCCCCGG-3'; *rp49*, forward 5'-GACGCTTCAAGGGACAGTATCTG-3', reverse 5'-AAACGCGGTTCTGCATGAG-3', probe 5'-CACATGCTGCCACCGGATTCAA-3'; *AcCoA*S, forward 5'-GAGCCACITTCAGTGATTTTCG-3', reverse 5'-ACTTCATGAGGCGACGAATC-3'; *FAS*, forward 5'-CAACAAGCCGAACCCAGATCTT-3', reverse 5'-CAAAGGAGTTCAGCCGATGAT-3'; *ACC*, forward 5'-TTAGTCAGCTGCAGGCAAAGG-3', reverse 5'-CGGAAGCTAACGCCACACA-3'.

Glyceroneogenesis Assay

Incorporation of pyruvate into lipids was measured essentially as described by Tordjman *et al.* (2003) using fat bodies. In brief, fat bodies obtained from a group of 10 third instar larvae were incubated with [¹⁴C₁]pyruvate (5 μCi/ml) in glucose-free RPMI 1640 medium (Invitrogen). After a 2-h incubation, fat tissues were frozen by liquid nitrogen before lipid extraction. Lipids were extracted by the method of Bligh and Dyer (1959), and radioactivity in the extracts was determined using a liquid scintillation counter. All assays were done at least six times.

Body Weight and Triglyceride Level on High-Calorie Diet

Newly eclosed males were collected. Batches of 20–30 male flies were placed into each vials with high-calorie diet (15% sucrose, 15% yeast, 2% agarose) and weighed every 4–5 d. The triglyceride level of male flies being fed a high-calorie diet was measured as described above.

Statistics

The p value given in the survival data are the result of a Log-rank test on the Kaplan-Meier data. For all other assays, error bars represent SE, and the p values are the results of the Student's *t* test.

Luciferase Reporter Assays

The DNA fragment containing the dPEPCK promoter (nucleotide -1043 to +109) was amplified by PCR and cloned into the luciferase reporter pGL3-basic vector (Promega, Madison, WI). The shorter version of dPEPCK promoter (dPck-2 or dPck-3) contained the region between nucleotide -743 or -393 and +109 of the dPEPCK promoter. The CRE half site is located at nucleotide -123, -618, -693, -799, -841, and -903 of the dPEPCK pro-

motor. The point mutation to all the CRE half sites was introduced using QuickChange mutagenesis kit (Stratagene, La Jolla, CA). S2 cells were transfected with the *dPEPCK-luc* reporter (100 ng) together with the internal control plasmid pRL-CMV (20 ng) and either pact5C-FLAG-dATF-2 (500 ng) or pact5C-FLAG-dATF-2 T59/61A (500 ng) by the CaPO₄ method. The *dAcCoAS* promoter (nucleotides -237 to +119) was prepared similarly to that described above. The CRE half site is located at nucleotide -123 and -227 of the *dAcCoAS* promoter. S2 cells were transfected with the *dAcCoAS-luc* reporter (100 ng) together with the internal control plasmid pRL-CMV (20 ng) and either pact5C-FLAG-dATF-2 (800 ng) or pact5C-FLAG-dATF-2 T59/61A (800 ng) by the CaPO₄ method. The total amount of plasmid DNA introduced was adjusted to 1 μ g with empty plasmid. Twenty-four hours after transfection, a kinase inhibitor was added to the medium. Transfected cells were collected at 48 h after transfection, and the luciferase activity was measured.

Western Blotting

S2 cells were transfected with the dATF-2 expression plasmids (FLAG-dATF-2 WT or FLAG-dATF-2 T59/61A) by the CaPO₄ method. Cells were cultured for 48 h after transfection and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 25 mM NaF, 25 mM β -glycerophosphate, and 0.1 mM Na₃VO₄) containing a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany), and the lysates were subjected to SDS-PAGE, followed by Western blotting with an anti-dATF-2 polyclonal antibody described previously (Sano *et al.*, 2005).

RESULTS

Generation of dATF-2 Transgenic Flies

There are no mutations identified in the *dATF-2* locus, and the *dATF-2* null mutant could be lethal, because the *Atf-2* null mutant mice die immediately after birth because of a respiration defect (Maekawa *et al.*, 1999). Therefore, we used the transgenic RNAi technique to analyze the function of dATF-2 in vivo (Kennerdell and Carthew, 2000). In this method, a hairpin RNA is expressed from a transgene exhibiting dyad symmetry under the control of GAL4 drivers. RNAi has been successfully used for functional analyses of numerous genes (Ueda, 2001). We established transgenic fly strains bearing IRs of 566-base pair DNA sequences corresponding to the amino terminus of *dATF-2* coding regions (Figure 1A) under the control of upstream activating sequences (UAS) for the GAL4 transcription factor (Brand and Perrimon, 1993). RT-PCR analysis showed that fat body-specific expression of *dATF-2* dsRNA with the *c564-GAL4* driver (Kambris *et al.*, 2006) reduced the *dATF-2* mRNA level to ~40% of the wild-type level (Figure 1B), whereas it had no effect on mRNA levels of ribosomal protein 49 (rp49; data not shown), confirming the target specificity of the transgenic RNAi technique. Expression of *dATF-2* using ubiquitous drivers such as *actin-GAL4* did not cause lethality during embryonic and larval stages at 25°C (data not shown). Furthermore, we also generated dATF-2 transgenic flies, which carried an extra copy of the genomic DNA fragment covering the entire *dATF-2* gene (Figure 1A), to analyze the effect of increased ATF-2 level. RT-PCR analysis showed that the *dATF-2* mRNA level in *dATF-2* transgenic flies was ~2.5-fold higher than the wild-type level (Figure 1C).

dATF-2 Knockdown Flies Have Impaired Survival under Starvation Condition

To examine whether dATF-2 plays any role in metabolic control, we first asked whether *dATF-2* knockdown flies have an impaired response to nutrient deprivation. *dATF-2* knockdown flies and control flies were grown under identical conditions, aged 7 d, and then deprived of all sources of energy. Ubiquitous expression of *dATF-2* dsRNA with the *da-GAL4* driver led to a more rapid death than in control flies under these conditions (Figure 2A). After 48 h of starvation, 86% of control flies were alive, whereas only 40% of *dATF-2* knockdown mutant flies were alive. This difference

Figure 1. Generation of dATF-2 transgenic flies for loss- or gain-of-function analysis. (A) The DNA constructs used to generate transgenic flies. The exon-intron structure of *dATF-2* gene is indicated below. We established transgenic fly strains bearing inverted repeat (IR) corresponding to the "Target for RNAi" region indicated in A. The double-stranded (dsRNA) transcripts encoded by this region are under the control of a UAS-binding site for the yeast GAL4 transcription factor. Thus, tissue-specific dsRNA expression could be directed by coexpression of GAL4 driven by an appropriate promoter. The DNA fragment covering the whole portion of the *dATF-2* gene that was used to generate transgenic flies is also shown. (B) Quantitative RT-PCR analysis of *dATF-2* mRNA in the fat bodies of control and *dATF-2* transgenic RNAi flies. Genotypes: *c564-GAL4/+* versus *c564-GAL4/+*; *UAS-dATF-2* IR. Error bars, average $\pm$ SE; *n* = 3. *** *p* < 0.0001. (C) Quantitative RT-PCR analysis of *dATF-2* mRNA in the fat bodies of control and transgenic flies harboring an extra copy of the *dATF-2* gene. Genotypes: *w* versus *w*; *dATF-2/dATF-2*. Error bars, average $\pm$ SE; *n* = 3. *** *p* < 0.0001.

was statistically highly significant (log-rank test, *p* < 0.0001). We next asked whether the down-regulation of *dATF-2* in specific tissue caused this impaired survival. We chose fat body and muscle as the tissues that have the critical role for metabolism in *Drosophila*. Fat body is an essential organ for energy storage, whereas muscle plays an important role for energy consumption. When *dATF-2* dsRNA was expressed in the fat body under the control of the *c564-GAL4* driver (Kambris *et al.*, 2006), the difference in survival between *dATF-2* knockdown flies and control flies was more clear (Figure 2B). At 40 h of starvation, 80% of control flies were alive, whereas only 30% of *dATF-2* knockdown flies were still alive (log-rank test, *p* < 0.0001). In the control experiment, we used the flies that expressed dsRNA for GFP (green fluorescent protein). There was no difference in survival between the transgenic GFP RNAi flies and control flies, indicating that the reduced survival of the *dATF-2* knockdown flies is due to reduced ATF-2 expression and is not a nonspecific effect of overexpressed dsRNA. In contrast,

Figure 2. A decrease of *dATF-2* in adipose tissue results in impaired survival under starvation conditions. In all the experiments, 7-d-old male flies were starved, and dead flies were counted every 8 h. Survival percentage is indicated as an average of three independent experiments $\pm$ SE. The log-rank test was used for statistical analysis. (A) *dATF-2* knockdown in whole body resulted in hypersensitivity to starvation. Control (total flies $n = 98$) versus *dATF-2* RNAi (total $n = 97$), $p < 0.0001$. Genotypes: *da-GAL4/+* versus *da-GAL4/+*; *UAS-dATF-2* IR/+ . (B) *dATF-2* knockdown in fat body, *Drosophila* adipose tissue, resulted in hypersensitivity to starvation. Control (total $n = 274$) versus *dATF-2* RNAi (total $n = 178$), $p < 0.0001$. *GFP* RNAi (total $n = 120$). Genotypes: *c564-GAL4/+* versus *c564-GAL4/+*; *UAS-dATF-2* IR/+ versus *c564-GAL4/+*; *UAS-GFP* IR/+ . (C) *dATF-2* knockdown in muscle did not result in hypersensitivity to starvation. Control (total $n = 190$) versus *dATF-2* RNAi (total $n = 182$), $p = 0.033$. Genotypes: *MHC-GAL4/+* versus *MHC-GAL4/+*; *UAS-dATF-2* IR/+ . (D) Transgenic flies harboring an extra copy of the *dATF-2* gene exhibited resistance to starvation stress. Control (total $n = 140$) versus *dATF-2* transgenic (total $n = 80$), $p = 0.0012$. Genotypes: *w* versus *w*; *dATF-2/dATF-2*.

when *dATF-2* dsRNA was expressed specifically in muscle using the myosin heavy-chain (*MHC*)-*GAL4* driver (Schuster *et al.*, 1996), there was no clear difference in survival between *dATF-2* knockdown flies and control flies (Figure 2C).

To perform the converse experiment, we used *dATF-2* transgenic flies. *dATF-2* transgenic flies died later than wild-type flies under conditions of nutrient deprivation (Figure 2D). This effect was statistically significant (log-rank test, $p = 0.0012$).

dATF-2 Does Not Control the Size and Morphology of the Cells in the Fat Body

To assess the contribution of *dATF-2* toward controlling tissue growth in flies, we compared the body weight of *dATF-2* knockdown flies with wild-type flies. The body weight of *dATF-2* knockdown flies, in which *dATF-2* dsRNA was expressed in the whole body, was slightly (4%) but significantly (t test < 0.0001) lower than that of wild-type flies (Figure 3A). We then asked whether *dATF-2* controls tissue morphology of the cells in fat body. Morphology of the cells in fat body was similar between *dATF-2* knockdown flies in which *dATF-2* dsRNA was expressed specifically in the fat body and wild-type flies (Figure 3, C and D). In addition, there was no significant difference in the cell size in the fat body between *dATF-2* knockdown flies and wild-type flies (Figure 3B).

dATF-2 Knockdown Flies Have Smaller Energy Reserves than Control Flies

Although the size and morphology of the cells of fat body in *dATF-2* knockdown flies were normal, *dATF-2* knockdown

flies had impaired survival under starvation conditions. One possible explanation for these results is that *dATF-2* knockdown flies have an abnormality in fat metabolism. Therefore, we examined the lipid storage level of *dATF-2* knockdown flies under standard conditions. The signal of Nile-Red staining in the *dATF-2* knockdown fat body was smaller than those in wild-type tissue, suggesting that lipid storage in *dATF-2* knockdown fat body is lower than that in wild type (Figure 4, A and B).

Because flies store energy predominantly as triglycerides in the fat body (Van der Horst, 2003), we measured the triglyceride levels in *dATF-2* knockdown and control flies. In fact, the triglyceride level of the whole body was 23% lower in the fat body-specific *dATF-2* knockdown flies than in wild-type flies (Figure 4C). In addition, the level of triglyceride in the fat bodies of third instar larvae was also 28% lower in the *dATF-2* knockdown flies than in wild-type flies (Figure 4D). We next asked whether the reduced *dATF-2* affects the utilizing rate of stored triglyceride. A time-course analysis of triglyceride levels in *dATF-2* knockdown and control flies during starvation indicated that the triglyceride stores of both *dATF-2* knockdown and control flies dropped during starvation with similar rates (Figure 4F). Taken together, these results suggest that the *dATF-2* knockdown flies store lower amounts of triglycerides and start out with smaller energy reserves than control flies, but do not have defects in utilizing the energy stored as triglycerides.

To verify that the lower level of triglycerides in *dATF-2* knockdown flies is due specifically to decreased *dATF-2* function, we measured the triglyceride levels of *dATF-2* transgenic flies harboring an extra copy of the *dATF-2* gene (Figure 4E). The triglyceride level in *dATF-2* transgenic flies was

Figure 3. The body weight and the size of fat body cells of dATF-2 knockdown flies. (A) The body weight of dATF-2 knockdown flies and control flies is shown as an average $\pm$ SE ($n > 200$ for each genotype). *** $p < 0.0001$. Genotypes: *da-GAL4/+* versus *da-GAL4/+; UAS-dATF-2 IR*. (B) The size of fat body cells from the fat body-specific dATF-2 knockdown flies and control flies is indicated. The cell size was measured from images of fat body cells stained with phalloidin-FITC as shown in C and D. Error bars, mean $\pm$ SD. NS, no significant difference. Genotypes: *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*. (C and D) Histology of fat body cells in control (C) and in the fat body-specific dATF-2 knockdown flies (D). Membranes were stained green with phalloidin-FITC, and nuclei were stained red with propidium iodide. Genotypes: *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*.

52% higher than that of wild-type flies (Figure 4E). Thus, the decreased triglyceride level in the dATF-2 knockdown flies can be attributed to the decrease in the dATF-2 activity.

In flies, the fat body plays an important role in metabolic control not only by serving as energy storage like mammalian WAT but also by assuming primitive liver functions such as gluconeogenesis (Sondergaard, 1993). Therefore, a decrease in dATF-2 activity may also affect the blood sugar level, which may affect the sensitivity to starvation stress. Trehalose is a disaccharide composed of two glucose molecules and is the principal blood sugar in insects (Bedford, 1977). Therefore, we examined whether expression of dATF-2 dsRNA in fat bodies affected trehalose levels. Hemolymph trehalose levels in dATF-2 knockdown flies were almost the same as that in control flies (Figure 4G). These results further support the notion that hypersensitivity of dATF-2 knockdown flies to starvation stress is mainly due to lower amounts of stored triglyceride.

dATF-2 Knockdown Flies Exhibit Decreased dPEPCK Expression and Decreased Glyceroneogenesis

Mammalian ATF-2 was previously reported to positively regulate PEPCK gene transcription (Cheong *et al.*, 1998). PEPCK plays an important role not only for gluconeogenesis but also for glyceroneogenesis, a pathway that produces glycerol-3-phosphate, a precursor of triglyceride, from gluconeogenic substrates (Figure 5D). Therefore, we compared the dPEPCK mRNA levels in the fat body-specific dATF-2

knockdown flies and control flies. The dPEPCK mRNA level in dATF-2 knockdown flies was about one-third of that of control flies (Figure 5A). We also examined the mRNA levels of some other enzymes that are critical for triglyceride metabolism. The mRNA level of acetyl-CoA synthetase (Ac-CoAS), which is a key enzyme in the synthesis of acetyl-CoA, which produces triglycerides, was also lower by $\sim 40\%$ in dATF-2 knockdown flies than in control flies. Fatty acid synthetase (FAS) and acetyl-CoA carboxylase (ACC) are two rate-limiting enzymes in fatty acid synthesis, a source of triglyceride (Figure 5A). The mRNA levels of these two enzymes was not lower than that of control flies; if anything, the FAS mRNA levels were slightly higher than in control flies (Figure 5A). On the other hand, the dPEPCK mRNA levels was ~ 2.1 -fold higher in the transgenic flies harboring an extra copy of the dATF-2 gene than that in wild-type flies (Figure 5B). These results suggest that a decrease in the dPEPCK mRNA in the dATF-2 knockdown flies is one of the key mechanisms leading to lower triglyceride stores.

To examine whether dATF-2 knockdown flies have impaired glyceroneogenesis, we directly measured the rate of glyceroneogenesis. The fat bodies of dATF-2 knockdown and wild-type flies at the third instar stage were prepared and incubated with [^{14}C]pyruvate, and the incorporation of [^{14}C]pyruvate into lipids was examined. The level of pyruvate incorporation into lipids in dATF-2 knockdown fat bodies was decreased by $\sim 30\%$ compared with control (Figure 5C). Thus, the activity of glyceroneogenesis is reduced in the fat bodies of dATF-2 knockdown flies. Reduced glyceroneogenesis may at least partly contribute to decreased triglyceride stores in the dATF-2 knockdown flies, although decreased AcCoAS levels may contribute as well.

The observation that dATF-2 knockdown flies had reduced glyceroneogenesis, raised the possibility that a high-calorie diet would enhance the phenotype of dATF-2 knockdown flies, such as the differences in body weight and triglyceride levels. Body weight of dATF-2 knockdown flies was compared with control flies at various time points after starting a high-calorie diet. Initially, dATF-2 knockdown flies had only 4.0% lower body weight than control flies, but after 7 d of feeding with a high-calorie diet the difference in body weight increased to 8.0% (Figure 6A). After 8-d on a normal diet, the triglyceride level of dATF-2 knockdown flies was lower by 23% than in wild type (Figure 6B). This difference increased to 34% when the flies were fed a high-calorie diet. Thus, a high-calorie diet enhances the phenotype of dATF-2 knockdown flies, suggesting that triglyceride synthesis via glyceroneogenesis is critical for the phenotype of dATF-2 knockdown flies.

dATF-2 Regulates dPEPCK Transcription

To investigate the mechanism of dATF-2 action, we examined the subcellular localization of dATF-2 in fat body cells. To clearly detect dATF-2, dATF-2 was ectopically expressed in the clones using the GAL4 driver and immunostained with anti-dATF-2 antibody. dATF-2 was found to be localized in the nucleus (Figure 7A), suggesting that like mammalian ATF-2 in the liver, dATF-2 mainly acts as a transcriptional regulator in fat body cells.

To examine whether dATF-2 directly regulates dPEPCK gene transcription, we performed luciferase reporter assays using the dPEPCK gene promoter-luciferase reporter. Co-transfection of the dATF-2 expression plasmid into S2 cells with a reporter containing the 1.1-kb dPEPCK gene promoter (dPck-1) enhanced luciferase expression ~ 3.7 -fold (Figure 7B). Truncation of the dPEPCK promoter region from 5', which generated the 0.7- (dPck-2) and 0.4-kb (dPck-3) pro-

Figure 4. *dATF-2* knockdown flies exhibit decreased triglyceride levels. (A and B) Nile-Red staining of fat bodies from the fat body-specific *dATF-2* knockdown flies (B) and control flies (A). Genotypes: *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*. (C–E) Triglyceride levels under normal conditions. Triglyceride levels in the whole body (C) of 7-d-old males or fat body of third instar larvae (D) of the fat body-specific *dATF-2* knockdown flies and control flies were measured. Triglyceride levels of the whole body (E) of 7-d-old males of transgenic flies harboring an extra copy of the *ATF-2* gene and control flies were also measured. Batches of three males were assayed, and the experiment was done in triplicate and repeated. Triglyceride levels were normalized to protein levels, and relative triglyceride level compared with that of control is indicated as an average $\pm$ SE ($n = 8-10$). ** $p < 0.01$; * $p < 0.02$. Genotypes: (C) *da-GAL4/+* versus *da-GAL4/+; UAS-dATF-2 IR*. (D) *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*. (E) *w* versus *w; dATF-2/dATF-2*. (F) Triglyceride levels during starvation. Triglyceride levels of the fat body-specific *dATF-2* knockdown flies and control flies were measured at various times during starvation as described above. Relative triglyceride level compared with that at the start of starvation is indicated as an average $\pm$ SE ($n = 8$). Genotypes: *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*. (G) A decrease in the *dATF-2* level does not affect the trehalose level. The hemolymph trehalose levels of third instar larvae were measured after controlled growth and aging of the fat body-specific *dATF-2* knockdown flies and control flies. Relative hemolymph trehalose level compared with that of control is indicated as an average $\pm$ SE ($n = 8$). NS, no significant difference. Genotypes: *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*.

motor constructs, decreased the luciferase activity to 3.3- and 2.4-fold, respectively, suggesting that multiple sites in the promoter region are responsible for *dATF-2*-induced activation. *ATF-2* forms a heterodimer with Jun (Hai and Curran, 1989) and also with C/EBP (Shuman *et al.*, 1997). *ATF-2* in these complexes recognizes the half-site of CRE, raising the possibility that the half-site of CRE in the *dPEPCK* promoter may be responsible for the *dATF-2*-dependent activation. To examine this possibility, we introduced mutations into six CRE half-sites in the *dPEPCK* promoter and used this

promoter in the reporter assay. Disruption of six CRE half-sites reduced the degree of *trans*-activation by *dATF-2* from 3.7- to 2.3-fold. These results suggest that *dATF-2* activates the *dPEPCK* promoter in part by directly binding to the CRE half-site.

Drosophila p38 (dp38) enhances *dATF-2* activity by phosphorylating *dATF-2* at Thr-59 and Thr-61 (Sano *et al.*, 2005). The *dATF-2* mutant in which Thr-59 and Thr-61 were mutated into Ala residues did not activate the *dPEPCK* promoter in the luciferase reporter assays (Figure 7C). The

Figure 5. *dATF-2* knockdown flies exhibit decreased *dPEPCK* expression. (A) *dPEPCK* mRNA levels were decreased in *dATF-2* knockdown flies. The levels of mRNA for key enzymes that control triglyceride levels were measured by quantitative RT-PCR. Total RNA was prepared from fat bodies of third instar larva. Relative mRNA levels were normalized to *rp49* mRNA. Relative mRNA levels are indicated as an average $\pm$ SE ($n = 3$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.02$; NS, no significant difference. (B) *dPEPCK* mRNA levels are increased in transgenic flies harboring an extra copy of the *ATF-2* gene. The *dATF-2* mRNA levels were measured using RNA from *dATF-2* transgenic flies and wild-type flies and is indicated as described above ($n = 3$). (C) Lower glyceroneogenesis activity in *dATF-2* knockdown flies. The third instar larval fat bodies from the fat body-specific *dATF-2* knockdown flies and control flies were incubated with [14 C]-pyruvate, and incorporation of pyruvate into lipid was measured. Four batches of 10 larvae were assayed, and the experiment was done twice. The results indicate an average $\pm$ SE ($n = 6-8$). ** $p < 0.01$. Genotypes: (A-C) *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*. (D) The pathway and some critical enzymes for triglyceride biosynthesis. Three enzymes that are critical for triglyceride metabolism are shown.

levels of wild-type and T59/61A mutant *dATF-2* expressed in the transfected cells were similar (Figure 7D). Furthermore, SB203580, an inhibitor of p38, significantly inhibited the *dATF-2*-induced activation of the *dPEPCK* promoter, whereas SP600125 and PD98059, inhibitors of JNK and Erk, respectively, did not (Figure 7C). Thus, the *dATF-2*-dependent activation of *dPEPCK* transcription is inhibited by the p38 inhibitor.

We also analyzed whether *dATF-2* also activates the *dAcCoAS* promoter. In the reporter assays using the *dAcCoAS* promoter-luciferase reporter, wild-type *dATF-2* activated the *dAcCoAS* promoter, whereas the T59/61A mutant did not (Figure 7E). Further, SB203580, an inhibitor of p38, significantly inhibited the *dATF-2*-induced activation of the *dAcCoAS* promoter, whereas SP600125, an inhibitor of JNK, did not.

Figure 6. A high-calorie diet enhanced the phenotype of *dATF-2* knockdown flies. (A) A high-calorie diet enhanced the difference in body weight between *dATF-2* knockdown and control flies. Body weight was measured at various time points after starting a high-calorie diet. Error bars, average $\pm$ SE ($n > 90$). High-calorie diets contain 15% Y-S (normal diets: 5% Y-S). (B) A high-calorie diet enhanced the difference in triglyceride levels between *dATF-2* knockdown and control flies. Triglyceride levels in the bodies of 7-d-old males of the fat body-specific *dATF-2* knockdown flies and control flies were measured after feeding with a normal diet and a high-calorie diet for 7 d. Triglyceride levels were normalized to total protein levels, and relative triglyceride levels were compared with control: the results are indicated as an average $\pm$ SE ($n = 8$). *** $p < 0.001$; ** $p < 0.01$. Genotypes: (A and B) *c564-GAL4/+* versus *c564-GAL4/+; UAS-dATF-2 IR*.

DISCUSSION

Knockdown Flies as a Model Suitable for Study of the Role of ATF-2 in Metabolism

Some members of ATF/CREB family and SAPK family have been demonstrated to regulate metabolism. Using the transgenic mice expressing dominant negative CREB, CREB was shown to control hepatic lipid metabolism and gluconeogenesis in response to a series of hormonal cues (Herzig *et al.*, 2001, 2003). In *Drosophila* cultured cells, box-B-binding factor-2 (BBF-2), a member of ATF/CREB family, binds specifically to the fat body-specific regulator element to activate transcription (Abel *et al.*, 1992). Obesity increases total JNK activity and *JNK1*-deficient mice exhibit reduced adiposity and improved insulin sensitivity (Hirosumi *et al.*, 2002). p38 mediates the free fatty acid-induced transcription of key gluconeogenic genes, including *PEPCK* (Collins *et al.*, 2006). Furthermore, mice lacking MKP-1, which is a member of MAPK phosphatase and inactivates p38, are resistant to diet-induced obesity due to energy expenditure (Wu *et al.*, 2006).

ATF-2 was also suggested to regulate metabolism. ATF-2 activated by p38 stimulates transcription of *PTEN* gene, leading to inhibition of insulin signaling (Shen *et al.*, 2006). ATF-2 also activates transcription of *PEPCK-C* gene (Cheong *et al.*, 1998). However, these data were obtained using cultured cells, and the role of ATF-2 in the regulation of metabolism remains elusive. This is partly due to a lack of appropriate model system to study the role of ATF-2 in a whole animal system. In this study, we have generated the fat body-specific *dATF-2* knockdown flies, in which the *dATF-2* mRNA level was reduced to ~40% of the wild-type level. *dATF-2* knockdown flies were not lethal and exhibited a reduced triglyceride storage compared with control flies. Thus, *dATF-2* knockdown flies can be thought as a good model suitable to study the role of ATF-2 in metabolism.

dATF-2 Affects Energy Reserve but not Energy Expenditure

Fat body and muscle are critical organs for *Drosophila* metabolism. Flies store energy as triglyceride in fat body, which is thought to resemble to mammalian WAT. In addition, the fat body functions like mammalian liver and has multiple enzymes that are required for glycolysis and lipid synthesis. Reduced dATF-2 activity in the fat body resulted in impaired survival under starvation condition, whereas *dATF-2* down-regulation in muscle did not. The fat body-specific *dATF-2* knockdown flies had a reduced reserve of triglyceride compared with control flies, suggesting that impaired survival of *dATF-2* knockdown flies may be due to reduced triglyceride in the fat body. Further, *dATF-2* knockdown flies did not affect either the hemolymph trehalose level or expenditure rate of triglyceride during starvation condition. Thus, dATF-2 may function in regulation of triglyceride store in the fat body by affecting the synthesis of triglyceride, but not its consumption.

dATF-2 Knock-down Flies Exhibited Reduced Glyceroneogenesis

In the fat body, many enzymes that control triglyceride level are expressed. Among these enzymes, *PEPCK* gene expression was strikingly reduced in the fat body of *dATF-2* knockdown flies. Mammalian ATF-2 was previously reported to regulate transcription of *PEPCK* gene in hepatoma cells (Cheong *et al.*, 1998; Lee *et al.*, 2002). *PEPCK* is a rate-limiting enzyme for gluconeogenesis in the mammalian liver, and the *Drosophila* fat body is an organ that has a function similar to both mammalian liver and WAT. Therefore, reduced

dPEPCK activity in the fat body would be expected to decrease the blood sugar trehalose level. However, hemolymph trehalose levels in *dATF-2* knockdown flies were similar to that of control flies. This may be due to the presence of multiple backup systems to keep the blood sugar levels steady because it is quite critical for the organism. In contrast to trehalose, the level of stored triglyceride in the fat bodies of *dATF-2* knockdown flies was reduced compared with control flies. These data suggest that stored triglyceride may convert to trehalose to maintain blood sugar level even under normal condition. Our results suggest that this was due to reduced activity of glyceroneogenesis, although a decrease in some other enzyme levels such as AcCoAS, which are involved in fatty acid synthesis, could also contribute. These results suggest that a 60% reduction of *dATF-2* mRNA reduces both glyceroneogenesis and triglyceride stores.

Although glyceroneogenesis was originally reported more than 35 years ago, it was not researched much until recently, when it has been the focus of many studies. The original discovery of glyceroneogenesis was that cytosolic *PEPCK*, which catalyzes the first step of hepatic and renal gluconeogenesis, is present in adipocytes (Ballard *et al.*, 1967). Mammalian adipose tissues do not have the gluconeogenic activity because adipocytes lack the terminal two enzymes of the pathway. This led to a finding that *PEPCK* converts gluconeogenic precursors such as pyruvate into glycerol-3-phosphate, the glycerol backbone of triacylglycerol, the major storage form of fat (Figure 8). The pathway is now described as glyceroneogenesis. Some reports described the important role of glyceroneogenesis to control the fat store. For instance, in fasting pregnant women, up to 60% of plasma glyceride-glycerol (the glycerol portion of triglyceride) was generated by glyceroneogenesis, primarily in the liver (Kalhan *et al.*, 2001). Similarly, more than 80% of the glyceride-glycerol in epididymal fat of rats fed a high-protein diet is produced by adipocyte glyceroneogenesis (Botion *et al.*, 1998).

Reduced PEPCK Level in dATF-2 Knockdown Flies Is Correlated with Decreased Glyceroneogenesis

In mammal, *PEPCK* is known to regulate glyceroneogenesis in addition to gluconeogenesis. The important role of *PEPCK* in glyceroneogenesis was demonstrated using the mice in which *PEPCK* was down- or up-regulated in WAT. Mice in which the PPAR γ -binding site in the *PEPCK-C* gene promoter exhibited reduced adipose tissue size and fat content (Olswang *et al.*, 2002). Conversely, transgenic mice expressing *PEPCK* in adipose tissue increased adipocyte size and fat mass (Franckhauser *et al.*, 2002). Consistent with these reports, we have demonstrated that reduced triglyceride levels are correlated with decreased glyceroneogenesis and *dPEPCK* gene transcription in *dATF-2* knockdown flies. Therefore, it is likely that a 60% decrease in *dATF-2* mRNA reduced triglyceride levels, at least partly by affecting glyceroneogenesis via *dPEPCK* gene transcription.

Glyceroneogenesis is thought to occur during fasting. However, adipose tissue-specific *PEPCK* knockdown mice exhibited lower triglyceride levels not only under fasted condition but also under fed condition (Olswang *et al.*, 2002). This observation is consistent with our results using *dATF-2* knockdown flies. The fat bodies of *dATF-2* knockdown flies fed under normal condition exhibited reduced activity of glyceroneogenesis compared with the control flies. These results suggest that *PEPCK* contributes to glyceroneogenesis not only under fasted condition but also normal fed condition. Important role of *PEPCK* in glyceroneogenesis was also

Figure 8. Model for the role of dATF-2 in triglyceride homeostasis. In *Drosophila* adipose tissue, dATF-2 is activated by dp38 and stimulates the expression of dPEPCK. dPEPCK is rate-limiting enzyme both in gluconeogenesis and in glyceroneogenesis. In gluconeogenesis, *Drosophila* PEPCK regulates biosynthesis of trehalose (Tre), analogous to mammalian glucose. dPEPCK also regulates biosynthesis of triglyceride (TG) via glycerol-3-phosphate (G-3-P) from pyruvate (Pyr) in glyceroneogenesis. PEPCK is known to function mainly during fasting. However PEPCK may be expressed and have the basal activity even under normal fed state. dATF-2 may be necessary for basal dPEPCK expression like a mammalian ATF-2 (Lee *et al.*, 2002). Decreased dPEPCK level in dATF-2 knockdown flies may affect both hemolymph trehalose and stored triglyceride. Blood sugar level, trehalose in *Drosophila*, is more essential for organism than stored lipid such as triglyceride. To maintain blood sugar level, triglyceride stored in fat body might be converted into trehalose. As a result, the stored triglyceride level may be reduced in dATF-2 knockdown flies.

demonstrated by the observations that thiazolidinediones, the antidiabetic drug, reduces serum fatty acid levels by enhancing glyceroneogenesis by activating PEPCK gene transcription (Tordjman *et al.*, 2003). If this result can be extended to mammals, ATF-2 may be a useful target for antidiabetic drugs like thiazolidinediones, although the role of ATF-2 in WAT has not been intensely studied.

dATF-2 Regulates dPEPCK Expression through Phosphorylation by p38

We have demonstrated that dATF-2 activates dPEPCK gene transcription in part via the CRE half-sites in the dPEPCK promoter. The p38 inhibitor, SB203580, suppressed the dATF-2-dependent activation of the dPEPCK promoter, suggesting that the dp38 signal positively regulates dPEPCK transcription in the fat body. These results are consistent with the observation in mammals that ATF-2 directly binds to and activates the PEPCK promoter through the p38 pathway (Cheong *et al.*, 1998). C/EBP was also reported to control PEPCK gene transcription in liver (Croniger *et al.*, 1998) and can form a heterodimer with ATF-2, which binds to an asymmetric sequence composed of one consensus half-site for each monomer (Shuman *et al.*, 1997). Therefore, ATF-2 could regulate PEPCK gene transcription together with other factors, such as C/EBP, which forms a heterodimer with ATF-2. If dp38 is required for dATF-2-dependent activation of dPEPCK transcription, dp38 would have to be constitutively activated in the fat body. In fact, p38 was reported to be constitutively active in the mammalian liver, which may be a result of metabolic oxidative stress (Mendelson *et al.*, 1996). Retinoic acid was also shown to activate the p38 pathway leading to ATF-2-dependent activation of PEPCK gene transcription (Lee *et al.*, 2002). Furthermore, dp38 mutants were shown to be more sensitive to starvation than wild-type flies (Craig *et al.*, 2004). If the p38 signaling pathway is important for triglyceride stores via glyceroneogenesis, this pathway may be a useful target for antidiabetic drugs, because various inhibitors for the kinases in this pathway have already been developed.

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