Original Research

ATF4 Responds to Metabolic Stress in Drosophila

Soonhyuck Ok1,†, Jung-Eun Park2,†, Seunghee Byun2,†, Kwonyoon Kang2, Jaekyoung Son3,4, Min-Ji Kang2,4, †

1 Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, 05505 Seoul, Republic of Korea
2 Department of Pharmacology, University of Ulsan College of Medicine, Asan Medical Center, 05505 Seoul, Republic of Korea
3 Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Asan Medical Center, 05505 Seoul, Republic of Korea
4 Biomedical Research Center, Asan Institute for Life Sciences, Asan Medical Center, 05505 Seoul, Republic of Korea
*Correspondence: mjkang@amc.seoul.kr (Min-Ji Kang)
†These authors contributed equally.

Abstract

Background: Activating transcription factor 4 (ATF4) is a fundamental basic-leucine zipper transcription factor that plays a pivotal role in numerous stress responses, including endoplasmic reticulum (ER) stress and the integrated stress response. ATF4 regulates adaptive gene expression, thereby triggering stress resistance in cells. Methods: To characterize the metabolic status of atf4−/− Drosophila larvae, we conducted both metabolomic and microarray analyses. Results: Metabolomic analysis demonstrated an increase in lactate levels in atf4−/− mutants when compared to wild-type flies. However, there was a significant reduction in adenosine triphosphate (ATP) synthesis in the atf4−/− flies, suggesting an abnormal energy metabolism in the mutant larvae. Microarray analysis unveiled that Drosophila ATF4 controls gene expression related to diverse biological processes, including lipase activity, oxidoreductase activity, acyltransferase, immune response, cell death, and transcription factor, particularly under nutrient-restricted conditions. In situ hybridization analysis further demonstrated specific augmentation of CG6283, classified as a gastric lipase, within the gastric caeca of nutrient-restricted flies. Moreover, overexpression of lipases, CG6283 and CG6295, made the flies resistant to starvation. Conclusions: These findings underscore the role of Drosophila ATF4 in responding to metabolic fluctuations and modulating gene expression associated with metabolism and stress adaptation. Dysregulation of ATF4 may detrimentally impact the development and physiology of Drosophila.

Keywords: ATF4; integrated stress response; nutrient restriction; lipase; microarray; Drosophila

1. Introduction

In Drosophila, the cryptocephal (crc) gene encodes the activating transcription factor 4 (ATF4) protein, which belongs to the family of a basic-leucine zipper transcription factors [1,2]. ATF4 regulates gene expression involved in endoplasmic reticulum (ER) stress, amino acid metabolism, and redox enzymes via a CCAAT-enhancer binding protein-activating transcription factor response element. Through this transcriptional activity, ATF4 protein is associated with developmental and disease processes, including axonix [3], long-term facilitation [4], stress response [5,6], apoptosis [7], and cancer [8,9]. Mutations in the crc (atf4) gene in Drosophila result in significant lethality during development. Specifically, the hypomorphic point mutation in crc, known as crc1, which involves a single amino acid change at residue 171 from glutamine to arginine, leads to delayed larval development and pupal lethality [10–13].

Integrated stress response (ISR) is a highly conserved homeostatic signaling pathway that is crucial in controlling translation, amino acid imbalance, and glucose homeostasis [14–16]. The common event in this pathway is the phosphorylation of eukaryotic translation initiation factor 2 on serine 51 of its alpha subunit (eIF2α), which reduces global protein synthesis and induces the expression of certain genes. GCN2 kinase mediates the ISR signaling pathway as an amino acid sensor by binding to the uncharged transfer tRNA. The activation of GCN2 kinase phosphorylates eIF2α, leading to ATF4 protein synthesis, and further triggering ATF4-mediated gene expression to protect cells from amino acid deprivation. Thus, ATF4 is presumed to be a main downstream component of the ISR.

Previously, we developed an assay tool to detect the in vivo ATF4 translational activity [17]. The study using this reporter indicates that Drosophila ATF4 protein synthesis increases in response to ER stress and ISR, and the translational regulatory mechanism of ATF4 is conserved among other species. Moreover, we demonstrated that the GCN2/ATF4/4E-BP pathway is required for lifespan extension upon the dietary restriction of amino acids [5,18]. In the present study, we analyzed the metabolic status in atf4−/− mutant flies. These flies appear to use glucose to produce lactate instead of producing adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle. Microarray analysis of atf4−/− mutant flies revealed that ATF4 regulates gene expression related to enzymes such as hydrolase, acyltransferase, and oxidoreductase, as well as related to immune response, cell death, and transcription factor.
Further, the overexpression of lipase, which is the transcriptional target of ATF4, manifests in increased starvation resistance. These results demonstrate that Drosophila ATF4 regulates gene expression in response to dietary restriction to resist metabolic stress.

2. Materials and Methods

2.1 Fly Strains

All Drosophila samples were conducted on standard Bloomington Drosophila Stock Center cornmeal food containing 1.6% yeast, 0.9% soy flour, 6.7% cornmeal, 1% agar, and 7% light corn syrup at 25 °C. The coding sequences for CG6283 and CG6295 were obtained via reverse transcription polymerase chain reaction (RT-PCR) from yw larvae. The HA-tag was added to the C termini of these coding sequences and subcloned into a pUAST. The following strains of flies have been previously described: atf4<sup> crc1, crc1</sup> [5], and Act5C<sup>GS</sup> [19]. UAS-lacZ flies were obtained from Bloomington Drosophila Stock Center (IN, USA). For gene induction, 100 µL of a 5 mg/mL solution of RU486 (Sigma, St. Louis, MO, USA; cat. #M4086) was added on top of food in a vial and dried overnight before feeding it to the flies.

2.2 Nutrient Restriction on Drosophila Larvae

Larvae were collected approximately 47–49 h after egg laying (AEL) on apple-juice plates (25% apple juice, 1.25% sucrose, and 2.5% agar) and then transferred to standard cornmeal food (5.9% glucose, 6.6% cornmeal, 1.2% baker’s yeast, and 1% agar in water) or to nutrient-restricted medium (5% sucrose and 1% agar in PBS) for 18 h at 25 °C.

2.3 Real-Time RT-PCR

Total RNA was isolated using TRIZol (Invitrogen, 15596018, Waltham, MA, USA), and 100 ng of RNA was transcribed with ReverTra Ace qPCR RT kit (TOYobo Co., Osaka, JAPAN). The real-time RT-PCR was run for 40 cycles using the TOPreal<sup>TM</sup> qPCR 2X PreMIX (SYBR Green with high ROX, enzynomics, Seoul, Republic of KOREA) and a LightCycler 480 Real-Time PCR system (Roche, Rotkreuz, Switzerland). The primer sequences are listed in Supplementary Table 1.

2.4 In Situ Hybridization

The full-length CG6283 cDNA was subcloned into pBluescript SK+. The T3 and T7 promoters of the pBluescript SK+ were used to generate DIG-labeled riboprobes for in situ hybridization using standard protocols [DIG RNA Labeling Kit (SP6/T7), 11175025910, Roche].

2.5 Microarray Analysis

Microarray experiments were performed using GeneChip® Drosophila Genome 2.0 Array (Applied Biosystems<sup>TM</sup>, Foster City, CA, USA). Total RNA from the flies was isolated using the Trizol reagent (Invitrogen, Waltham, MA, USA). Thereafter, cDNA was amplified from a 100-ng aliquot of total RNA from each sample using the GeneChip WT (whole transcript) amplification kit, as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). The sense cDNA was then fragmented and biotin-labeled with terminal deoxynucleotidyl transferase using the GeneChip WT Terminal labeling kit (Thermo Fisher Scientific, Waltham, MA, USA).

Specifically, the fold change (fc) was determined as follows: First, log<sub>2</sub>fc was calculated as the difference between the normalized values of the Test and Control samples (log<sub>2</sub>fc = Normalized value of Test – Normalized value of Control). Second, the calculated log<sub>2</sub>fc value was computed as 2 raised to the power of log<sub>2</sub>fc (fc = 2<sup>log<sub>2</sub>fc</sup>) to convert it into a linear scale. If the calculated fc value fell within the range of 0 to 1, it was interpreted as downregulated. If the value was ≥1, it was considered upregulated. To effectively represent values between 0 and 1, the fc value was transformed into its negative reciprocal (1/fc) (Macrogen, Seoul, Korea).

2.6 Metabolic Profiling

Fifty larvae of each genotype were collected at approximately 47–49 h AEL and homogenized using TissueLyzer (Qiagen, Hilden, Germany) with MeOH. Internal standard solutions (malonyl-L-<sup>13</sup>C<sub>3</sub> CoA, 5 µM Gln-d4) were added to the samples. The samples were centrifuged at 15,700 × g for 10 min (Eppendorf Centrifuge 5415R). The precipitate was stored for further measurement of the protein amount by the Bradford assay. For the supernatant, the aqueous phase after liquid–liquid extraction was collected and used for subsequent analysis. Metabolites were analyzed via liquid chromatography with tandem mass spectrometry (LC-MS/MS) [1290 HPLC (Agilent)-Qtrap 5500 (ABSciex)]. For metabolites related to energy metabolism, Synergi Fusion RP 50 × 2 mm was used. Here, 5 mM CH<sub>3</sub>COONH<sub>4</sub> in H<sub>2</sub>O and in MeOH served as mobile phases A and B, respectively. The separation gradient was as follows: hold at 0% B for 5 min, 0%–90% B for 2 min, hold at 90% for 8 min, 90%–0% B for 1 min, and then hold at 0% B for 9 min. The LC flow was 70 µL/min, except for 140 µL/min between 7–15 min, at 23 °C. For fatty acyl CoAs, a Zorbax 300 Extend-C18 column (2.1 × 150 mm) was used. Mobile phase A comprised acetonitrile (ACN)–H<sub>2</sub>O (10:90) with 15 mM NH<sub>4</sub>OH, and mobile phase B comprised ACN containing 15 mM NH<sub>4</sub>OH. The separation gradient was as follows: hold at 0% B for 3 min, 0%–50% B for 2 min, 50%–80% B for 5 min, 80%–0% B
Fig. 1. Metabolic reprogramming in atf4−/− mutants. Control (yw) and atf4cr1/R6 (atf4−/−) mutant larvae were collected at approximately 47–49 h after egg laying. The metabolites were monitored via liquid chromatography-tandem mass spectrometry (LC-MS/MS). (A) The relative amount of the intermediates of glycolysis. The values of each metabolite were normalized to the total protein level. (B) The relative levels of the intermediates of the tricarboxylic acid (TCA) cycle. (C) The relative amount of the intermediates of the pentose phosphate pathway (PPP). (D,E) The levels of fatty acyl CoA and diacylglycerol (DAG). These experiments were conducted in triplicate. Data are presented as mean ± standard error of the mean (SEM). *p < 0.05, **p < 0.01, and ***p < 0.001. Abbreviation: GLU, Glucose; G6P, Glucose-6-phosphate; F6P, Fructose-6-phosphate; FBP, Fructose-1,6-bisphosphate; 3PG, 3-phosphoglycerate; PEP, Phosphoenolpyruvate; PYR, Pyruvate; LAC, Lactate; NADH, reduced nicotinamide adenine dinucleotide; NAD, Nicotinamide Adenine Dinucleotide; CIT, Citrate; ISO, Iso citrate; AKG, alpha-ketoglutarate; SUC, Succinate; FUM, Fumarate; ATP, Adenosine triphosphate; 6PG, 6-phosphogluconate; R5P, Ribulose-5-phosphate; r5P, Ribose-5-phosphate; R15BP, Ribose-1,5-bisphosphate; S7P, Sedoheptulose-7-phosphate; NADPH, reduced Nicotinamide Adenine Dinucleotide Phosphate; NADP, Nicotinamide Adenine Dinucleotide Phosphate; DAG, diacylglycerol.

for 0.1 min, and then hold at 0% B for 4.9 min. The LC flow was 200 µL/min, and the column was kept at 25 °C. Multiple reaction monitoring was employed for analysis. The quantitative value of each metabolite was normalized to the total protein amount.

2.7 Starvation Assay on Adults

Twenty female flies (5 days old) of each genotype were transferred to vials containing 1% agar in PBS. The flies were supplied fresh food every 12 h and maintained at 25 °C; deaths were recorded at 96 h after starvation.

3. Results

3.1 Metabolomic Analysis Revealed the Metabolic Status in atf4−/− Mutant Larvae

To investigate the potential involvement of ATF4 in metabolic homeostasis, we assessed the nutrient reserves in the atf4−/− mutant larvae. Specifically, we measured the level of intermediates of major metabolic pathways, including glycolysis, the TCA cycle, and the pentose phosphate pathway, as well as coenzymes related to fatty acid metabolism. As shown in Fig. 1, there were notable differences in the levels of intermediates in the major metabolic pathways between the yw (atf4+/+, control) flies and the
Table 1. The analysis of genes regulated by ATF4 in response to nutrient restriction.

<table>
<thead>
<tr>
<th>Function</th>
<th>Probe ID</th>
<th>Drosophila gene</th>
<th>Human othologs</th>
<th>Fold</th>
<th>Function</th>
<th>Probe ID</th>
<th>Drosophila gene</th>
<th>Human othologs</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase activity</td>
<td>1635045</td>
<td>CG86271 (triglyceride lipase)</td>
<td></td>
<td>–8.61 Acyltransferase</td>
<td>1625325</td>
<td>CG13325</td>
<td></td>
<td></td>
<td>–4.22</td>
</tr>
<tr>
<td></td>
<td>1629367</td>
<td>CG15534 ( sphingomyelin SMPD1 phosphodiesterase)</td>
<td></td>
<td>–6.14</td>
<td></td>
<td>1626764</td>
<td>CG10182</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1623775</td>
<td>CG31089 ( triglyceride lipase)</td>
<td></td>
<td>–5.02</td>
<td></td>
<td>1631234</td>
<td>CG18173</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1636343</td>
<td>CG6293 (triglyceride lipase)</td>
<td></td>
<td>–2.81</td>
<td></td>
<td>1632163</td>
<td>CG8481</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1635868</td>
<td>CG6295 (serine hydrolase)</td>
<td></td>
<td>–2.76</td>
<td></td>
<td>1629934</td>
<td>CG14219</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1633709</td>
<td>CG2772 (triglyceride lipase)</td>
<td></td>
<td>–2.51</td>
<td>Hydrolase activity</td>
<td>1635812</td>
<td>CG16965</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1632120</td>
<td>CG15533 ( sphingomyelin SMPD1 phosphodiesterase)</td>
<td></td>
<td>–2.30</td>
<td></td>
<td>1637357</td>
<td>CG9463</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidoreductase activity</td>
<td>1640566</td>
<td>Cyp4p2 cytochrome P450</td>
<td></td>
<td>–62.81</td>
<td></td>
<td>1637602</td>
<td>CG32801 /// Edem1</td>
<td>EDEM1</td>
<td>–3.02</td>
</tr>
<tr>
<td></td>
<td>1625436</td>
<td>Uro</td>
<td></td>
<td>–4.16</td>
<td></td>
<td>1639401</td>
<td>Mal-A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1634623</td>
<td>Cyp6a14</td>
<td></td>
<td>–3.88</td>
<td>Immune response</td>
<td>1626319</td>
<td>CG33470 /// IM10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1630000</td>
<td>CG33093</td>
<td></td>
<td>–3.82</td>
<td></td>
<td>1627986</td>
<td>PGRP-SC1a /// PGRP-SC1b</td>
<td>PGLYRP</td>
<td>–5.26</td>
</tr>
<tr>
<td></td>
<td>1622906</td>
<td>Sod3 SOD1, CCS</td>
<td></td>
<td>–3.82</td>
<td></td>
<td>1627613</td>
<td>Mtk</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1633471</td>
<td>Ptx2540-2</td>
<td></td>
<td>–3.73</td>
<td></td>
<td>1636490</td>
<td>PGRP-SB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1626401</td>
<td>Cyp6a2</td>
<td></td>
<td>–3.53</td>
<td>Cell death</td>
<td>1630010</td>
<td>pnt</td>
<td>ETS1, ETS2</td>
<td>–3.75</td>
</tr>
<tr>
<td></td>
<td>1626503</td>
<td>CG2254 /// DsecGM11216 DHRS3, HSD17B11, RDH10, SDR16C5</td>
<td></td>
<td>–3.45</td>
<td></td>
<td>1625981</td>
<td>rhab3-GEF</td>
<td>MADD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1624101</td>
<td>Cyp6a23</td>
<td></td>
<td>–3.23</td>
<td></td>
<td>1627463</td>
<td>Damm</td>
<td>Caspase-like domain</td>
<td>–3.13</td>
</tr>
<tr>
<td></td>
<td>1633401</td>
<td>Cyp12d1-d /// Cyp12d1-p</td>
<td></td>
<td>–3.00</td>
<td>Transcription factor activity</td>
<td>1626392</td>
<td>Mef2</td>
<td>MEF2</td>
<td>–6.59</td>
</tr>
<tr>
<td></td>
<td>1639892</td>
<td>Sodh-1</td>
<td></td>
<td>–2.88</td>
<td></td>
<td>1641365</td>
<td>Jim</td>
<td>–3.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1629745</td>
<td>CG6439 IDH3G</td>
<td></td>
<td>–2.75</td>
<td></td>
<td>1625195</td>
<td>Cyp6v1 /// shn</td>
<td>–2.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1635110</td>
<td>Cyp6a13</td>
<td></td>
<td>–2.72</td>
<td>Peptidase activity</td>
<td>1634477</td>
<td>CG42335</td>
<td>ERAP1-like C-terminal domain</td>
<td>–8.57</td>
</tr>
<tr>
<td></td>
<td>1630244</td>
<td>CG31809 /// CG31810 HSDL1, HSD17B3</td>
<td></td>
<td>–2.43</td>
<td></td>
<td>1627156</td>
<td>CG33225</td>
<td>GZMB (granzyme B), CTSG (cathepsin G)</td>
<td>–3.69</td>
</tr>
<tr>
<td></td>
<td>1631452</td>
<td>CG8665 ALDH1L1</td>
<td></td>
<td>–2.41</td>
<td></td>
<td>1635453</td>
<td>Bace</td>
<td>–3.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1630359</td>
<td>CG31810 HSDL1, HSD17B3</td>
<td></td>
<td>–2.16</td>
<td></td>
<td>1632493</td>
<td>CG12717</td>
<td>–2.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1624159</td>
<td>Cyp9h1 CYP3A4</td>
<td></td>
<td>–2.07</td>
<td></td>
<td>1635398</td>
<td>CG10587 /// Snp2</td>
<td>–2.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1639391</td>
<td>CG17109 /// PM20D1</td>
<td></td>
<td>–2.19</td>
</tr>
</tbody>
</table>

# Fold: the value of atf4−/− starved/the value of atf4+/+ starved.
**3.2 ATF4 Regulates Gene Expression Upon Nutrient Restriction**

Considering that ATF4 is known to respond to nutrient restriction in various species [5,20,21], we conducted a genome-wide expression profiling of control (atf4+/+) and atf4−/− larvae subjected to dietary restriction for 18 hours using GeneChip® Drosophila Genome 2.0 Array (see Material and Methods). The microarray analysis was performed three times using independent biological replicates, and we compared the gene expression between the wild type (yw, atf4+/+) and atf4−/− mutant larvae subjected to 18 hours of starvation (Supplementary Fig. 1 and Supplementary Table 2). The differentially expressed genes (the value of atf4−/− starved/the value of atf4+/+ starved) with \( p < 0.05 \) and a fold change >2 were selected for subsequent gene ontology analysis. Based on these criteria, we identified 141 genes that were downregulated in atf4−/− mutant larvae compared to the control (Supplementary Table 3), and 132 genes that were upregulated (Supplementary Table 4). Out of the 141 downregulated genes, 110 genes could be grouped into significantly enriched functional categories, which included lipase activity, oxidoreductase activity, acyltransferase, immune response, cell death, and transcription factor functions (Table 1 and Supplementary Table 3).

To validate the microarray results, we performed quantitative RT-PCR. As shown in Fig. 2, the expression of lipase genes (CG6271, CG15533, CG15534, and CG31089) increased in response to 18 hours of larval starvation. However, this increase in gene expression due to nutrient restriction was suppressed in atf4−/− mutants, indicating that these lipases are induced by ATF4 in response to nutrient restriction. Similarly, the expression of CG11893 and Cyp4p2, which exhibit transferase and oxidoreductase activities, respectively, was also increased by starvation, and their regulation was dependent on ATF4. In contrast, the expression of CG33039 (with oxidoreductase activity), CG31436 (with transferase activity), and nol (involved in neuroblast proliferation) were regulated by ATF4 independently of the nutrient status.

**3.3 Increased Expression of Lipase, the Transcriptional Target of ATF4, Enhances Resistance to Starvation**

To investigate the localization of lipase gene expression regulated by ATF4, we performed in situ hybridization. Among the lipases in Drosophila, CG6283 is classified as a gastric lipase, but is not well characterized. Under fed conditions, we detected mRNA expression of CG6283 in the midguts of both atf4−/− mutant flies and the wild-type flies (Fig. 3A). However, upon starvation, the mRNA levels of CG6283 significantly increased. Specifically, we observed an induction of CG6283 in the gastric caeca (arrow in Fig. 3A), which is involved in enhancing digestive enzyme secretion and nutrient absorption. Notably, this increase in CG6283 expression was absent in atf4−/− mutants. To further explore the physiological function of lipase under nutrient restriction, we induced the expression of CG6283 or CG6295 using Act5C^{GS-gal4}, which led to the expression of these genes throughout the entire body only when RU486 was added to the diet. We subjected the flies to complete starvation for 4 days and monitored their survival rate. Flies overexpressing lipases (Act5C^{GS} > CG6283 or Act5C^{GS} > CG6295; 71.4% and 83.3%, respectively) exhibited longer survival than control flies (Act5C^{GS} > lacZ; 26.7%) (Fig. 3B). In summary, these findings suggest that Drosophila ATF4 plays a critical role in promoting organism survival during nutritional starvation by inducing the expression of enzymes such as lipases, which provide essential energy sources for the organism.

**4. Discussion**

In our previous work, we developed an in vivo reporter to detect ATF4 translational activity, which demonstrated that ATF4 responds to ER stress and ISR [17]. In this study, we performed a metabolomic analysis to investigate the metabolic status of Drosophila atf4−/− mutants. Most of the analyzed metabolites were found to be higher in the atf4−/− flies compared to the control (yw) flies. Notably, the lactate level was elevated in the atf4−/− flies, although not significantly, while ATP production was reduced.

Lactate is a product of glucose metabolism and is produced in highly glycolytic tissues, such as the skeletal muscle. It can be converted to pyruvate by lactate dehydrogenase and utilized in mitochondria in various tissues, including the liver and kidney. Under certain anaerobic conditions (hypoxia) and hypostasis, lactate levels can substantially increase (hyperlactatemia), potentially leading to cell injury. Hyperlactatemia is associated with various diseases, such as heart disease, severe anemia, and diabetes mellitus [22,23]. Moreover, the increase in lactate levels in the atf4−/− mutants resembles the Warburg effect, characterized by increased glucose uptake and lactate accumulation even under aerobic conditions. The Warburg effect is a metabolic reprogramming observed in cancer cells and is essential for cancer progression. Cancer cells primarily employ the glucose → pyruvate → lactate pathway for their proliferation.
Fig. 2. Relative expression levels of the selected transcripts identified as the transcriptional targets of ATF4. Control (yw) and atf4−/− larvae at 48 h after egg laying were collected and subjected to a restricted diet. After 18 h, total mRNA was extracted and used for quantitative reverse transcription polymerase chain reaction (RT-PCR). The values were normalized to the Rp49 data. (A) Lipase activity. (B) Transferase activity. (C) Oxidoreductase activity. (D) Neuroblast proliferation. The data represent the mean and SEM from at least four independent experiments. p-values were determined using Student’s t-test. *p < 0.05, **p < 0.01, and ***p < 0.001.

Fig. 3. Lipase gene contributes to the survival of flies from starvation. (A) the transcripts of CG6283 are at the basal level in the midgut of wild type (atf4+/+) and atf4−/− mutants but are significantly enhanced only in the gastric caeca of wild type by starvation. The induction of CG6283 by starvation was not detected in atf4−/− mutants. (B) lipase overexpressing flies are more resistant to starvation. Five-day-old flies (20 flies in each vial) were cultured in a complete starvation medium for 4 days. The percentage indicated the number of surviving flies (n = 3). The data represent the mean and SEM from at least three independent experiments. p-values were determined using Student’s t-test. *p < 0.05 and **p < 0.01.

However, when they require energy for metastasis, similar to normal cells, they undergo a metabolic shift to produce ATP in mitochondria [24,25]. Interestingly, the atf4−/− mutant flies seem to preferentially use glucose to produce lactate rather than generating ATP through the TCA cycle, similar to the Warburg effect. This is supported by the decreased ATP levels and relatively low or similar levels of TCA cycle intermediates in the atf4−/− mutants (Fig. 1B). These metabolic characteristics are typically observed in proliferating cancer cells but not in differentiating cells. Although some TCA cycle metabolites, such as AKG and SUC, were increased in the atf4−/− mutants, they could po-
tentially be produced through a salvage pathway. Overall, it appears that the \( \text{atf}^{4-/-} \) mutants experience dysregulation of energy metabolism.

Furthermore, these mutants die during the pupal stage [10]. They exhibit certain developmental defects, such as the absence of head eversion and abnormal differentiation of the abdomen but show normal eye pigmentation and proper differentiation of wings and legs. Considering that metamorphosis at the pupal stage requires substantial energy [26], it is plausible that the dysregulation of energy metabolism in the \( \text{atf}^{4-/-} \) mutants may play a role in their lethality. However, additional research is necessary to comprehensively understand this phenomenon.

The findings from numerous previous studies have reported \( \text{ATF}4 \) as a transcriptional regulator of genes involved in various cellular processes under different stress conditions. For instance, \( \text{ATF}4 \) promotes the expression of genes related to amino acid import, glutathione biosynthesis, and resistance to oxidative stress during ER stress in eukaryotes [20]. Another study in \( \text{Drosophila} \) S2 cells subjected to ER stress revealed that \( \text{ATF}4 \) controls the gene expression of glycolytic enzymes [27].

In our study, we observed that genes categorized as redox/detoxification and secretion/transmembrane transport were upregulated under nutrient restriction and that their expression was regulated by \( \text{ATF}4 \) (Table 1 and Fig. 2A). As demonstrated in Table 1, the expression of genes with oxidoreductase activity was significantly reduced in \( \text{atf}^{4-/-} \) mutants. When considering the lower levels of NADH/NAD and NADPH/NADP observed in \( \text{atf}^{4-/-} \) mutants compared to that in control flies (Fig. 1), it suggests that NADH and NADPH could serve as compensatory reducing agents, given the insufficient reductase activity in \( \text{atf}^{4-/-} \) mutants flies. In contrast, when we subjected larvae to nutrient restriction, we specifically found that the gene expression related to lipid catabolism was increased, which was suppressed in \( \text{atf}^{4-/-} \) mutants (Table 1 and Fig. 2A). Additionally, we found that gene expression related to lipid catabolism was specifically increased during nutrient restriction and suppressed in \( \text{atf}^{4-/-} \) mutants (Table 1 and Fig. 2A). These genes, including \( \text{CG}6271, \text{CG}15533, \text{CG}31089, \text{and CG}6283, \) showed significant homology to human \( \text{LIPH, SMPD1, LIPA, and LIPH, respectively.} \) The human orthologs of these genes are known to be associated with various metabolic diseases, such as type 2 diabetes mellitus, hypotrichosis 7, lysosomal acid lipase deficiency, and Niemann-pick disease. According to the modENCODE project (http://www.modencode.org), these genes are highly expressed in \( \text{Drosophila} \) larvae and adults, although their expression levels differ between the two developmental stages.

In \( \text{Drosophila} \), \( \text{CG}6283 \) is specifically expressed in the gut and is induced by nutrient restriction in the gastric caeca (Fig. 3A). Gastric caeca are finger-like projections in the gut found in several insects and play a crucial role in secreting digestive enzymes and facilitating nutrient absorption. Additionally, gastric caeca contain lysosomes, multivesicular bodies, autophagosomes, and lipid droplets, all of which are essential for energy metabolism [28]. Based on these observations, we hypothesized that \( \text{ATF}4 \) may aid the organism’s survival under starvation by increasing the expression of various genes, including \( \text{CG}6283 \) and \( \text{CG}6295 \), as illustrated in Fig. 3B. In our previous studies, we demonstrated that the GCN2/\( \text{ATF}4/4E-BP \) pathway controls the lifespan of flies under dietary amino acid restriction by regulating stress-response protein synthesis [5,18]. Collectively, we believe that \( \text{ATF}4 \) plays a pivotal role in controlling lifespan under nutrient restriction by upregulating the gene expression of lipases to provide the required energy sources.

5. Conclusions
Considering that \( \text{ATF}4 \) in mammals is involved in metabolic diseases [29–31], understanding the function of \( \text{ATF}4 \) in relation to the regulation of lipase activity under excess energy conditions would be beneficial for further research.

Availability of Data and Materials
The datasets utilized and/or examined during the present study can be obtained from the corresponding author upon reasonable request.

Author Contributions
SO, JEP, and MJK designed the research study. SO, JEP, SB, KK and MJK performed the research. JS provided help and advice on metabolic analysis. SO, JEP, SB, KK, JS, and MJK analyzed the data. MJK wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate
Not applicable.

Acknowledgment
The authors are grateful to the Bloomington \( \text{Drosophila} \) Stock Center at Indiana University (NIH P40OD018537) for providing fly strains.

Funding
This research was funded by grants from the National Research Foundation of Korea, NRF-2022R1A2C1003431, and from the Asan Institute for Life Sciences (Seoul, Republic of KOREA; 2022IL0010 and 2023P0121).
Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2812344.

References


