Biochemical and Biophysical Research Communications 468 (2015) 312–318

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# CCAAT/enhancer-binding protein CEBP-2 controls fat consumption and fatty acid desaturation in *Caenorhabditis elegans*

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ARTICLE INFO

*Article history:*
Received 18 October 2015
Accepted 20 October 2015
Available online 23 October 2015

*Keywords:*
*Caenorhabditis elegans* (*C. elegans*)
CEBP-2
Beta-oxidation
Lipid synthesis

ABSTRACT

Mammalian CCAAT/enhancer-binding proteins (C/EBPs) are generally known as regulators in adipocyte differentiation. However, more understanding of the role of C/EBPs in lipid and glucose metabolism remains to be discovered. In this study, we verified the effect of CEBP-2, the homolog of CEBPs, on fat storage in *Caenorhabditis elegans*. Expressions of 85 genes that encode the major enzymes in energy metabolic pathways were then screened in *cebp-2*-deficient worms using a quantitative real-time polymerase chain reaction (QRT-PCR). Our data implied that loss of function of CEBP-2 displayed a low-fat phenotype in *C. elegans* owing to increased expression of *ech-1.1* and decreased expression of *fat-5*. Our findings indicated that *cebp-2* controls total body fat content by governing fatty acid mitochondrial β-oxidation and desaturation in *C. elegans*. These data provide insights into how C/EBPs may affect lipid metabolism in mammals in addition to regulating adipocyte differentiation.



## 1. Introduction

Obesity, which results from excessive accumulation of triglycerides in the body's adipocytes, has become a worldwide problem. In 2005, it was estimated that approximately 2.3 billion adults worldwide would be overweight or obese in 2015 by WHO [1]. Obesity is a significant risk factor for major diseases, including hypertension, type II diabetes, and coronary heart disease [2,3], which reduce the quality of life for those patients. The detailed molecular mechanisms of obesity remain unclear; therefore, the study of lipid metabolism has become one of the most important topics in the medical field.

CCAAT/enhancer-binding proteins (C/EBPs) are a family of basic-region leucine zipper transcription factors that are widely expressed in various species, such as humans, mice, *Caenorhabditis elegans*, and Mediterranean fruit flies. They are a transcription factor family proved to play an important role in the process of adipocyte differentiation and are expressed in a specific sequence [4–6]. In addition, some members of this family have been identified to regulate the expression of lipogenesis [7,8]. Thus, more understanding of the roles of C/EBPs in lipid and glucose metabolism should be encouraged to be found.

The nematode *C. elegans* has no dedicated adipocytes [9]. The fat in this species, which is stored in the form of lipid droplets in hypodermal cells and intestinal cells, can be easily stained and visualized because of the species' translucent body. *C. elegans* is the first multicellular organism with its genome completely sequenced [10]. In addition, the major metabolic pathways and transcription factors are highly conserved in *C. elegans* [11]. By using the RNA-mediated interference (RNAi) method, researchers have identified 417 gene inactivations that either reduce or increase body fat [12]. Because of its specific genetic characteristics, *C. elegans* has become a prominent model in the study of metabolic diseases [9,11,13].

Using Oil-Red-O (ORO) staining and quantitative real-time polymerase chain reaction (QRT-PCR), *cebp-2*, the homolog of C/EBPs genes, was found to regulate fat storage by governing the expression of *ech-1.1* and *fat-5* in *C. elegans*. These findings show that CEBP-2 also influences fatty acid mitochondrial β-oxidation and

*Abbreviations:* C/EBPs, CCAAT/enhancer-binding proteins; CGC, Caenorhabditis Genetics Center; GFP, green fluorescent protein; NGM, nematode growth medium; ORO, Oil-Red-O; QRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA-mediated interference; RT, room temperature; SCD, stearoyl-CoA desaturase; WT, wild type.

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http://dx.doi.org/10.1016/j.bbrc.2015.10.106
0006-291X/

fatty acid desaturation, which provides insights into the study of functions of C/EBPs in fat metabolism in addition to the regulation of adipocyte differentiation.

## 2. Materials and methods

### 2.1. Worms and culture

*C. elegans* was cultured at 20 °C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50. Strains used were wild-type (WT) N2, *cebp-2* (gk509377), *cebp-2* (gk112657), *cebp-2* (gk112658), *cebp-2* (gk112659), and BX150 (*fat-5*::green fluorescent protein [GFP]) were obtained from Caenorhabditis Genetics Center (CGC), University of Minnesota, College of Biological Science, Minneapolis, Minnesota, USA, funded by the National Institutes of Health—Office of Research Infrastructure Programs.

### 2.2. Synchronization of the growth stage

Batches of adult worms were washed with M9 buffer and treated with bleaching solution (600uL NaClO, 150uL 5.0 M NaOH, 3.0 mL M9 buffer). Worms were vortexed at the maximum speed until the skin of 80% of the worms was destroyed. Bleaching solution was washed off. Eggs were then collected and hatched in M9 buffer overnight at room temperature (RT). Synchronized L1 larvae were obtained the next day.

### 2.3. RNA-mediated interference constructs

The *ech-1.1* and *fat-5* RNAi constructs were obtained from the *C. elegans* RNAi feeding library provided by Source Bioscience Geneservice (Nottingham, Nottinghamshire, UK). The *cebp-2* RNAi construct was created by cloning *cebp-2* cDNA (forward primer: 5′−3′ATGAGTGGAAATCGGAAGCG; reverse primer: 5′−3′ACACG GCGGAAGAAGATGGT) into vector L4440 (Addgene, Cambridge, MA, USA).

### 2.4. Feeding RNA-mediated interference

Bacteria producing the desired double-stranded RNA were inoculated to 3.0 mL LB medium containing 25ug/mL carbenicillin and 12.5ug/mL tetracycline. Grow cultures were shaken at 300 rpm at 37 °C overnight. The solution was then moved to fresh LB medium containing 25ug/mL carbenicillin at 1:100 and shaken at 300 rpm at 37 °C for 4.0–6.0 h. The bacterial cultures were then seeded on NGM agar plates containing 1.0 mM IPTG (RNAi plate) and induced at RT for 1.0–2.0 d. After synchronization of the growth stage, hatched L1 larvae were grown on RNAi plates at 20 °C until the worms developed into the L4 stage.

### 2.5. Oil-Red-O staining of fixed worm

Batches of worms at L4 stage were washed from plates with 1 × PBS. To permeabilize the cuticle, worms were suspended in 1 × PBS to which an equal volume of 2 × MRWB buffer (160 mM KCl, 40 mM NaCl, 14 mM Na2EGTA, 1.0 mM spermidine-HCl, 0.4 mM spermine, 30 mM Na-PIPES pH 7.4, 0.2% β-mercaptoethanol) containing 2.0% paraformaldehyde were added. The worms were rocked for 30 min at RT. Samples were then frozen at −80 °C for 15 min and thawed at RT three times. After removal of the paraformaldehyde, worms were suspended in 60% isopropanol and incubated for 15 min at RT to dehydrate. ORO was prepared as follows: 0.5 g/100 mL isopropanol stock solution equilibrated overnight was freshly diluted to 60% with water and rocked for at least 1.0 h and filtered using a 0.22-μm filter. After the isopropanol was removed, 1.0 mL 60% ORO stain was added and the worms were incubated on the bench overnight at RT. Next morning, the dye was removed as much as possible by washing with 1 × PBS (0.01% Triton X-100 was added). The worms were counted using the Nikon SMZ1500 (Nikon Corporation, Tokyo, Japan) and imaged using the Olympus IX51 (Olympus Corporation, Shinjuku, Japan).

### 2.6. Oil-Red-O quantification

A standard curve was drawn with different concentrations of ORO stain (0 ng/uL, 100 ng/uL, 200 ng/uL, 300 ng/uL, 400 ng/uL, and 500 ng/uL). To extract ORO stain from the animals, 200 worms were collected in the tube treated with 20uL isopropanol and incubated at 37 °C for 2.0 h. The density of the extraction was then tested according to the absorption peak at a wavelength of 490 nm with the NanoDrop2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA).

### 2.7. Movement analysis

Fifteen animals were chosen for plating using fairly thin lawns with one worm per plate. The pumping rate was measured by counting the grinder movement for 10 s. When the area just behind the worm's pharynx reached a maximum bend in the opposite direction from the bend last counted, the count was advanced by one. Body bending rate was counted for 20 s.

### 2.8. Overexpression of CEBP-2::GFP and FAT-5::GFP

To construct a transgenic plasmid, a genomic fragment comprising 2.0 kb *cebp-2* or *fat-5* upstream sequence and full-length *cebp-2* or *fat-5* DNA was cloned into the pPD95.75 GFP expression vector (a gift from Kai Li, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). For rescue experiments, 40 ng/uL of *cebp-2* or *fat-5* transgenic plasmid was injected into the syncytial gonad of the *cebp-2* (gk509377) mutants along with a coinjection of 20 ng/uL marker pRF4 *rol-6* (su1006). Transgenic worms derived from the $F_1$ generation were selected for rolling behavior and were analyzed by GFP microscopy.

### 2.9. Preparation of nematode total RNA and QRT-PCR

Batches of worms were harvested at L4 stage. For RNA preparation, worms mixed with TRIzol were frozen in liquid nitrogen, thawed at 37 °C, and rocked for 30 s three times. RNA was then extracted using the TRIzol method. cDNA was synthesized from 1.0 ug total RNA in a 25-uL reaction using M-MLV reverse transcriptase (TaKaRa Bio Inc., Shiga, Japan). Formation of a double-stranded DNA product was monitored using FastStart Universal SYBR Green Master (Rox) (F. Hoffmann-La Roche Ltd., Basel, Switzerland). All QRT-PCR reactions were conducted and analyzed on a 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using each primer (Supplementary Table 1). *act-1* was measured as a reference gene because it is expressed at constant levels throughout development.

### 2.10. Statistical analyses

Results are presented as the means ± SD of triplicate cultures, and statistical differences were assessed using t-test or 2-way analysis of variance.