Biochemical and Biophysical Research Communications 463 (2015) 693–698

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Constitutive activation of *Drosophila* CncC transcription factor reduces lipid formation in the fat body

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ARTICLE INFO

Article history:
Received 20 May 2015
Accepted 31 May 2015
Available online 3 June 2015

Keywords:
Lipid homeostasis
Gene regulation
Drosophila model
Fat body
Innate immunity

ABSTRACT

Accumulating evidence indicates that the vertebrate stress-response transcription factors Nrf1 and Nrf2 are involved in hepatic lipid metabolism. However, the underlying molecular mechanisms of Nrf1-and Nrf2-mediated lipid metabolism remain unclear. To elucidate the precise roles of Nrfs in this process, we analyzed the physiological role of CncC in lipid metabolism as a *Drosophila* model for vertebrate Nrf1 and Nrf2. We first examined whether CncC activity is repressed under physiological conditions through a species-conserved NHB1 (N-terminal homology box 1) domain, similar to that observed for Nrf1. Deletion of the NHB1 domain (CncCΔN) led to CncC-mediated rough-eye phenotypes and the induced expression of the CncC target gene *gstD1* both *in vivo* and *in vitro*. Thus, we decided to explore how CncCΔN overexpression affects the formation of the fat body, which is the major lipid storage organ. Intriguingly, CncCΔN caused a significant reduction in lipid droplet size and triglyceride (TG) levels in the fat body compared to wild type. We found that CncCΔN induced a number of genes related to innate immunity that might have an effect on the regulation of cellular lipid storage. Our study provides new insights into the regulatory mechanism of CncC and its role in lipid homeostasis.

1. Introduction

Lipids are a major source of cellular energy. Our bodies precisely maintain the homeostasis of lipid uptake, synthesis, storage and utilization. Perturbations in any of these steps lead to pathological conditions such as obesity, diabetes, liver steatosis and lipodystrophy [5,6,13]. Therefore, understanding the etiological mechanisms that lead to energy imbalance is important. Increasing evidence suggests that gene regulation in lipid metabolism is tightly controlled by a transcription factor network that includes PPARγ and C/EBP [26].

NF-E2-related factor 1 and 2 (Nrf1 and Nrf2) belong to the basic leucine zipper transcription factor CNC family [20,21,31]. Nrf2 is a conserved global regulator of cellular protection against reactive oxygen species that induces antioxidant and detoxifying genes. Nrf1 regulates proteasome recovery by inducing the expression of proteasome subunit genes in response to proteasome inhibition [24,28]. Recently, Nrf1 and Nrf2 have also been reported to be involved in lipid metabolism. Nrf2-deficient mice show the rapid onset and progression of hepatic steatosis [29]. Hepatocyte-specific *Nrf1*-knockout mice develop hepatic steatosis and inflammation [10,22,33,37]. Despite the severe impairment of lipid metabolism in these knockout mice, the precise molecular and signaling connections between metabolic signals and the Nrf pathway remain poorly explored.

The *Drosophila Cnc* (Cap'N' collar) gene, which is orthologous to the vertebrate Nrf transcription factor gene family, acts as a cellular defense mechanism against oxidative stress by inducing oxidative stress response genes such as *gstD1* via the antioxidant response element (ARE) [30–32]. The *cnc* gene encodes 16 different polypeptides, with evolutionarily conservation between CncC and vertebrate Nrf1 and Nrf2 [9,31]. CncC has been reported to share sequence similarity with the N-terminal region of vertebrate Nrf1 and another CNC family member, Nrf3 [9]. This region is known as the NHB1 (N-terminal homology box 1) domain, which sequesters Nrf1 and Nrf3 to the endoplasmic reticulum (ER), thereby inhibiting their nuclear translocation and transcriptional activity [35,38]. However, the functional significance of the NHB1 domain in CncC *in vivo* remains to be elucidated.

The *Drosophila melanogaster* larval fat body, which is composed of adipose tissue, liver and hemocytes, is an ideal system to study

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http://dx.doi.org/10.1016/j.bbrc.2015.05.126
0006-291X/

lipid metabolism [14]. Thus, we analyzed the physiological role of CncC in lipid metabolism as a *Drosophila* model for vertebrate Nrf1 and Nrf2. Here, we show a dramatic reduction of lipid storage in flies overexpressing a *CncCΔN* mutant that lacks the NHB1 domain of CncC. This result clearly documents two points: First, the NHB1 domain represses CncC function *in vivo*, just as it does in vertebrate Nrf1. Second, CncC plays roles in lipid metabolism in *Drosophila*. We found that *CncCΔN* overexpression in the fat body induces the expression of genes related to Immune-induced molecules (IMs), antimicrobial peptides (AMPs), and tumor necrosis factor (Eiger). These results might suggest that CncC regulates lipid homeostasis by regulating the immune system. Collectively, these *in vivo* genetic analyses provide a powerful tool to decipher the regulatory function of CncC, as well as other Nrf1/2 orthologs, in controlling lipid homeostasis.

2. Materials and methods

2.1. Fly husbandry and lines

The following fly lines were used in this study: Oregon-R-modENCODE (Bloomington #25211) as a wild-type, *Sep-Gal4*, *gstD1-GFP* (a reporter line that was a gift from Dirk Bohmann), *Cg-Gal4* (Bloomington #7011), *UAS-CncC FL* (full length), and *UAS-CncCΔN* (N-terminal truncated). Both *UAS-CncC* lines were kind gifts from Dirk Bohmann. We used the *Dfd-GMR-YFP* balancer line to select for the correct genotype. All fly lines were maintained at 25 °C on commercially available Formula 4–24 food (Instant Drosophila medium, Carolina Biological Supply Company, Burlington, North Carolina 27215).

2.2. Staining of larval fat body and quantification of triglycerides

Whole fat bodies from wandering third instar larvae were dissected in PBS on a Sylgard plate [3] and fixed in 4% paraformaldehyde for 20 min at room temperature (RT). The tissues were then rinsed with 1 × PBS (3 min each) and incubated for 20 min in 0.5 mg/ml Nile red diluted 1:2500 in PBS. After five washes in PBS (5 min each), the tissues were mounted in 80% glycerol. For DAPI staining, tissues were incubated in 1 μM DAPI for 5 min and washed with PBS before mounting on slides. Lipid droplets were visualized on a Zeiss confocal microscope using 568-nm-wavelength and DAPI filters. For quantification of total triglycerides, the fat body from a single larva was homogenized in 50 μl PBS with 0.05% Tween 20, followed by immediate heat inactivation at 70 °C for 5 min. Triglyceride levels were measured

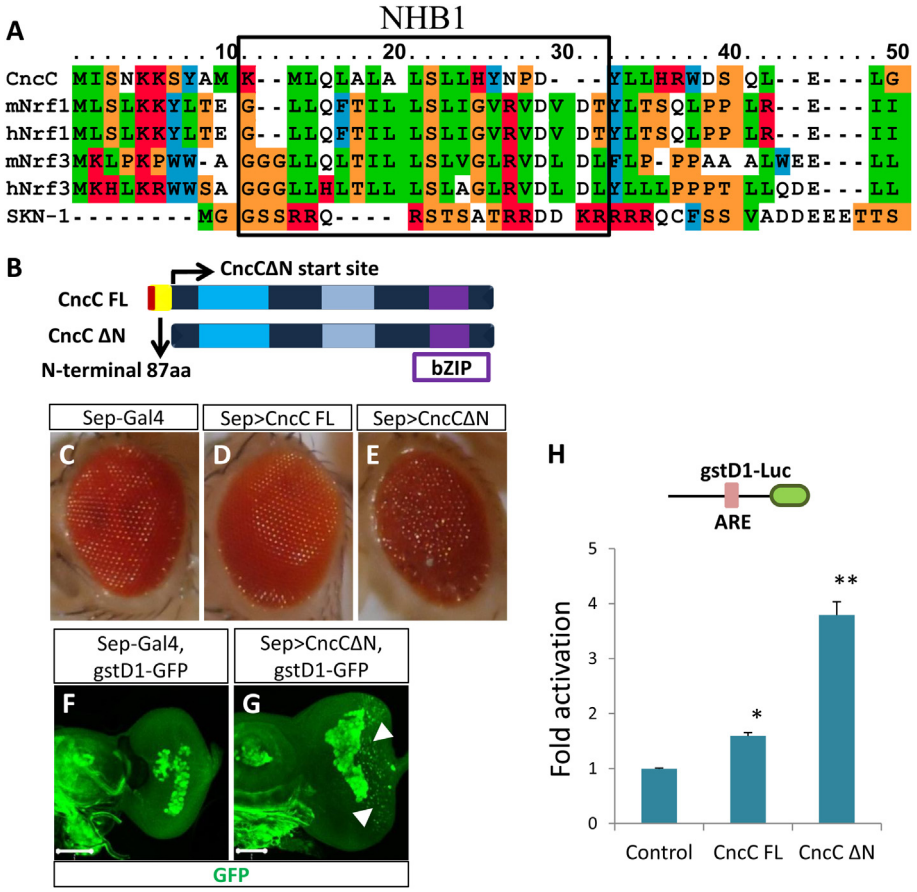

Fig. 1. Deleting the NHB1 domain of CncC triggers its transcriptional activity. (A) Species conservation of the N-terminal NHB1 domain among CNC-family proteins. The residues that share similarities are placed on colored backgrounds. (B) Schematic structures of full-length CncC (CncC FL) and the CncC mutant lacking the N-terminal region (87 aa) that includes the NHB1 domain (CncCΔN). (C–E) Overexpression of *UAS-CncCΔN* in fly eyes under the control of a *Sep-GAL4* driver results in a rough-eye phenotype (E) compared with the control, *Sep-Gal4* (C) and forced expression of *CncC FL* (D). (F, G) Eye imaginal discs of wandering 3rd instar larvae from the *gstD1-GFP* reporter transgene genetic background stained with anti-GFP antibody. Arrowheads show the *CncCΔN*-mediated induction of *gstD1* expression (G). Fluorescence observed in both flies is background. The scale bar represents 50 μm. (H) Validation of *gstD1* upregulation by *CncCΔN* in *Drosophila* Schneider 2 (S2) cells using a *gstD1-Luc* reporter. Error bars show standard deviation (n = 4). $p < 0.05$, paired Student's t-test. * = control vs *CncC FL*, ** = *CncC FL* vs *CncCΔN*.

using a LabAssay™ Triglyceride kit (Wako) according to the manufacturer's protocol. The absorbance of the sample at 595 nm was determined on a microplate reader v max (Molecular devices).

2.3. Immunostaining of eye discs

Eye discs from third instar larvae were fixed and stained as per standard immunohistochemical procedures [1]. A rabbit anti-GFP antibody (Invitrogen) at a 1:100 dilution was used as the primary antibody. FITC-labeled secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a 1:300 dilution. Immunofluorescence was visualized on a Zeiss confocal microscope.

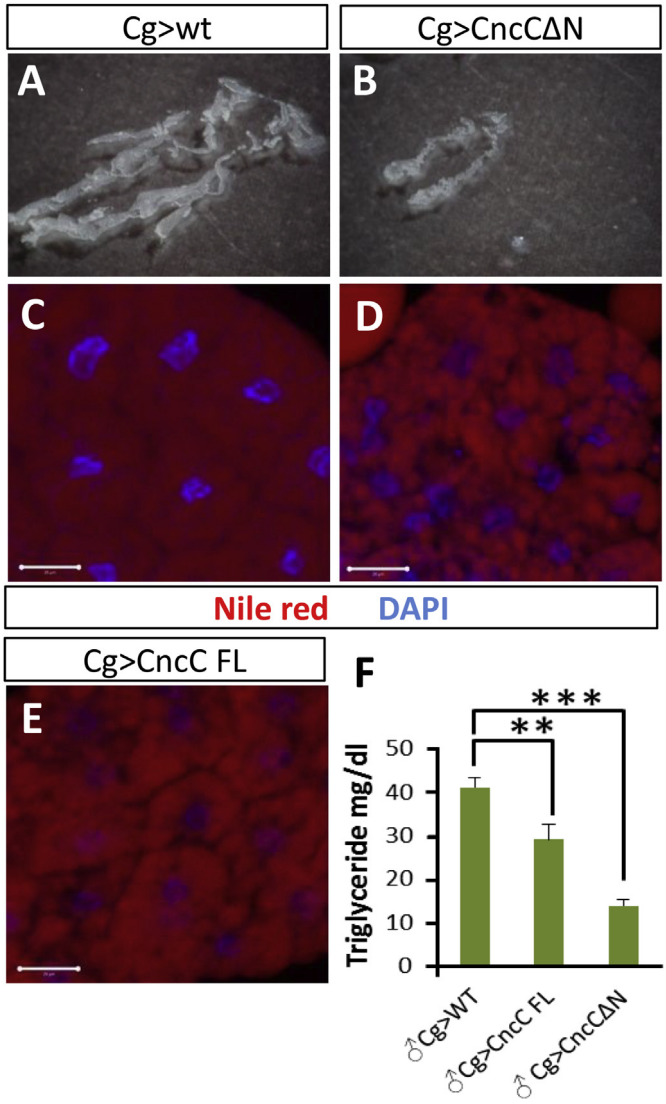

Fig. 2. Overexpression of *CncCΔN* in the fat body disrupts cell morphology and reduces triglyceride (TG) content. (A, B) A significant reduction of the entire fat body structure by forced expression of *CncCΔN* (B) compared with control (A). (C, D, and E) Staining of fat body structure with Nile Red (red) and DAPI to detect neutral lipid droplets and DNA, respectively, within cells. Overexpression of *CncCΔN* (D) causes much more severe disruption of fat body cell morphology than *CncC FL* (E) compared with the control (C). The scale bar represents 50 μm. (F) Quantification of total fat body triglyceride (TG) content shows a significant reduction of TG in *CncCΔN*-overexpressing flies (*Cg > CncCΔN*). Error bars show standard deviation (n = 4). $^{**}p < 0.005$, $^{***}p < 0.001$, paired Student's t-test.

2.4. Female vs male progeny survival ratio

The survival rate of female *vs* male *UAS-CncCΔN*-overexpressing adult progeny was determined by comparing the number of progeny collected for 8 days. The cross was set up at 25 °C, with 5 males and 5 females in a standard vial, and the adults were transferred to a new vial 5 days after the cross.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from the fat bodies of 5–8 larvae using the QIAGEN RNeasy Mini Kit. Oligo-dT primers and M-MLV reverse transcriptase (Invitrogen) were used to synthesize the complementary DNA. Real-time PCR was performed using FastStart Universal SYBR Green Master (Roche) and analyzed on a Thermal Cycler Dice Real Time System II (Takara). The results are shown as the average ± standard error of at least three independent samples, and gene expression levels were normalized to *GAPDH* mRNA. The following primers were used for qRT-PCR: *CncC*, 5′-GAGGTGGAAATCGGAGATGA-3′ and 5′-CTGCTTGTAGAGCACCTCAGC-3′; *IM14*, 5′-TGTCTGAAGATCTGCGGCTT-3′ and 5′-ACTGCGAGCGATCAGTTTGA-3′; *Drs*, 5′-CTGGGACAACGAGACCTGTC-3′ and 5′-ATCCTTCGCACCAGCACTTC-3′; *Eiger*, 5′-AGCTGATCCCCCTGGTTTTG-3′ and 5′-GCCAGATCGTTAGTGCGAGA-3′.

2.6. Cell culture and gstd1 luciferase reporter assay

Drosophila Schneider 2 (S2) cells were cultured in Schneider's medium (Life technologies), supplemented with 10% fetal bovine serum (FBS) (Nichirei), 40 μg/ml streptomycin, and 40 units/ml penicillin (Life technologies) in a humidified incubator at 27 °C. cDNAs encoding full-length CncC and the N-terminal CncC deletion mutant were cloned into pMT/V5-HisA (Invitrogen). For the reporter assay, the *glutathione S-transferase D* gene regulatory region was subcloned into the pGL3-mini luc vector (kindly provided by Dr. Alena Krejici) [2] using the following primers: Forward 5′-GGGGTACCCTCTTCGTCGTATCCATTCTC-3′, Reverse 5′-GAAGATCTG ATTAATACACTGTTGTGTGGCCATGG-3′. The effector plasmid was transfected into S2 cells along with the gstD1 gene luciferase reporter using Lipofectamine 2000 according to the manufacturer's protocol. Induction of *CncC* gene expression by $CuSO_4$ was carried out using a previously reported method [2]. The luciferase activities were measured using the PicaGene luciferase assay system (Toyo Ink) and an ARVO plate reader (Perkin Elmer).

2.7. Microarray analysis

More than three independent RNA samples of the male 3rd instar larval fat bodies from each genotype were mixed and used for the microarray analyses. Microarray analysis using *D. melanogaster* (FruitFly) Oligo Microarray slides (Agilent Technologies, G2519F#21791) was carried out by Oncomics Co., Ltd. (Nagoya, Japan).

3. Results and discussion

3.1. Deletion of the NHB1 domain from CncC constitutively activates its transcriptional activity

Prior to overexpressing *CncC* in the fat body, we first examined whether the N-terminal NHB1 domain of CncC represses its transcriptional activity *in vivo*. This highly conserved domain represses Nrf1 function by sequestration to the ER membrane (Fig. 1A) [9,35,38]. We used two transgenic flies carrying full-length *UAS-CncC* (hereafter referred to as CncC FL) and *UAS-CncCΔN*, in which

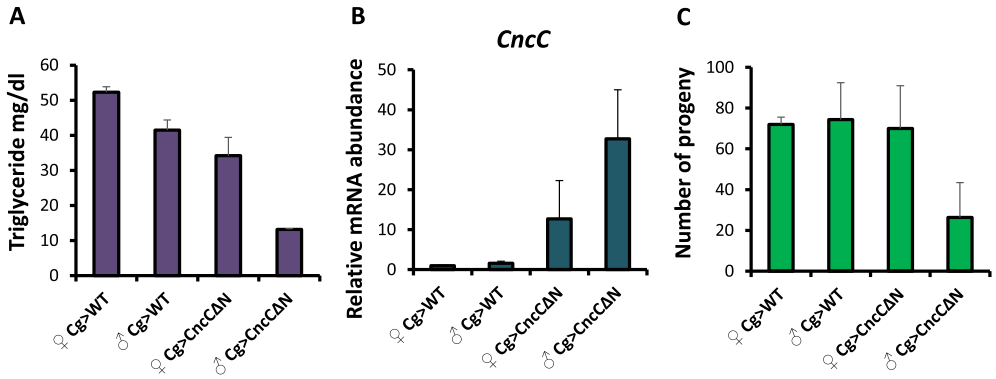

Fig. 3. Male *CncCΔN* flies show a severe reduction in lipid storage compared with female *CncCΔN* flies. (A) Triglyceride levels in female vs male *CncCΔN* flies. *CncCΔN* males have significantly lower triglyceride levels. (B) Female vs male *CncCΔN* transgene expression levels determined by qRT-PCR. (C) Survival rates of female vs male *CncCΔN* flies. Adult progeny were counted for 8 days after they started to hatch. The error bars represent standard deviation.

the 87-amino-acid N-terminal region containing the NHB1 domain is deleted (Fig. 1B). *CncC FL* and *CncCΔN* were expressed in adult eyes using *Sep-Gal4*, an eye-specific driver. As expected, the *CncCΔN* mutant dramatically induced rough eye-phenotype compared with *CncC FL*-expressing and control flies (*Sep-Gal4*) (Fig. 1C–E). These results indicate the repressive function of the NHB1 domain in CncC *in vivo*. We next investigated whether CncCΔN induces the expression of CncC target genes in flies. To this end, we used transgenic reporter flies containing a GFP gene fused to the regulatory region of the *gstd1* gene, which is a CncC target, and monitored the CncCΔN-mediated induction of *gstD1* gene expression via GFP expression in the larval eye disc. Indeed, a clear upregulation of *gstD1-GFP* reporter expression was observed upon *CncCΔN* overexpression compared with the control (Fig. 1F, G, arrowheads). Furthermore, we examined CncCΔN transcriptional activity through luciferase reporter analysis using the *gstD1 gene-luciferase* reporter (Fig. 1H). Transfection of the CncCΔN expression vector into *Drosophila* S2 cells strongly augmented luciferase reporter expression. Taken together, these findings clearly suggest that the NHB1 domain serves an important repressive function in CncC *in vivo*. Thus, we decided to decipher the physiological function of CncCΔN during lipid metabolism in the fat body.

3.2. Overexpression of CncCΔN reduces lipid storage in the fat body

We investigated the effect of *CncCΔN* on the fat body as a lipid reservoir by using the fat body-specific driver *Cg-Gal4*, which is active throughout larval development (Fig. 2, *Cg > CncCΔN*). As controls, we also analyzed a *Cg-Gal4/+* line (*Cg > WT*) and a *CncC FL*-expressing line (*Cg > CncC FL*). The morphology of the fat body was analyzed by staining with the lipophilic dye Nile Red. *CncCΔN* overexpression disrupted fat body cell morphology and lowered the cell number compared with control flies (Fig. 2A–D). In addition, we observed a severe disturbance of lipid droplet size and number in the *CncCΔN* larval fat body, which corresponds to lower lipid storage compared with control *Cg > wt* flies (Fig. 2D). *CncC FL* also showed a disrupted lipid droplet phenotype that was comparatively less severe than *CncCΔN* (Fig. 2E). However, this effect is more pronounced in *CncCΔN* than in *CncC FL*, indicating that the NHB1 domain also represses CncC function in the fat body (Fig. 2D, E). We measured total triglyceride (TG) levels in the fat bodies of each line (Fig. 2F). TG levels in the *CncCΔN* line were significantly reduced compared with *Cg > CncC FL* and *Cg-Gal4/+* control animals. Taken together, our observations indicate that *CncCΔN* overexpression leads to a developmental defect in larval fat body formation and severely reduced lipid storage levels.

Intriguingly, we found that the TG levels of *CncCΔN* males were significantly reduced compared with *CncCΔN* females (Fig. 3A). We speculated that this effect may be due to differences in *CncCΔN* transgene expression levels. Indeed, quantitative RT-PCR (qRT-PCR) analysis indicated that *CncCΔN* transgene expression is more pronounced in males compared with their female counterpart (Fig. 3B). Moreover, by quantifying adult progeny, we observed that *CncCΔN* males showed a significantly lower survival rate compared with their female counterparts (Fig. 3C). No such differences were observed between *Cg > WT* and/or *Cg > CncC FL* male and female

Table 1
Gene expression induced by CncCΔN overexpression in the male fat body.

Gene ID	Gene symbol	Probe	Gene name	Function	Fold change
FBgn0040653	IM4	A_09_P182905	Immune induced molecule 4	Defense response	6912.2
FBgn0025583	IM2	A_09_P045161	Immune induced molecule 2	Defense response	6547.1
FBgn0067905	IM14	A_09_P225510	Immune induced molecule 14	Defense response	1156.9
FBgn0034328	IM23	A_09_P009076	Immune induced molecule 23	Defense response	697.4
FBgn0040736	IM3	A_09_P063476	Immune induced molecule 3	Defense response	462.4
FBgn0034329	IM1	A_09_P009081	Immune induced molecule 1	Defense response	336.9
FBgn0034511	CG13422	A_09_P025711	Dmel CG13422	Defense response	263.7
FBgn0063667	CG32335	A_09_P002011	Dmel CG32335	Ion binding	172.0
FBgn0013772	Cyp6a8	A_09_P029911	Cytochrome P450-6a8	Secondary metabolites biosynthesis, transport, and catabolism	167.1
FBgn0010385	Def	A_09_P114595	Defensin	Antimicrobial	153.9
FBgn0033835	IM10	A_09_P007281	Immune induced molecule 10	Defense response	116.3
FBgn0039911	CG1909	A_09_P061886	Dmel CG1909	Cell–cell signaling	104.5
FBgn0010381	Drs	A_09_P012336	Drosomycin	Antimicrobial	76.2
FBgn0052274	Drsl1	A_09_P015151	Drosomycin-like	Defense response	76.0
FBgn0004431	LysX	A_09_P010571	Lysozyme X	Antimicrobial	71.5

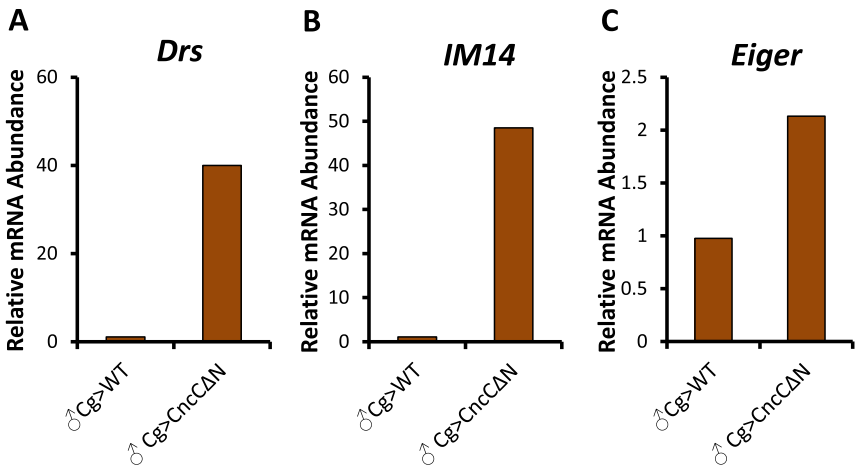

Fig. 4. *CncCΔN* induces the expression of genes involved in the innate immune response. mRNA expression of *Drosomycin* (*Drs*), an antimicrobial peptide (A), an Immune-induced molecule *IM14* (B), and tumor necrosis factor *Eiger* (C) in male *CncCΔN* 3rd instar larval fat bodies were determined by qRT-PCR analysis. Data shown are representative of three independent experiments.

progeny (data not shown). Preliminary, we found that *CncCΔN* male larvae could not pass their 3rd instar stage and that larvae died at the end of the 3rd instar stage (data not shown). Together, these data clearly indicate that CncC regulates lipid storage in the fat body.

3.3. *CncCΔN induces gene expression related to innate immunity in the fat body*

To identify genes whose expression correlates with *CncCΔN*-mediated lipid disruption, we initially compared the gene expression profiles between 3rd instar larval fat body samples from two males overexpressing either *CncCΔN* or *Cg-Gal4/+* (control) using *Drosophila* microarrays. There were no significant alterations in gene expression related to lipid synthesis and lipolysis (data not shown). However, we found that a number of genes are highly expressed in the *CncCΔN* fat body, particularly immune-associated genes such as antimicrobial peptides (AMPs) and immune-induced molecules (IMs) (Table 1). Both AMPs and IMs play an essential role in activating the innate immune system in *Drosophila* by exhibiting a wide range of activities against bacteria, viruses and/or fungi [7,8,18,34]. We also observed the upregulation of *Eiger*, the sole fly ortholog of Tumor Necrosis Factor (TNF), which induces programmed cell death through the activation of JNK and/or caspases in *Drosophila* [16,17,23,36]. To confirm these observations in different samples, we performed qRT-PCR analysis to quantitate the changes in mRNA expression levels of AMPs such as *Drosomycin* (*Drs*), the Immune-induced molecule *IM14*, and Tumor necrosis factor *Eiger*. The expression levels of these genes were normalized to GAPDH mRNA levels. CncCΔN overexpression in the fat body caused a notable increase in the expression levels of *Drs*, *IM14*, and *Eiger* (Fig. 4A–C). Thus, these data indicate that CncC is involved in activating the innate immune system.

The immune system has been shown to maintain lipid homeostasis by transporting endogenous lipids to a cell-surface protein called CD1, which can then regulate NK T cells, the key regulator of the immune system [4]. Disruption of this process can cause autoimmune diseases such as diabetes, as well as cancer. In this study, we found that the activating stress-responsive gene CncC causes the upregulation of genes relate to innate immunity and reduction of lipid storage, although how these processes are linked molecularly is unknown. Previous studies have shown that the activation of Eiger elicits the upregulation of the Toll pathway in adipocytes, which can lead to cell death [23]. JNK, the known downstream effector of Eiger, regulates lipid homeostasis in *Drosophila* [19]. Investigating the association between Eiger-induced JNK signaling and lipid storage could be of further interest to identify the possible molecular link between the immune system and lipid homeostasis.

The present study reveals a conserved inhibitory function of the NHB1 domain between *Drosophila* CncC and vertebrate Nrf1 and Nrf3. Accumulating evidence suggests that Nrf1 is sequestered at the ER through the NHB1 domain and that upon exposure to certain stresses/signals, Nrf1 is released from the ER via proteolysis at the N-terminal end [25]. Furthermore, it has been reported that the proteasome cleaves Nrf1 [27], though this effect remains to be confirmed *in vivo*. In this regard, our *Drosophila* genetic study system serves as a powerful tool to investigate the molecular basis of not only CncC but also Nrfs that could not be identified previously using conventional biochemical and molecular biological analyses.

Acknowledgments

We would like to thank Dr. Dirk Bohmann and Masayuki Miura for flies that have been used in this study. This research was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (26860198, MRK), Grants-in-Aid for scientific research (26116725, AK) and the Strategic Research Foundation at Private Universities (2012–2016) (S1201009, AK) from the Ministry of Education, Sports, Science and Technology.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.126.

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