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Ecdysone receptor (EcR) suppresses lipid accumulation in the *Drosophila* fat body via transcription control

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ABSTRACT

Lipid metabolism drastically changes in response to the environmental factors in metazoans. Lipid is accumulated at the food rich condition, while mobilized in adipocyte tissue in starvation. Such lipid mobilization is also evident during the pupation of the insects. Pupation is induced by metamorphosis hormone, ecdysone via ecdysone receptor (EcR) with lipid mobilization, however, the molecular link of the EcR-mediated signal to the lipid mobilization remains elusive. To address this issue, EcR was genetically knocked-down selectively in 3rd instar larva fat body of *Drosophila*, corresponding to the adipocyte tissues in mammalians, that contains adipocyte-like cells. In this mutant, lipid accumulation was increased in the fat body. Lipid accumulation was also increased when knocked-down of taiman, which served as the EcR co-activator. Two lipid metabolism regulatory factor, E75B and adipose (adp) as well as cell growth factor, dMyc, were found as EcR target genes in the adipocyte-like cells, and consistently knock-down of these EcR target genes brought phenotypes in lipid accumulation supporting EcR function. These findings suggest that EcR-mediated ecdysone signal is significant in lipid metabolism in insects.

1. Introduction

Adipose tissue is an organ to deposit lipids as energy source to meet demands under shortage of food intake in metazoans. In nutrient-enriched condition, lipids are accumulated in adipocytes, and lipid accumulation is under metabolic regulators by many factors like hormones, nutrients and environmental conditions [1]. Such regulatory systems appear well conserved in metazoans [2,3], and insulin signal cascade is representative of conservation of metabolic control since the insulin signaling factors functionally resemble from *Caenorhabditis elegans* to human [4]. Energy mobilization from adipose tissue is stimulated by nutrient conditions like starvation in mammalians, but similar metabolic is seen in insect at pupal stage. In larval stages, insect intakes plenty of foods and deposits excess energy as lipid in fat body of adipocyte-like cells, but ceases food intake at pupal stage. As pupation requires energy, preserved lipids are evident to be catabolized. However, the molecular basis and its regulators are largely unknown. In this respect, ecdysone is presumed as a pivotal hormone to stimulate lipid mobilization, because this hormone is best established to trigger dynamic metamorphosis like pupation and eclosion [5]. The ecdysone target genes like E74 and E75B are considered to mediate the wide variety of ecdysone actions in many organs at distinct developing stages.

Most of the ecdysone actions mediate its nuclear receptor, ecdysone receptor (EcR) [6]. EcR is a ligand-inducible transcription regulator and belongs to the nuclear receptor gene superfamily. Activated EcR by ecdysone binding controls expression of a set of target genes at transcriptional level, and such ligand-dependent transcriptional control requires a number of transcriptional co-regulators. Recent studies of co-regulators for mammalian nuclear receptors (NRs) have uncovered that histone modifying enzymes and chromatin remodelers transcriptionally co-regulates NRs [7]. Similar to the mammalian system, insect NRs are known to require histone modifying enzymes like taiman [8] and chromatin remodeler such as Nurf complex [9,10]. The present study was undertaken to ask if lipid metabolism in the fat body of *Drosophila* is under control by EcR. Selective knock-down of EcR in adipose-like tissues at 3rd instar larvae resulted in lipid accumulation in fat body without cell number increase. Likewise, knock-down assays of EcR co-activators and target genes have shown that lipid accumulation is expectedly modulated. Thus, these findings suggest that lipid mobilization in fat body is under positive control by EcR-mediate ecdysone signal.

Abbreviations: EcR, ecdysone receptor; IR, Inverted repeat; 20-HE, 20-hydroxyecdysone; adp, adipose; NR, nuclear receptor.

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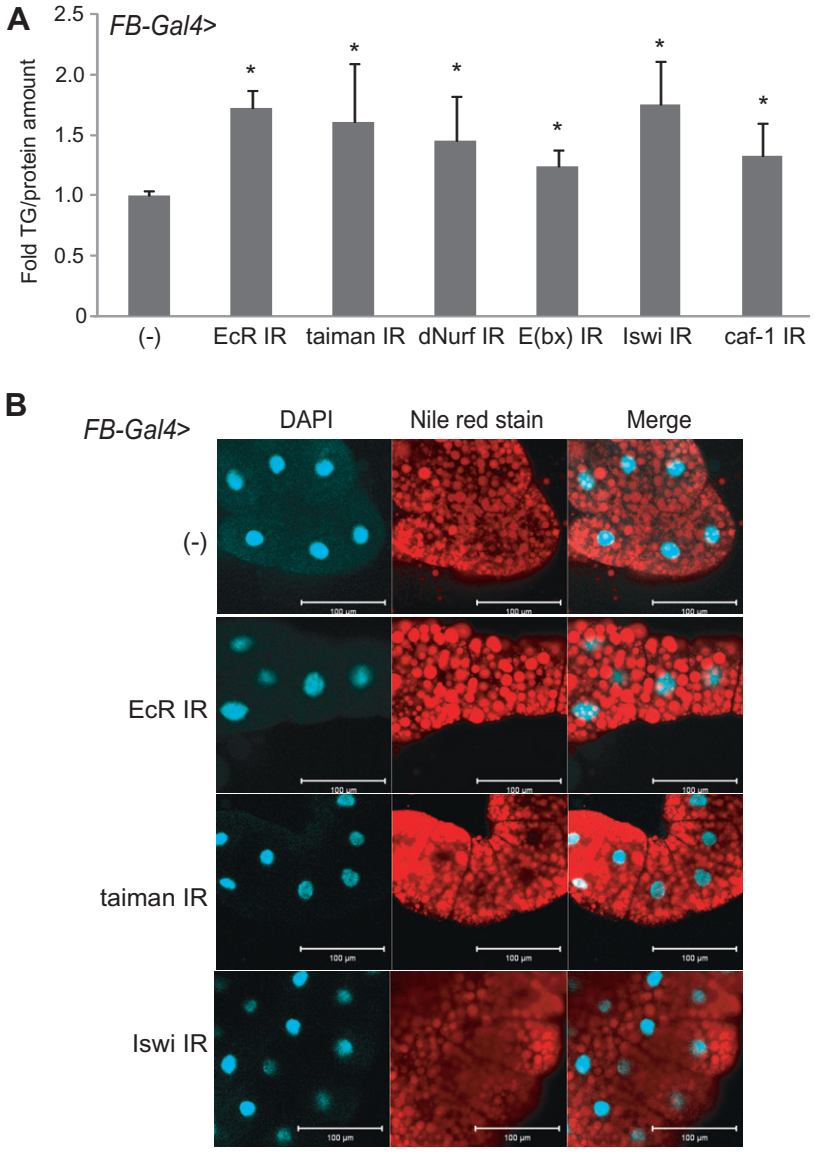

Fig. 1. Activated EcR was inhibitory for lipid accumulation in fat body of *Drosophila*. (A) Quantitative determination of TG amounts in EcR knock-down (*FB-Gal4 > UAS-EcR IR*), EcR co-regulators knock-down (*FB-Gal4 > UAS-taiman IR*, *FB-Gal4 > UAS-dNurf IR*, *FB-Gal4 > UAS-E(bx) IR*, *FB-Gal4 > UAS-Iswi IR* and *FB-Gal4 > UAS-caf-1 IR*) or control 3rd instar larvae. Total TG amounts of each 3rd instar larvae were quantified by TG test-E Wako and normalized with total protein amounts. TG amounts were normalized to control sample. Results are given as means ± SD of at least three independent experiments. $^{*}p < 0.05$. (B) Nile red staining of fat bodied of EcR knock-down (*FB-Gal4 > UAS-EcR IR*), EcR co-regulators knock-down (*FB-Gal4 > UAS-taiman IR* and *FB-Gal4 > UAS-Iswi IR*) or control 3rd instar larvae. DAPI was used as a nuclear marker. Bars, 100 μm.

2. Material and methods

2.1. Fly stocks

We used yw^{67C} as a wild type line. *FB-Gal4* line was provided from Dr. Kühnlein. *UAS-taiman IR* (Inverted repeat) expression line was provided from Dr. Ueda. *UAS-EcR IR* expression line was obtained from Bloomington *Drosophila* Stock Center. Other *UAS IR* expression lines were obtained from the Vienna *Drosophila* RNAi Center. For analysis of knock-down target genes in fat body, *FB-Gal4* females were crossed to male *UAS-IR* lines. All flies were cultured and crossed on cornmeal–yeast–agar medium at 25 °C. In all experiments, we used progeny of *FB-Gal4* line crossed with yw^{67C} as a control.

2.2. Metabolic assay

Metabolic assay was performed as described at [11] with a little modification.

For TG (Triglyceride) assays, 20 whole 3rd instar larvae were homogenized in 100 μl PBS (1.37 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 0.5% Tween 20 and immediately incubated at 70 °C for 5 min.10 μL of heat-treated homogenates were incubated with Triglyceride test-E Wako (Wako) for 5 min at 37 °C, after which the samples were centrifuged at maximum speed for 5 min. Then, samples were assayed using a Gene Quant 100 (GE healthcare) at 600 nm. TG levels were normalized to total protein amounts in each homogenates using a BCA Protein assay kit (Thermo scientific), and analyzed using a Student's *t* test.

For Nile red stain, 3rd instar larvae were dissected in PBS and fat bodies were stained with 0.00005% Nile Red (SIGMA) in PBS for 10 min. After incubation, samples were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Slides were imaged with Zeiss 510 laser confocal microscope.

2.3. RNA Isolation and real time RT-PCR

Third instar larvae were dissected in PBS and fat bodies were cultured for 3 h with or without 0.25 μM Muristerone A (MurA)

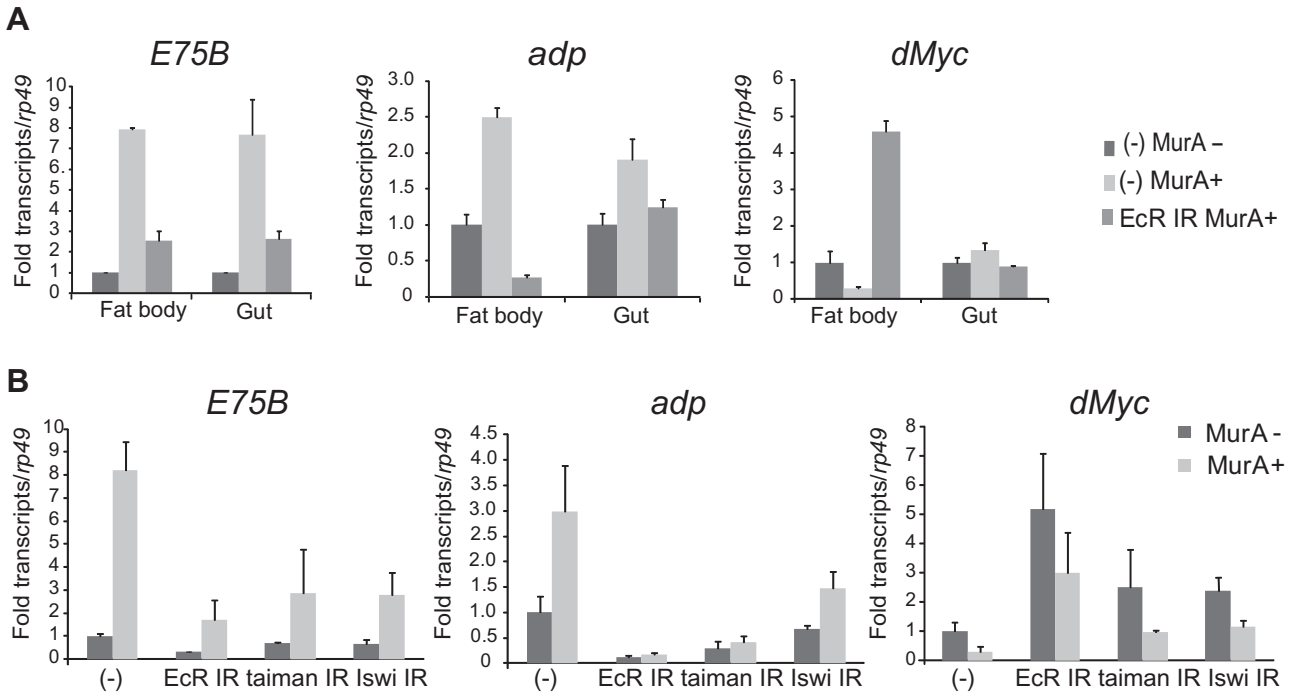

Fig. 2. E75B, adp and dMyc are EcR target genes in fat body. (A) Quantitative RT-PCR of E75B, adp and dMyc mRNA in EcR knock-down (*FB-Gal4 > UAS-EcR IR*) or control 3rd instar larvae fat bodies or guts. Total RNA was extracted from fat bodies and gut harvested from each knocked-down 3rd instar larvae and cultured with or without 0.25 μM MurA, a synthetic EcR ligand for 3 h. (B) Quantitative RT-PCR of E75B, adp and dMyc mRNA in EcR knock-down (*FB-Gal4 > UAS-EcR IR*), EcR co-regulators knock-down (*FB-Gal4 > UAS-taiman IR* and *FB-Gal4 > UAS-Iswi IR*) or control 3rd instar larvae fat bodies. Total RNA was extracted from fat bodies harvested from each knocked-down 3rd instar larvae and cultured with or without 0.25 μM MurA, a synthetic EcR ligand for 3 h. Each mRNA levels were normalized with *rp49* expression levels. mRNA expression levels were normalized to control sample without MurA. Results are given as means ± SD of at least three independent experiments.

(Wako) in Schneider medium (GIBCO). Total RNA was isolated with using Isogen (Nippon gene). Reverse transcription (RT) was performed with using Prime script RT Master mix (Takara). cDNA was quantified by Real time RT-PCR using KAPA SYBR Fast qPCR kit (KAPA BIOSYSTEMS) and Thermal Cycler TP800 (Takara). All experiments were performed according to the manufacturer's instruction. The sequences of primers are as followed: *rp49* (f) 5'-atgaccatccgcccagcatac-3′ (r) 5′-ctgcatgagcaggacctccag-3′, E75B (f) 5′-gcagcagcagatcggaatactc-3′ (r) 5'-ccgactcaatgcccgaatcc-3′, adp (f) 5′-atcaacgacccgaacgagac-3′ (r) 5′-tctagctgcaggatgcaacc-3′, dMyc (f) 5′-gagcaacaacaggccatcgatatag-3′ (r) 5′-ccttcagactggatcgtttgcg-3′.

3. Result

3.1. Activated EcR was inhibitory for lipid accumulation in fat body of Drosophila

To address if EcR is a regulatory factor in lipid metabolism in adipocyte-like cells of *Drosophila*, EcR was selectively knocked-down by a Gal4 driver system on the adipose-like tissue, fat body. By crossing a transgenic line (*UAS-EcR IR*) expressing EcR siRNA with a transgenic line (*FB-Gal4*) selectively expressing Gal4 driver by a CG1516 promoter [12] in the adipocyte-like cells, EcR gene was disrupted. In the adipose-like tissues of 3rd instar larva, more lipid accumulation was seen in the knocked-down mutant when triglyceride amounts were measured (Fig. 1A). To ask if this increased lipid accumulation is attributed to fat body number increase or more lipid accumulation in a single fat body, the adipose-like tissues were stained with Nile red to detect neutral lipids (Fig 1B). Though an increase in either adipocyte-like cell number or fat body size was not obvious, staining in single lipid droplet looked more visible and expanded, suggesting that EcR is suppressive in lipid accumulation in fat body.

We then similarly knocked-down the genes of known EcR co-activator (Taiman) [8] and chromatin remodeler [dNurf, E(bx), caf-1 and Iswi] [9,10] to ask if whether activated or inactivated EcR exert its repressive function. Like the mutant knocked-down EcR, all of the tested mutants displayed lipid accumulations, implying that transcriptional controls mediate the suppressive function of EcR (Fig. 1A and B). Presumably, EcR is activated by endogenous ligands in adipocyte-like cells.

3.2. Identification of EcR target genes in fat body

To address this issue further, transcriptional response to an EcR ligand was tested for known EcR target gene in the fat body. When an EcR synthetic ligand, MurA, was added for 3 h in an explant culture of the gut, the expected ligand response in mRNA induction was seen for E75B gene (Fig. 2A). Under the same experimental condition, E75B was inducible upon the ligand stimulation also in the adipose-like tissue, fat body (Fig. 2A), establishing that adipocyte-like cells are target cell of EcR. In this condition, we found that the gene expression of adipose (adp), reported to regulate lipid metabolism in mouse and *Drosophila* was induced by EcR ligand stimulation. However, ligand-induced repression of dMyc gene seen in the adipose-like tissue was not evident in the gut (Fig. 2A). When EcR was knocked-down, the ligand response was expectedly abolished (Fig. 2A). The adipose-like tissue may exhibit a tissue specific response to EcR ligand in gene expression.

Moreover, ligand responses of the EcR target genes were aborted also when either Taiman or Iswi was knocked-down (Fig. 2B).

3.3. The identified target genes mediated the suppressive function of EcR in the lipid accumulation

The roles of the EcR target genes in the adipose-like tissue were assessed by the selective knock-down approach in intact flies. Lipid accumulation was potentiated by knock-down of either E75B or adp, whereas dMyc knock-down brought decrease in lipid accumulation (Fig. 3A). Consistently, staining of lipid droplets was obvious in the mutants knocked-down of either E75B or adp (Fig. 3B). However, the number and size of adipocyte-like cells looked to be decreased in the dMyc-knocked-down mutant (Fig. 3B). All together, activated EcR is presumed to orchestrate expressions of a set of target genes, thereby exerting its suppressive function in lipid accumulation for fat body.

4. Discussion

The present study have shown that activated EcR-mediated ecdysone signal is suppressive for lipid accumulation in fat body

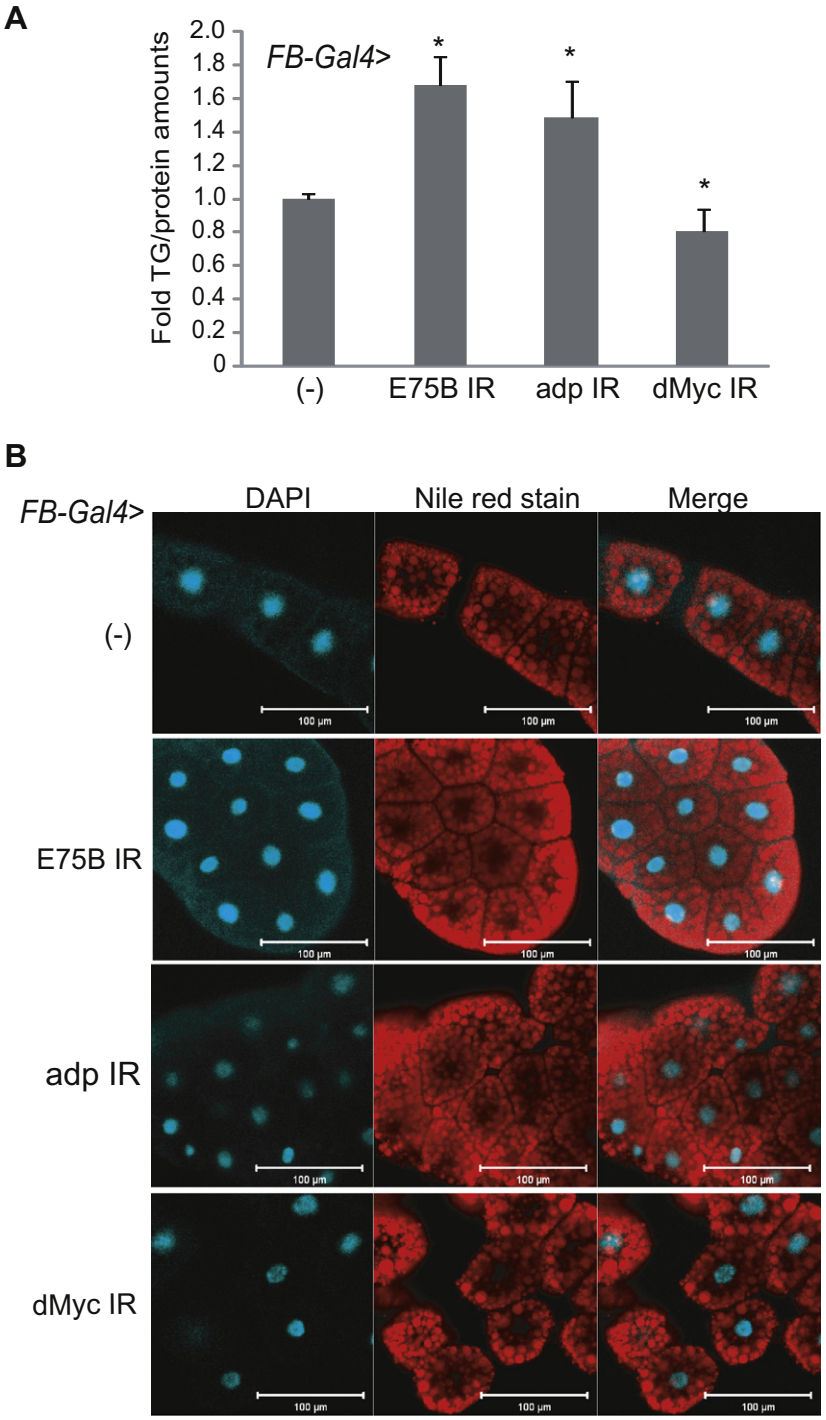

Fig. 3. The identified target genes mediated the suppressive function of EcR in the lipid accumulation. (A) Quantitative determination of TG amounts in EcR target gene knock-down (*FB-Gal4 > UAS-E75B IR*, *FB-Gal4 > UAS-adp IR* and *FB-Gal4 > UAS-dMyc IR*) or control 3rd instar larvae. Total TG amounts of each 3rd instar larvae were quantified by TG test-E Wako and normalized with total protein amounts. TG amounts were normalized to control sample. Results are given as means ± SD of at least three independent experiments. $^{*}p < 0.05$. (B) Nile red staining of fat bodied of EcR target gene knock-down (*FB-Gal4 > UAS-E75B IR*, *FB-Gal4 > UAS-adp IR* and *FB-Gal4 > UAS-dMyc IR*) or control 3rd instar larvae fat bodies. DAPI was used as a nuclear marker. Bars, 100 μm.

of *Drosophila*. As ecdysone is a pivotal hormone for metamorphosis in fly [6] as well as the other insects, lipid mobilization induced by ecdysone is indispensable for supplying enough energy to achieve metamorphosis. In this regard, the present findings are supportive for this idea, and are consistent with the previous report, in which activated EcR by 20-hydroxyecdysone (20-HE) induced feeding suppression and lipolysis through activating gene expression of lipase, *brummer* [13,14]. The observation that selective knock-down of EcR in fat body caused pupal lethality also supports our idea (Data not shown). Nevertheless, EcR target genes identified from *Drosophila*-derived cell line in the presence of an EcR ligand (20-HE) did not contain the genes related with lipid metabolism [15]. The applied cell line may not possess a regulatory system in lipid metabolism in response to ecdysone.

Three genes could be identified as EcR target genes in adipose-like tissue from the present study. Though these genes were shown to be responsive to ecdysone in gut, we could provide a genetic evidence that the ecdysone responses in the gene regulator mediate EcR in intact tissues. Moreover, by knocking-down of the genes of transcriptional co-regulators for EcR, we assume that activated EcR by ligand binding regulates the gene expression.

Physiologically, the function of identified three genes (*E75B, adp and dMyc*) look to reflect the ecdysone actions in lipid metabolism in fat body. E75B is also a nuclear receptor, and thereby regulates expression of its target genes [5]. As one of the E75B target genes encode NO synthesis (NOS) and NOS in prothoracic gland was suppressive for lipid accumulation in fat body [16], it is likely that E75B works as a suppressor for lipid accumulation in a variety of tissues/cells. Similarly, adp appears suppressive for lipid accumulation. In adp null mutant fly, enhanced lipid accumulation was reported [17]. In mice, the adp homolog acted as a repressor for PPARγ, leading attenuating PPARγ-mediated lipid synthesis through HDAC recruitment [18]. Though it is unclear at this stage if adp function is conserved across species, adp might co-repress nuclear receptors for suppression of lipid accumulation in *Drosophila*. Different from the function of E75B and adp in lipid metabolism, dMyc appeared to stimulate proliferation of adipocyte-like cells in fly, in consistent with its known potency in cell proliferation [19]. Since activated EcR repressed the dMyc expression, activated EcR is presumed to indirectly repress proliferation of adipocyte-like cells. Thus, from the observed regulators of the three target genes by activated EcR, activated EcR is likely to act as a master regulator to stimulate lipid mobilization, at least in adipose-like tissue of *Drosophila* in pupation.

Nuclear receptors are considered to require a number of co-regulators directing dynamic chromatin reorganization to generate proper promoter environment for efficient transcriptional controls [7]. In facts, many regulators associated with chromatin reorganization have emerged as NR co-regulators, however, it appears that still numerous factors remain to be identified. In this respect, genetic screening of NR co-regulators using *Drosophila* like previous studies [20–22] must be valuable, because this approach based on functional assessment of candidates is potentially possible to overcome limitation of biochemical materials. It is also quite feasible that a tissue-specific NR co-regulator will be identified, as seen in the ecdysone response in the adipose-like tissue of *Drosophila*.

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