Ph.D Thesis

Drosophila as a genetic model for studying lipid storage droplet-associated genes

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Contents

Abbreviations

ABHD5	Abhydrolase domain-containing protein 5
ATGL	Adipocyte triglyceride lipase
ATP	Adenosine triphosphate
Bmm	Brummer
cAMP	Cyclic adenosine monophosphate
CM-H2DCFDA acetyl ester	5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate,
DAPI	4′, 6-diamidino-2-phenylindole
DG or DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DIAP1	Death-associated inhibitor of apoptosis 1
Dm	*Drosophila melanogaster*
DRSC	*Drosophila* Genomics Resource Center
FFA	Free fatty acid
Foxo	Forkhead box, subgroup O
GFP	Green fluorescent protein
HDAC	Histone deacetylase

IR	Inverted repeat
LD	Lipid droplet
LRO	Lysosome-related organelles
Lsd1	Lipid storage droplet 1
Lsd2	Lipid storage droplet 2
NCBI	The National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PBST	Triton X-100 in phosphate-buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SPV	Sulfo-phospho-vanillin
TG	Triacylglycerol
TOR	Target of rapamycin
UAS	Upstream Activation Sequence
VDRC	Vienna Drosophila Rnai Center

General introduction

Drosophila melanogaster is a flexible genetic model organism

Drosophila belonging to the family *Drosophilidae* is well known as the genus of small fruit flies. Particularly, *Drosophila melanogaster* is a primary model organism in modern biology and has been commonly used for research in genetics [1]. Small in size, easy growing in the traditional laboratory, high productivity, and a ten-day generation are obvious advantages seen in *Drosophila*. Additionally, there are four homologous pairs of *Drosophila* chromosomes: three pairs of autosomes carry the bulk of the genome in which salivary gland chromosomes are giant and easily observed, and one pair of sex chromosomes. Basic development processes are conserved well from flies to vertebrates. Especially, Man database from the Online Mendelian Inheritance shows human disease genes, including neurological disorders, cancer, developmental and metabolic disorders, and *Drosophila* gene have related 75% in sequence [2-4]. *Drosophila* web resources, such as Flybase, Berkeley *Drosophila* Genome Project, Genome annotation database for *Drosophila*, NCBI *Drosophila* genome resources, Database of human disease gene homologs in *Drosophila* and many *Drosophila* stocks kept in Bloomington (Indiana, USA), *Drosophila* genetic Resource Center (Kyoto, Japan), and Vienna Drosophila RNAi Center, are enormously available for performing studies on *Drosophila*. Moreover, many developed methods have been established in *Drosophila* studies.

Since biomedical fields using humans and higher animals in experiments have been met obstacles of ethics and application, mouse, zebrafish, *Drosophila*, and silkworm, ect, have been ideal model organisms used to discover the mysteries of cells and tissues.

From the beginning up to now many studies of the diversity of biology patterns, such as genetics and inheritance, embryonic development, learning, behavior, and aging, using *Drosophila* as a model organism have been done remarkably. Even though humans and flies are extremely different in look, a huge number of fundamentals of biological mechanisms and pathways to regulate development and survival are discovered through evolution between these species [5].

Thomas Hunt Morgan is definite the father of biology of *Drosophila*, but the members of William Castle at Harvard brought *Drosophila* to the laboratory and marked it as the first record in use in 1901 [6]. Morgan used *Drosophila* to develop the inherited theory originated from Gregor Mendel. He defined genes locate within chromosomes while DNA was previously defined is the genetic material. Morgan was awarded The Nobel Prize for his outstanding works in Physiology or Medicine in 1933 *"for his discoveries concerning the role played by the chromosome in heredity*" [5]. Following Morgan, in 1946 his juniors received the Nobel Prize in the same field *"for the discovery of the production of mutations by means of x-ray irradiation"* [5]. In the 1920s a discovery found by Muller using *Drosophila* was X-rays could increase a wide rate of mutation in genes and destroy chromosomes [7]. The appearance of irradiated flies did not change, but there were effects of mutation in the offspring. This concludes the explosion of radiation caused by human is extremely harmful to genetic defects in the offspring [5].

In recent decades to discover the function of genes on the embryo development from a single cell to a multicellular organism, *Drosophila* has been chosen as a high priority

model. A Nobel Prize in Physiology or Medicine "*for their discoveries concerning the genetic control of early embryonic development"* was given to Christiane Nüsslein-Volhard, Eric Wieschaus, and Ed Lewis in 1995 [5]. Findings showed many genes play important roles in fly development and have been vitally related to the development in both animals and humans. Though fly and human are completely different in architecture, they have many structures and engineering processes remarkably similar. A genetic test using the *Drosophila* genome was processed by Craig Venter and colleagues to verify the practicality of the "shot-gun" sequencing strategy on the human genome in 1999 [5]. Based on the success of this approach, in March 2000, the first sequence of the *Drosophila* genome was been public, and then following the human genome after 11 months [8]. Anyone who is interested in the *Drosophila* genome sequence can freely access the Flybase homepage; this indicates *Drosophila* database is marvelous and universal [9]. The number of *Drosophila* genes is currently predicted around 14,000 [8] and each gene containing sequence and its gene product, mutation products, and related literature has an honor page on Flybase for practical applications [5].

Drosophila **as a useful model for medicinal invention**

In clinical field, *Drosophila* is an outstanding model used for targeting the drug discovery process [10,11]. Most importantly *Drosophila* can be used to conduct how new drugs act on the biochemical pathways preserved within humans that regulate a variety of key cellular activities for tissue regeneration such as cell division,

differentiation, and movement. The process of using *Drosophila* to test novel drugs is much more effective and faster than using mammalian models. They may be definitely used for the high-throughput screening process as an option to cell culture. The purpose of applying screening technique in a whole organism is to collect potential compounds for further researches in high mammalian models more safety. In addition, using *Drosophila* may be uncomplicated for testing drug efficiency in a diseased state since it is quiet easy to manage the genetic background for mimicked purpose [5].

Triacylglyceride synthesis pathway

Triacylglyceride (TG or TAG) is known as a primary type of neutral lipid which is composed by a heterogeneous group of molecules with a glycerol back bone and three fatty acids (FAs) bonded by ester bonds. The difference of FAs attached to glycerol would generate diversity in the physical and chemical properties of TAGs. TAGs participate in a variety of substantial functions in living organisms. One of them is a molecule reservoir of FAs for energy utilization, namely lipid droplets (LDs). Besides, they are responsible for the synthesis of membrane lipids. TAGs store more than six times the amount of energy compared to hydrated glycogen because of their high reduction and anhydrous compounds [12]. TAGs are normally necessary for physical processes, but the uncontrolled production of TAGs in human adipose tissue causes obesity and nonadipose tissues and is related to organ dysfunction. There are lots of examples demonstrated for this relationship, including the excessiveness of TAGs

deposition in skeletal muscle and in the liver associated with insulin resistance, in the liver with nonalcoholic steatohepatitis, and in the heart with cardiomyopathy [13,14].

TAG biosynthesis is under controlled by two major pathways, the glycerol phosphate, also called the Kennedy pathway [15], and the monoacylglycerol (MG) pathway [16-18] (Fig.1), that had been identified in the 1950s and 1960s. Fatty acyl-CoAs, known as the activated forms of FA synthesized by intracellular acyl-CoA synthases, are involved in both pathways as acyl donors [19]. The glycerol pathway can be found in most cells whereas the MG pathway is presented particularly in some cells, such as enterocytes, hepatocytes, and adipocytes in which the re-esterification of hydrolyzed TAG may be participated [20]. Moreover, the MG pathway is the dominant type of TAG synthetic process in humans who have small intestines, and the TAG synthesis is taken from components of hydrolyzed dietary fats in those small intestines [21].

To achieve the final products in both pathways, a fatty acyl-CoA is covalently combined with diacylglycerol (DG or DAG) molecule to form TAG which needs acyl-CoA: diacylglycerol acyltransferase enzymes (DGAT) present to catalyze. The endoplasmic reticulum (ER) is considered as the place where TAG biosynthesis generally takes place [22]. It has a belief that new TAGs are just synthesized releasing into the lipid bilayer where they are transferred into cytosolic LDs, or in cells that conceal TAGs, nascent lipoproteins. The fact is that the mechanism used to deposit TAGs into LDs is under investigated process [23].

Triacylglycerol mobilization and the function of *bmm* and *Lsd1*

Neutral lipids are the sources of energy stored in lipid droplets. They metabolize accessibly through lipolysis; for example, the hydrolytic cleavage of the three fatty acids is successively synthesized from the glycerol backbone of triacylglycerols [24]. Remarkably, DGs, the abundant lipid components of *Drosophila* lipoprotein particles, are the major sources supplying energy to peripheral organs and lipid building blocks through hemolymth, the fly body fluid [25]. Particularly, metabolizing process of *Drosophila* lipid makes a prediction that TGs hydrolyzation is partially supported by TG lipases to become DGs before they are loading into preformed lipoprotein particles, describing the distance related to the migratory locust [26]. Otherwise lipid transportation could be achieved based on a complete deacylation of TGs in the fat body that a DGs resynthesized process is following. Understanding insights into the entire lipolysis of *Drosophila* fat body cells is the priority that should be done before studying TG mobilization in the fly.

There are two different types of lipolysis presented in the fat storage tissue of the fly. Basal lipolysis takes the role to keep steady-state lipogenesis balance in storage lipid homeostasis. On the other hand, stimulated lipolysis affects TG mobilization excessively in negative energy balance periods or unpredictable stages of biosynthetic activity [24]. The brummer (BMM) lipase, a homolog of adipocyte triglyceride lipase (ATGL), is essential in both basal and stimulated lipolysis in *Drosophila* [27,28]. Lipid droplet associated TAG lipase brummer is encoded by *brummer* (*bmm*) gene. Any risk

associated to food deprivation or chronic *bmm* overexpression quickly causes organism fat store depletion *in vivo* while lack of BMM activity causes obesity in flies. These studies emphasize insect energy homeostasis system plays the central role in obesity control. Their evolution reveals human obesity involves brummer/ATGL family members and modifies obesity disease's modeling mechanistic and therapeutic aspects in fly [27]. Flies without BMM lipase activity show unchanged development; however, excessive body storage fat accumulation happens compared to control flies. On the other hand, fed flies bring transgene-dependent *bmm* overexpression in fat body cells showed a similar effect to starvation-induced upregulation of *bmm* transcription, depletes the TAG content of immature and mature adults accounted for 96% and 46%, respectively [27]. There is no effects found out over transgenic expression of the enzymatically inactive *bmm* mutant, and this demonstrates TAG mobilization is under controlled of the BMM lipase activity. *Bmm*-dependent differences of organismal TAG content are also reflected by the lipid storage phenotype of fat body cells. This induced in the difference of sized storage droplets in *bmm* mutant fat body cells, and their reduction in size and number upon overexpression of the gene showed. BMM specifically affects on the fat-based aspect of energy storage because *bmm* mutant or even *bmm* overexpressing flies show unaffected glycogen content [27]. *Bmm* transcription consisting with the critical dose-dependency of the gene is tightly under controlled by nutritional system. Consequently, direct activation via the Forkhead box, subgroup O (Foxo) transcription factor [29] is the aspect by which *bmm* expression shows its possible effect under starvation [27] in response to downregulation of the insulin signaling pathway in the fly

[30]. Likewise, the *target of rapamycin* (TOR) gene function [31] plays the role as a nutrient sensor in the fly [32] to reserve *bmm* transcription. Furthermore, the *bmm* gene is directed by antagonistic transcriptional control of stimulated lipolysis, one of the lipolysis pathways in *Drosophila* [28].

Fly perinilin 1, one of two members of the evolutionarily conserved protein family of lipid droplets [33], regulates storage lipid homeostasis in the fly [34-36]. *In vitro* studies provide evidence that perilipin 1/Lipid storage droplet 1 (Lsd1) is a PKA phosphorylation target [37], additionally, analyzing *in vivo* mutants verifies *Lsd1* essentially acts as prolipolytic effector of the AKH/AKHR pathway on the LDs surface [35]. PKA-dependent phosphorylation of perilipin and translocation of HSL to LDs are remarkable points of stimulated lipolysis in mammalian adipocytes, which emphasizes the function of TG mobilization module conserving between flies and mammals. An *in vitro* assay reveals binding Lsd1 with PKA-dependent phosphorylation of LD enhances the activity of lipolysis in the *Manduca sexta* CG8552 ortholog *Ms*TGL (triglyceride lipase) [37]. Besides, *Ms*TGL is the fat body triglyceride hydrolase that is the major lipase purified from this insect species [38,39]. The function of *Lsd1* in lipid metabolism in *Drosophila* is well known. For example, analyses with GFP (green fluorescent protein)-tagged *Lsd1* displayed its presence on the surface of LDs in *Drosophila* fat body cells [40]. In addition, loss of function or overexpression of *Lsd1* in *Drosophila* indicated that *Lsd1* probably facilitates lipid mobilization [41].

GAL4/UAS system

The binary GAL4/UAS (upstream activating sequence) system is one the most useful tools targeting in gene expression in *Drosophila.* The system includes two main components, GAL4 and UAS. GAL4 structured by 881 amino acid protein found in yeast is an activator for transcription in *Drosophila.* UAS is a short part of the promoter section at which GAL4 is combined to activate gene transcription [42]. *Drosophila* GAL4 lines are crossed to the target gene lines in which five GAL4-binding sequences are used to compose sub-cloned downstream of UAS. In the progeny of such a cross, GAL4 is present and activates the transcription of the target gene only in those cells and tissues [43]. For example, expressing of *Drosophila* line shows GAL4 protein crossed to *Drosophila* carrying RNAi gene that targets in the sub-cloned downstream of UAS, and in the progeny the target gene specially knocks tissues down (Fig.2).

Outline of the thesis

Lipid plays an essential function in living organisms. However, the accumulation of lipid leads to risks of diseases. *Bmm* and *Lsd1* are conserved in eukaryotes and play the important role in lipid metabolism. The *bmm* has been identified as a regulator of fat storage in *Drosophila* as *bmm* mutants caused obesity, while *Lsd1* mutants cause the increase of lipid droplet size in flies. In my thesis study, I used *Drosophila melanogaster* as a genetic model to establish a transgenic model for screening anti-obesity agents (Chapter 1) and discover new function of *Lsd1* gene in wing development of *Drosophila melanogaster* (Chapter 2).

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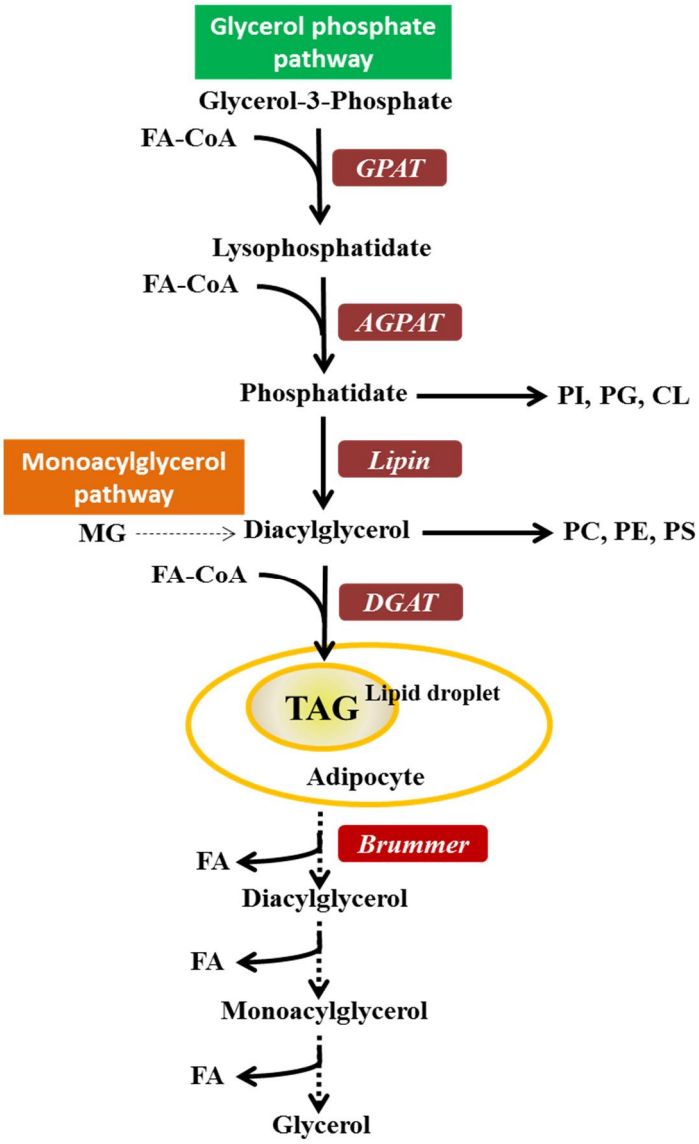

Figure 1. The pathway of triacylglycerol (TAG) synthesis (line arrow) and lipolysis (dotted arrow). FA-CoA; fatty acid coenzyme A ester, GPAT; glycerol-3-phosphate acyltransferase, AGPAT; 1-acylglycerol-3-phosphate acyltransferase, PI; phosphatidylinositol, PG; phosphatidylglycerol, CL; cardiolipin, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidylserine, DGAT; diacylglycerol acyltransferase, MG; monoacylglycerol, FA; fatty acid.

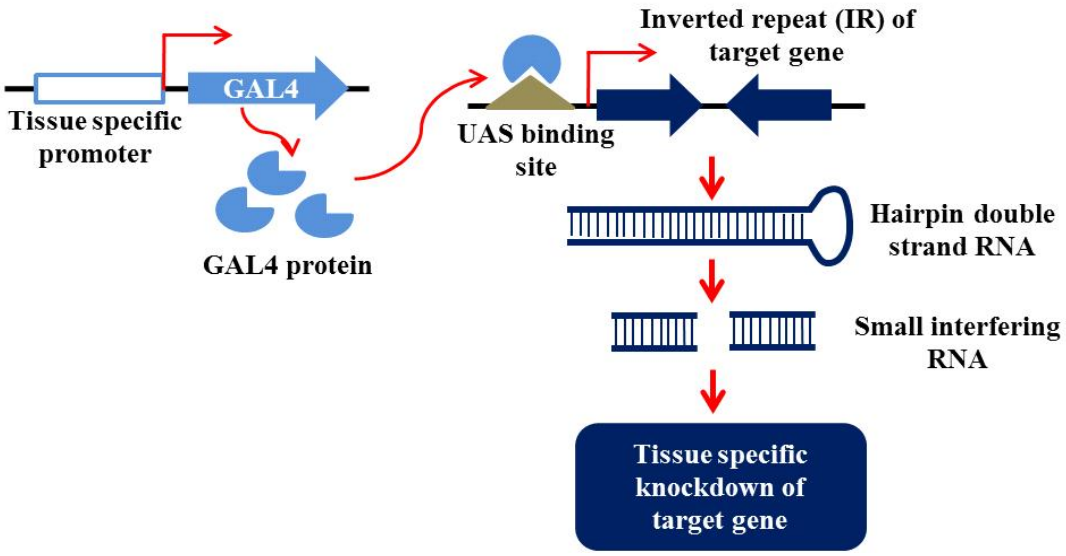

Figure 2. Tissue specific knockdown by GAL4-UAS system. Fly strains expressing GAL4 proteins are regulated under the control of tissue specific promoter, which are genetically crossed with fly strains bearing a target gene expressing hairpin double strand RNA downstream of an UAS binding site. In the progeny, RNAi is expressed in a regulated pattern and induces its target gene silencing.

Chapter 1

A *Drosophila* model for screening anti-obesity agents

1. Introduction

Triglyceride (TG or triacylglyceride, TAG) is a main type of neutral lipid structured by a heterogeneous group of molecules with a glycerol back bone and three fatty acids (FA) bonded by ester bonds. TAGs serve multiple important functions in living organisms. One of them is the major storage molecules of FAs for energy utilization; these molecules are stored in lipid droplets (LDs). Besides, they also have the function in the synthesis of membrane lipids. TAGs store six times the amount of energy more than hydrated glycogen because of their high reduction and anhydrous compounds. TAGs are normally necessary for physical processes, but the uncontrolled production of TAGs in human adipose tissue that causes obesity and nonadipose tissues and is related to organ dysfunction [1]. Obesity is a complex disorder, involving an abnormal or excessive fat accumulation that presents a risk to human health. It is the main cause of the cluster of metabolic diseases such as insulin resistance, atherosclerosis, and cancer, all of which can lead to the premature death of patients [2]. Obesity usually results from a combination of factors, the major ones of which are an unhealthy diet and physical inactivity. In addition, genetics plays an important role in how an individual's body converts and burns energy. Heritability of obesity is related to not only monogene but also multigene [3,4]. The recent investigations elucidate that the heritability of obesity tends to be high compared to other complex, polygenic diseases such as schizophrenia and autism. Additionally, its heritability is significantly higher than for other complex

traits such as hypertension and depression [5]. However, obesity-causing genes are complex and not yet fully understood.

In order to study the metabolic syndrome, *Drosophila melanogaster* might be the evaluable nominee because it shares most of the same basic metabolic functions with vertebrates. Many analogous organ systems in humans that direct the uptake, storage, and metabolism of nutrients are found in fruit flies [6]. Moreover, the rapid growth of flies, their inexpensive breeding costs, and their small genome size facilitate screening for therapeutics or preventive agents of obesity.

The primary sites of fat storage in cells are the lipid droplets (LDs), which are organelles with a phospholipid monolayer membrane coated by numerous proteins that surround a lipid core [7]. Recently, a gene homolog of human adipocyte triglyceride lipase (ATGL) was discovered in *Drosophila* as a controller of lipid storage; namely, brummer (*bmm*). The *bmm* gene encodes a LD-associated triacylglycerol (TG) lipase, which controls the systemic TG levels of flies in a dose-dependent manner. Mutation of the *bmm* gene was reported to induce obesity in flies [8].

Previously, BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) and Nile red (9-diethylamino-5-benzo[α]phenoxazinone) were used to visualize intracellular fats in *D. melanogaster* [9,10]. However, Nile red was reported to label lysosome-related organelles (LRO) instead of fat-storing LDs. Similarly, under the same conditions, BODIPY stained LRO strongly but stained LDs weakly [11]. These discoveries are

increasing concerns about the results obtained from vital staining methods, which may not reflect the real *in vivo* situation. Therefore, the combination of LD staining with biochemical quantitation of TG is needed to evaluate fat storage in a body [10,12]. Green fluorescent protein (GFP)-tagged markers have been broadly applied in the analysis of *D. melanogaster* to reveal the localization of LD-associated proteins, such as hormone-sensitive lipase, lipid storage droplet 1 and 2, and BMM [8,9]. GFP was also used as a fat indicator to study new fat storage regulators in *Caenorhabditis elegans* [13]. However, these studies revealed difficulties in achieving easy and rapid screening for anti-obesity drug candidates, since so many LDs are contained in a cell.

In this study, I introduced the *bmm* promoter fused with the *GFP* gene into *Drosophila* to reveal whether the transgenic fly could be used as a lipid storage indicator and serve as a marker for the effective screening of anti-obesity agents. Because GFP contains a nuclear localization sequence, its signal is expected to be easily detected in the nucleus of the *Drosophila* salivary gland, which is very large owing to endoreplication. Therefore, I revealed the relationship between lipid accumulation and *bmm* expression, by observing the GFP signal in the salivary gland. Furthermore, I examined the effects of oral administration of histone deacetylase (HDAC) inhibitors and vegetable powders on *bmm* expression using the transgenic fly.

2. Materials and Methods

Materials

NCC-149 (an HDAC8 inhibitor) and T302 (an HDAC9 inhibitor) were provided by Professor Takayoshi Suzuki (Kyoto Prefectural University of Medicine, Kyoto, Japan) [14,15]. The following edible portions of vegetables were provided by Designer Foods Co. Ltd. (Nagoya, Japan): leaves of spinach and komatsuna; leaf heads of cabbage and lettuce; leaves and bud/flower of nabana (*Brassica* flower), broccoli, and edible flower; bulbs of onion; fruits of red paprika and tomato; and roots of Japanese radish. These vegetables were lyophilized and ground in a mill before use. Mulberry leaves harvested in Kyotango city (Kyoto, Japan) were dried and ground by air flush at 180°C for 7 s.

Recombinant plasmid construction

DNA fragments containing the *bmm* promoter were used for checking the promoter activity. The 2 kbp fragment from −1655 to +345 with the expected transcription initiation site was amplified by PCR using genomic DNA of yellow white (*yw*) *Drosophila*. The primers *bmm*F-1655 and *bmm*R+345 containing a *Not*I site (underlined) were employed:

bmm F-1655:

5′-ATCAGATCCGCGGCCGCTTGAAGTGATTGGTAGTGGGTG-3′

bmm R+345:

5′-GCTCACCATGCGGCCGCGCTTTGGACTCGGCGTTAGATT-3′

The fragment obtained was inserted into the *Not*I site of plasmid pOBP-GFP (in which GFP was inserted into the pOBP vector derived from transposable P element) with the

aid of the In-Fusion PCR cloning kit (Clontech, Mountain View, CA, USA) [16]. Thus, the plasmid pOBP-*bmm* promoter-GFP for generating transgenic flies was obtained. A recombinant plasmid carrying the p53 promoter region of *Drosophila* (pOBP-p53 promoter-GFP) was used as the positive control [16].

Transfection of Drosophila cells

The recombinant vector was transfected into S2-DRSC *Drosophila* cells (*Drosophila* Genomics Resource Center, Bloomington, IN, USA) with HilyMax transfection reagent (Dojindo, Kumamoto, Japan). At 48 h after transfection, the cells were collected, fixed with paraformaldehyde, and then stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). The cells were mounted on glass slides using Vectashield Mounting Medium (Vector Laboratories Ltd.) and were observed for DAPI and GFP signals under a confocal laser scanning microscope (Model Fv10i; Olympus Corp., Tokyo, Japan).

Establishment of transgenic flies

According to previous studies, P-element-mediated germ-line transformation was carried out by microinjecting pOBP-*bmm* promoter-GFP into fertilized eggs. Based on rescue of the white eye color, F1 transformants (*yw*; +; *bmm* promoter-GFP) were selected [17]. Because the *bmm* promoter activities in several independent transgenic lines were almost the same, line 17 carrying the *bmm* promoter-GFP gene on the third chromosome was used for further experiments.

Glucose diet feeding

The standard food supplement contained 0.8% agar (w/v), 9% cornmeal (w/v), 4% dry yeast (w/v), 0.05% (w/v) ethyl *p*-hydroxybenzoate, and 0.5% propionic acid (v/v). For preparation of the glucose diet, glucose was added to the standard food supplement to a final concentration range of 2.5–20% (w/v). Five male and 5 female transgenic flies were mated and allowed to lay eggs on the glucose-supplemented diet for 2 days at 25°C. The hatched larvae were grown on the same diet. Then, the third instar larvae were used for lipid measurement or were dissected to analyze the GFP signal as described below.

Image analysis

The third instar larvae were dissected in phosphate-buffered saline (PBS), and the tissues collected were fixed in 3% paraformaldehyde for 30 min at room temperature. The tissues were washed 3 times with PBS and then set onto the glass slides using the Vectashield Mounting Medium. Images were acquired at 589 nm excitation/510 nm emission under a fluorescence microscope (Model BX-50; Olympus) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K.K., Shizuoka, Japan). The intensity of the GFP signal from the nucleus of the salivary gland was analyzed by using the MetaMorph software (version 7.7.7.0; Molecular Devices, Sunnyvale, CA, USA). The average intensity was used for calculating the GFP signal after being normalized by the background intensity.

Measurement of lipid content

The lipid content in flies was measured by the colorimetric sulfo-phospho-vanillin (SPV) method as described previously [18,19]. Ten third instar larvae were homogenized in 2% sodium sulfate, and then chloroform-methanol (1:1) was added. The supernatant was collected by centrifugation (1000 ×*g*, 1 min), mixed with distilled water, and centrifuged again (1000 ×*g*, 1 min). For lipid measurement, the chloroform layer was first dried at 90°C for 10 min, and then 98% sulfuric acid was added and the solution was incubated for 10 min at 90°C. After cooling to room temperature, SPV reagent was mixed into the solution and the color development was measured at 530 nm.

HDAC inhibitors and vegetable-powder feeding

HDAC inhibitors were dissolved in ethanol and then diluted with distilled water. The solution was mixed with instant medium Formula 4–24 (Carolina Biological Supply Co., Burlington, NC, USA) to obtain a final HDAC inhibitor concentration of 0.5% (w/w). The control medium was prepared by mixing instant medium Formula 4–24 and distilled water together with the same concentration of ethanol used to dissolve the HDAC inhibitors.

After lyophilization, the vegetable powder was mixed with instant medium Formula 4–24 to obtain a final concentration of 10% (w/w). Water was mixed well into the powder mixture to constitute the vegetable diet. The instant medium dissolved with water was used as a control medium.

Five male and 5 female transgenic flies were mated on the diet containing HDAC inhibitors or various vegetable powders for 2 days. Newborn larvae were continuously fed on the same mediums until they reached the third instar larval stage at 25°C. The salivary glands of the third instar larvae were used to analyze the GFP intensity as described above.

Quantitative RT-PCR

Total RNAs were extracted from the whole body of third instar larvae of transgenic flies and analyzed by a LightCycler Nano System with SYBR Green (FastStart SYBR Green Master Mix; Roche Diagnostics Corp., Indianapolis, IN, USA), as described previously [20]. The *Rp49* gene was used as an endogenous reference gene. Primers used were as follows.

Rp49-F76: 5′-AATCTCCTTGCGCTTCTTGG-3′

Rp49-R214: 5′-TTACGGATCGAACAAGCGC-3′

bmm-F466: 5′-CTGCTGTCTCCTCTGCGATTT-3′

bmm-R606: 5′-TTCTGTAGACCCTCCAGCAG-3′

Experiments were performed in triplicate for each of three RNA batches isolated separately. The results collected from the real-time PCR were analyzed by the $2^{-\Delta\Delta}$ (Livak) method, based on the observation of fold changes in mRNA levels [21].

3. Results

Establishment of the fly model for bmm expression monitoring

We constructed a pOBP-*bmm* promoter-GFP plasmid carrying the 2 kbp 5′-flanking region of the *bmm* gene. To evaluate the *bmm* promoter activity, the plasmid was transfected into *Drosophila* S2-DRSC cells and the GFP signals were inspected by fluorescence microscopy. The cells transfected with pOBP-*bmm* promoter-GFP showed the GFP signal (Fig. 1C), similar to the cells transfected with pOBP-p53 promoter-GFP as a positive control (Fig. 1D). The results indicated that the 5′-flanking region of *bmm* amplified in this study was functional for promoter activity. Therefore, pOBP-*bmm* promoter-GFP was used to establish the transgenic flies.

D. melanogaster was transformed with pOBP-*bmm* promoter-GFP and then the GFP expression in the third instar larvae was analyzed. Under the fluorescence microscope, all observed tissues such as brain lobe, gut, salivary gland, wing disc, eye disc, and lipid tissue of transgenic flies displayed the green fluorescence of GFP (Fig. 2A–F). In contrast, there was no detectable GFP signal in the brain lobe (Fig. 2G) and wing disc (Fig. 2H) of control flies, indicating that the *bmm* promoter had functioned as expected in live flies.

Relationship between lipid storage and GFP intensity

In order to check the usefulness of the transgenic fly model in evaluating obesity and screening anti-obesity agents, the relationship between *bmm* expression and lipid content in flies was analyzed. First, we measured the lipid content of flies that had been orally administered different concentrations of glucose (2.5–20%). As shown in Fig. 3, the third instar larvae fed higher glucose diets showed significantly increased lipid storage, whereas flies fed lower glucose diets had a lower lipid content. This indicated that *Drosophila* on higher glucose diets had become obese, whereas those on lower glucose diets remained thin.

The GFP expression driven by the *bmm* promoter was localized to the nucleus of transgenic flies, as shown in Fig. 4, because it contained the nucleus localization signal sequence [16]. Since the nucleus of the *Drosophila* salivary gland is very large as a result of endoreplication, the salivary gland of flies fed a glucose diet was used to detect the GFP signal by fluorescence microscopy (Fig. 5A–D). The intensity of the GFP signal decreased with increasing dietary glucose concentration (Fig. 5E). Furthermore, quantitative RT-PCR analysis of the mRNA expression of *bmm* in the whole body of transgenic flies found the levels to be reduced upon increasing glucose amount in the diet (Fig. 5F). These results correlated well with a previous report that had demonstrated the up-regulation of *bmm* expression upon starvation and its down-regulation upon re-feeding [8], suggesting that this transgenic fly can be used for screening anti-obesity drug or food candidates.

HDAC inhibitors as potential anti-obesity agents

To evaluate the effects of HDAC inhibitors on *bmm* expression, we fed transgenic flies with 0.5% (w/w) of NCC-149 (an HDAC8 inhibitor) or T302 (an HDAC9 inhibitor). As shown in Fig. 6, the GFP intensities increased 1.4-fold and 1.8-fold relative to the control after administration of NCC-149 and T302, respectively. These results indicated that HDAC inhibitors can potentially be new anti-obesity drugs.

Effect of vegetables on bmm expression

Drosophila that had been orally fed with nabana, spinach, lettuce, red paprika, cabbage, broccoli, Japanese radish, edible flower, or mulberry leaf showed increased GFP intensity in the salivary gland (Table 1). This suggests that these vegetables may suppress obesity. In contrast, flies fed with tomato showed a 0.74-fold decrease in the GFP signal relative to control flies, indicating enhanced lipid storage, whereas flies fed with komatsuna or onion showed no difference in GFP signal strength from that of control. Taken together, our screening identified several vegetables that have potential for preventing lipid accumulation in *Drosophila*.

4. Discussion

The development of a simple and rapid screening system using a living organism is important to screen new anti-obesity substances. In the present study, the transgenic *Drosophila* fly carrying the fused genes of the *bmm* promoter and *GFP* showed fluorescence in all examined tissues, including salivary gland nuclei (Fig. 2). In the past few years, GFP has been broadly used as a marker in fat storage studies in *C. elegans*.

For instance, GFP was used as an indicator for RNAi screening to identify uncharacterized fat storage regulatory genes in *C. elegans* [13], and GFP fused with ATGL (a *C. elegans* ortholog and a key lipolytic enzyme) was used to mark LDs in *C. elegans* [22]. GFP-fusion proteins are also widely applied for analyzing gene expression and protein localization in various *Drosophila* cell lines [6,23]. The transgenic *bmm*-expressing *Drosophila* model established in this study has many advantages. First, the easy detection of the GFP signal due to the large size of the salivary gland nucleus enables the efficient screening for anti-obesity candidate drugs. Second, fixing and staining of salivary gland tissue are not needed. Third, the GFP intensity in the salivary gland nucleus can be quantified easily by the MetaMorph software.

Obesity is associated with elevated levels of lipid content in tissues. Animals fed low- or high-calorie food can experience a decrease or an increase of their lipid level, respectively. In this study, we designed an experiment to culture *Drosophila* under different food conditions that could affect their lipid storage, while keeping the other compositions constant. By using the SPV method to measure total lipids, we proved that the lipid content in the fly body was elevated with increasing glucose content in the food diet. The transgenic *bmm*-expressing flies on low-glucose diets showed enhanced GFP signals; conversely, flies fed on high-glucose diets showed significantly decreased GFP signal intensities, indicating enhanced lipid storage. These results suggest that the expression of *bmm* is up-regulated with low-calorie foods and down-regulated with high-calorie foods, in good concordance with previous reports [8]. The inverse correlation

between lipid level and the GFP intensity indicates that this transgenic fly could be a useful model for screening novel anti-obesity drug or food candidates.

The present study indicates that HDAC inhibitors might be remarkable candidates for further obesity-therapy studies. In addition, our finding that those vegetables are potential to prevent obesity is coincided well to previous report [24]. By adding 0.5% (w/w) HDAC8 and HDAC9 inhibitors into the culture medium, the GFP intensity in the transgenic *bmm*-expressing flies increased significantly compared with the control, indicating that obesity was suppressed. In previous studies, HDAC9 was proposed as a potential therapeutic target for obesity, since lipid accumulation was prevented in HDAC9-knockdown mice fed chronic high-fat diets [25]. In contrast, the potential of HDAC8 inhibitors for diabetes treatment has not yet been reported. Our results indicate that HDAC inhibitors not only of HDAC9 but also of HDAC8 may be new potential candidates as anti-obesity drugs. In addition, when the transgenic *bmm*-expressing flies were fed with vegetable-powder-containing food, some of the vegetables induced the GFP signal, suggesting a reduction in lipid storage. Interestingly, cabbage and broccoli, which showed potential for preventing obesity in this study, both contain sulforaphane [26], a compound that has been reported to inhibit HDAC activity [27,28]. Other groups have reported that tomato contains 9-oxo-10(E),12(E)-octadecadienoic and its derivatives, which are potent PPARα agonists that decrease triglyceride accumulation in mouse primary hepatocytes [29,30]. In contrast, our data suggested that tomato may

induce lipid accumulation in *Drosophila*. The possible reason for this discrepancy is that the glucose and fructose in tomato can suppress *bmm* expression.

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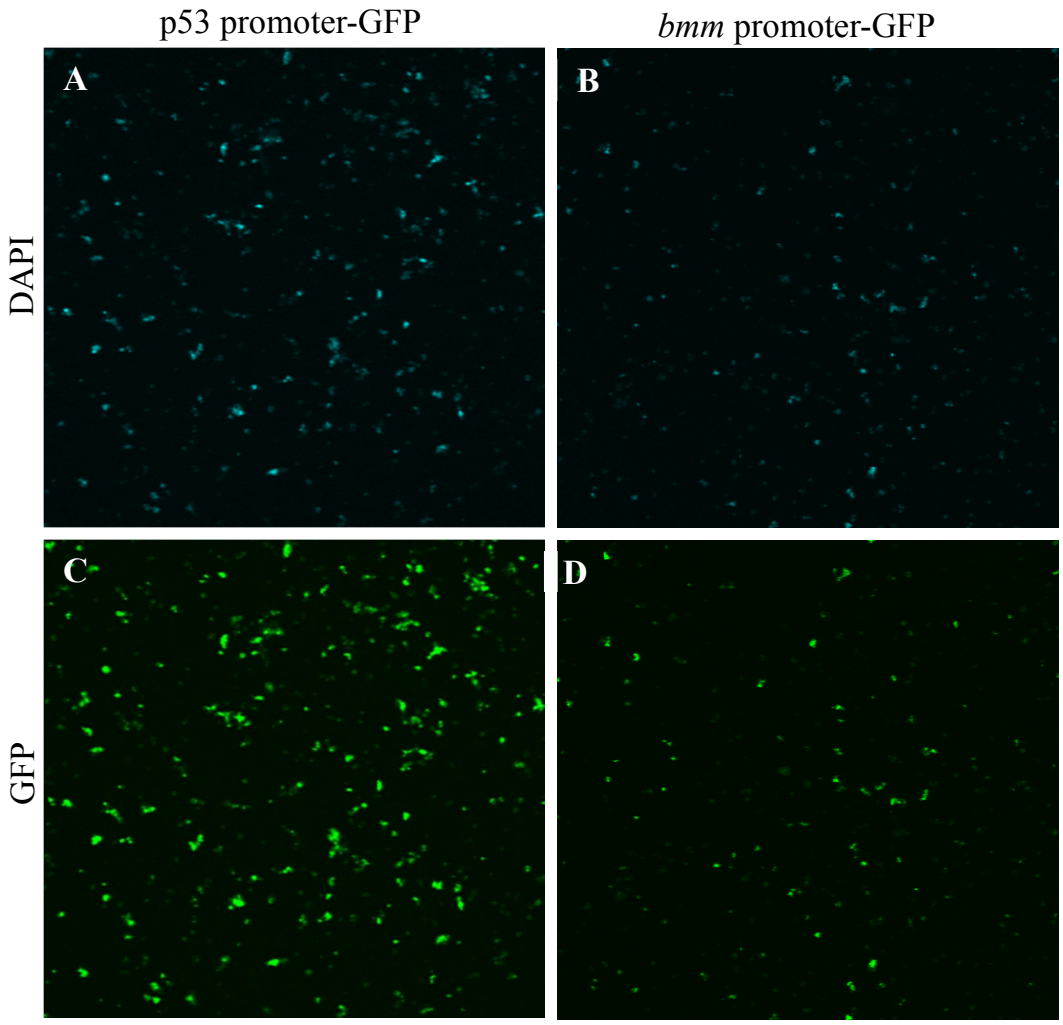

Figure 1. Transfection of S2-DRSC *Drosophila* cells. S2-DRSC *Drosophila* cells were transfected with pOBP-*bmm* promoter-GFP (B, D) or with pOBP-p53 promoter-GFP as a positive control (A, C). After staining with DAPI, cells were observed for DAPI signals (A, B) and GFP signals (C, D) under a fluorescence microscope. Cells transfected with pOBP-*bmm* promoter-GFP showed GFP signals, indicating that the *bmm* promoter functioned as expected.

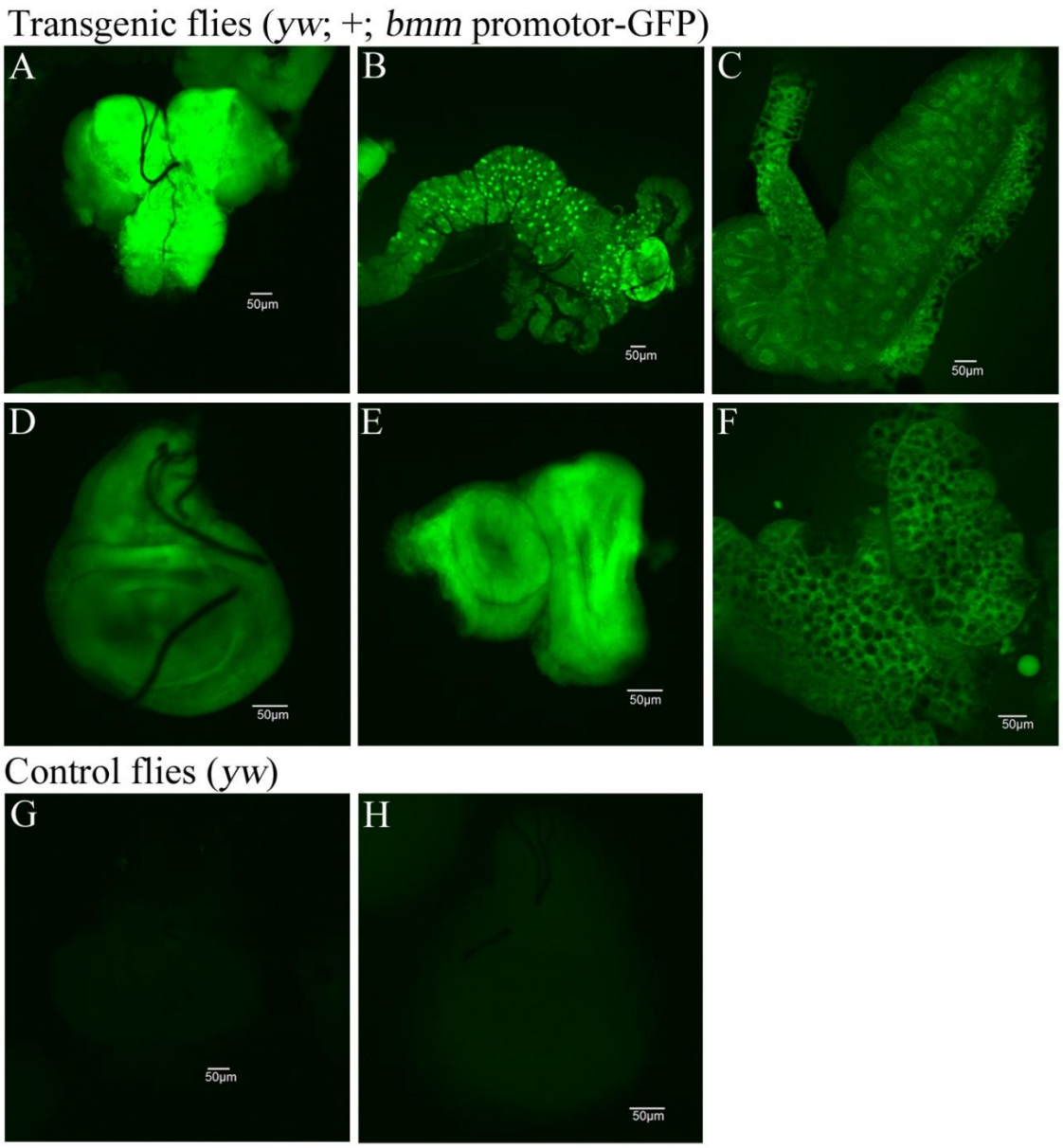

Figure 2. The *bmm*-promoter-driven expression of GFP in transgenic *Drosophila* (*yw*; +; *bmm* promoter-GFP). The various tissues of the third instar larvae of transgenic flies were observed by fluorescence microscopy. The sections of brain lobe (A), gut (B), salivary gland (C), wing disc (D), eye disc (E), and lipid tissue (F) showed GFP signals. In contrast, the control fly (*yw*) showed no detectable GFP signal (G, brain lobe; and H, wing disc).

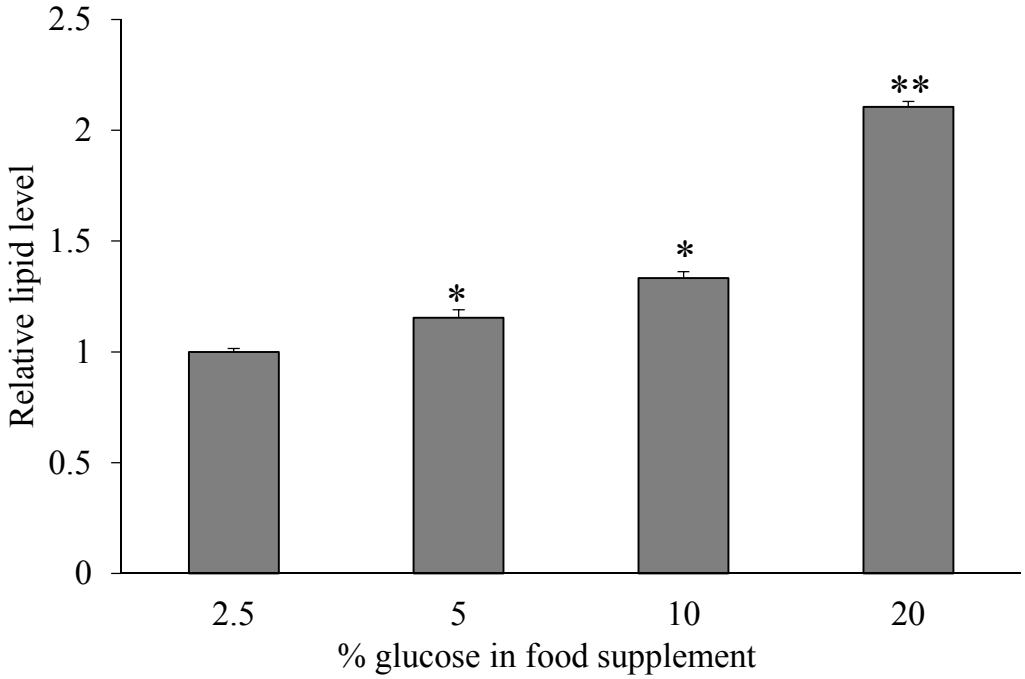

Figure 3. Relative lipid level in transgenic flies fed different glucose diets. The lipid level was measured by the sulfo-phospho-vanillin method and normalized with body weight. The lipid content increased with increasing glucose content in the food supplements. $n = 30$ for each treatment. The error bars represent the standard deviation. *, $p < 0.05$; **, $p < 0.001$.

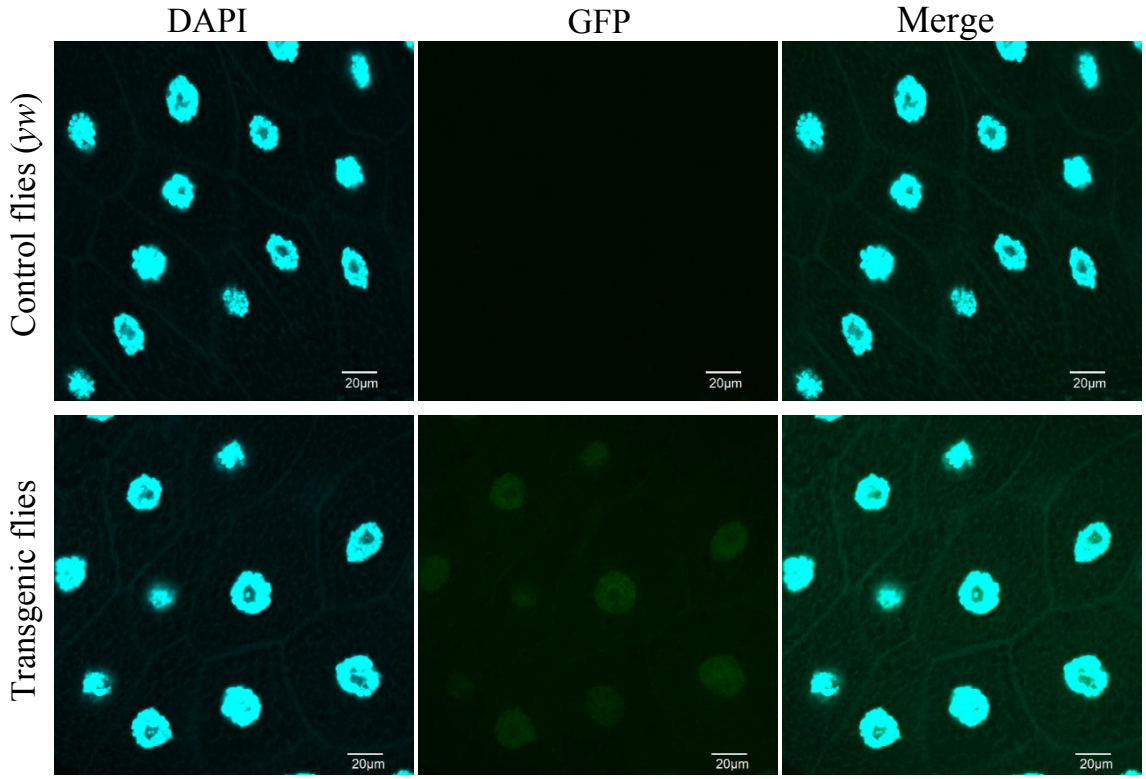

Figure 4. Intracellular localization of the GFP signal in the salivary gland. After staining with DAPI, the salivary glands of the third instar larvae were observed by fluorescence microscopy. There was no detectable GFP signal in control flies (*yw*), whereas GFP carrying the nuclear localization sequence was detected in the nucleus of transgenic *Drosophila* (*yw*; +; *bmm* promoter-GFP).

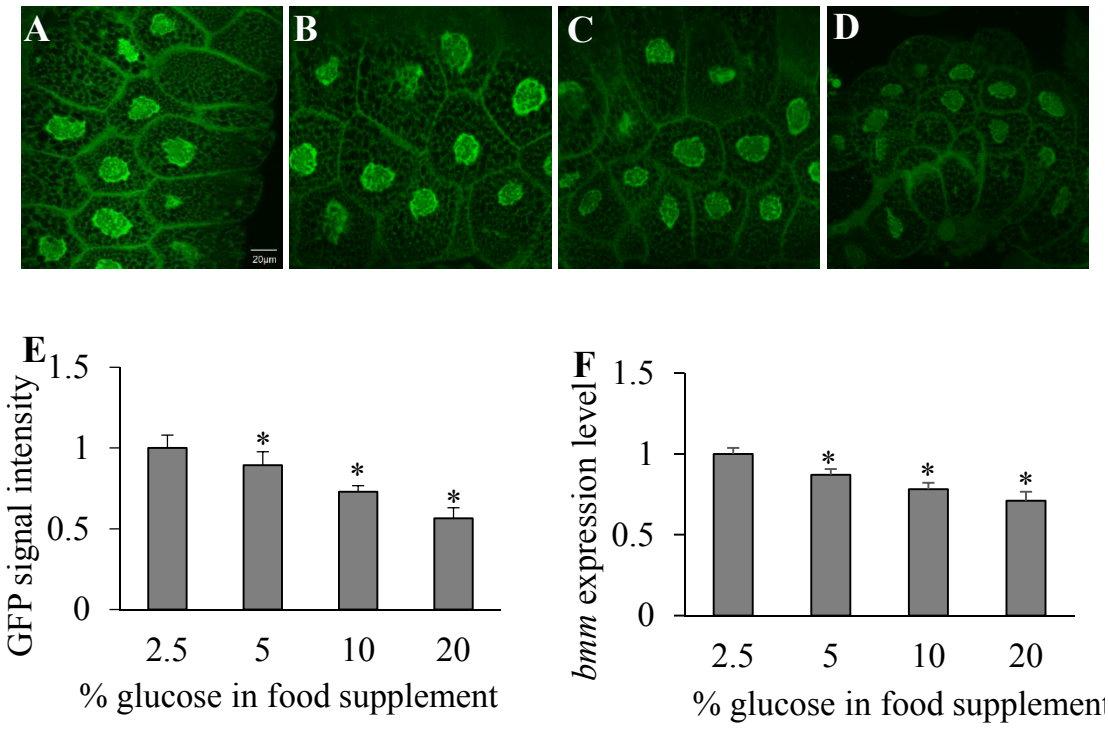

Figure 5. Relationship between *bmm* expression and glucose contents in diets. The transgenic flies were fed with diet containing different amounts of glucose, and the salivary glands of the third instar larvae were observed by fluorescence microscopy. GFP signal intensities measured by the MetaMorph software (E) showed that flies fed 2.5% glucose (A) showed stronger signals than flies fed 5% (B), 10% (C), and 20% (D) glucose, respectively. Real-time PCRs were used to measure the endogenous *bmm* mRNA expression levels of transgenic flies (F). $n = 30$ for each treatment. The error bars represent the standard deviation. *, $p < 0.05$.

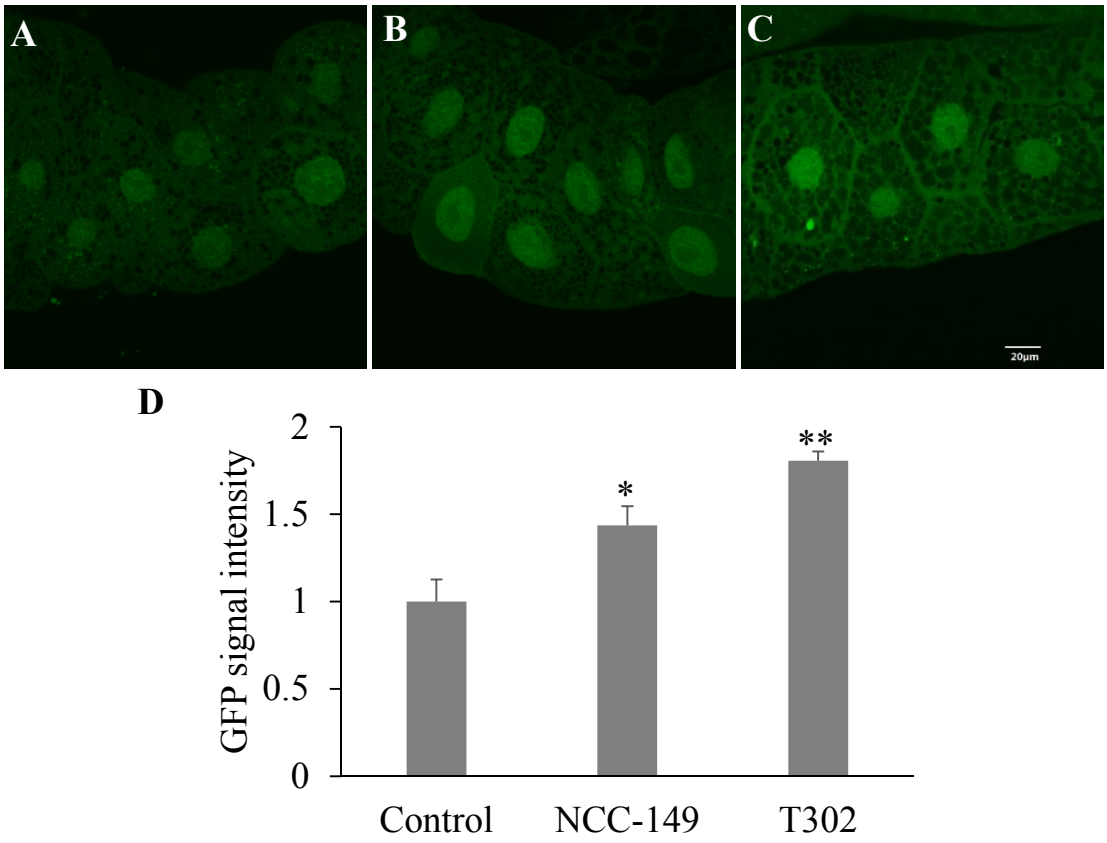

Figure 6. Effects of oral administration of histone deacetylase (HDAC) inhibitors on *bmm* expression in *Drosophila*. The transgenic flies were fed a diet containing HDAC inhibitors, and the salivary glands of the third instar larvae were observed by fluorescence microscopy to evaluate *bmm*-promoter-driven GFP signaling as a marker. GFP signals of *Drosophila* fed with instant food alone (A), instant food with NCC-149 (an HDAC8 inhibitor) (B), or instant food with T302 (an HDAC9 inhibitor) (C). The GFP intensity measured by the MetaMorph software was enhanced in flies fed the HDAC inhibitors, and significant differences were observed relative to the control (D). $n = 20$ for each treatment. The error bars represent the standard deviation. *, $p < 0.05$; **; $p < 0.001$.

Table 1. Effects of oral administration of vegetables on *bmm* expression in *Drosophila*.

Vegetable		Relative GFP intensity
Nabana	*Brassica napus* L.	$1.49^{**} \pm 0.07$
Spinach	*Spinacia oleracea* L.	$1.33^{*} \pm 0.12$
Lettuce	*Lactuca sativa*	$1.79^{**} \pm 0.10$
Red paprika	*Capsicum annuum* L.	$1.92^{**} \pm 0.13$
Cabbage	*Brassica oleracea* L. var. *capitata*	$2.18^{**} \pm 0.31$
Tomato	*Solanum lycopersicum* L.	$0.74^{*} \pm 0.07$
Komatsuna	*Brassica rapa* L. var. *perviridis* cv. Komatsuna	$1.11^{ns} \pm 0.22$
Onion	*Allium cepa* L.	$1.16^{ns} \pm 0.23$
Broccoli	*Brassica oleracea* L.	$1.60^{**} \pm 0.11$
Japanese radish	*Raphanus sativus* L.	$1.44^{**} \pm 0.32$
Edible flower	*Brassica rapa* L. var. *nippo-oleifera*	$1.83^{**} \pm 0.25$
Mulberry	*Morus alba* L.	$2.25^{**} \pm 0.19$

The transgenic flies were fed with a vegetable diet, and green fluorescent protein (GFP) signals in the salivary gland nuclei of the third instar larvae were detected. The GFP intensity was analyzed using the MetaMorph software and was subtracted by that of the background signal; then the GFP intensity of the vegetable group relative to that of the control group was calculated. $n = 6$ for each sample. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

Chapter 2

Function of lipid storage droplet 1 (Lsd1) in wing development of *Drosophila melanogaster*

1. Introduction

Triacylglycerols (TAG) in adipose tissue are the body's major energy storage source [1]. Lipid storage organelles, known as lipid droplets (LDs), are abundant in the adipose tissue and play a role in controlling the body fat banks of animals [2]. LDs are composed of a neutral lipid core coated by a lipid monolayer with proteins, the best known of which is a protein family named PAT domain proteins. PAT domain proteins include ADRP and TIP47, and are collectively named perilipins [3]. The interaction between perilipins and lipases in LDs is related to the regulation of lipid homeostasis. Moreover, perilipin 1 is the most well-characterized member of the PAT family, and regulates basal and stimulated lipolysis in opposite ways. In normal conditions, perilipin 1 blocks the access of lipases to LDs and suppresses adipose triglyceride lipase (ATGL) activation by forming a complex with CGI-58, an activator of ATGL. In times of energy deficit, perilipin 1 is phosphorylated by protein kinase A (PKA) in response to hormonal signals, and phosphorylated perilipin 1 facilitates maximal lipolysis by recruiting hormone-sensitive lipase (HSL) and allowing ATGL to access the LD [4-7]. The mutation of perilipin 1 in mice results in a lean phenotype, and a lack of perilipin 1 combined with a mutation in the leptin receptor gene in mice reverses obesity. These phenotypes are derived from loss of the anti-lipase protective effect of perilipin 1 under normal conditions [8]. In humans, genetic variations in the perilipin 1 gene have been associated with metabolic disorders, including type 2 diabetes and partial lipodystrophy [9,10]. In addition to perilipin 1, there are four other mammalian perilipins: ADRP/perilipin 2,

TIP47/perilipin 3, S3-12/perilipin 4, and OXPAT/perilipin 5 [9]. Perilipins are evolutionarily conserved from *Drosophila* to humans. *Drosophila* has only two perilipins, perilipin 1/Lipid storage droplet 1 (Lsd1), and perilipin 2/Lipid storage droplet 2 (Lsd2) [9]. More importantly, *Drosophila* and higher animals share the same basic metabolic functions and lipid metabolism-related genes [11,12]. The function of *Lsd1* in lipid metabolism in *Drosophila* is well known. For example, analyses with GFP (green fluorescent protein)-tagged *Lsd1* displayed its presence on the surface of LDs in *Drosophila* fat body cells [13]. In addition, loss of function or overexpression of *Lsd1* in *Drosophila* indicated that *Lsd1* probably facilitates lipid mobilization [8]. *In vitro* studies identified *Lsd1* as a PKA phosphorylation target [14], while *in vivo* mutant analysis demonstrated an essential role of *Lsd1* as a pro-lipolytic effector of the AKH/AKHR pathway on the LD surface [2]. To date, other functions and genetic regulatory mechanisms of this gene are still under investigation.

In this study, the function of *Lsd1* was further investigated in *Drosophila* by selective knockdown of the *Lsd1* gene using the GAL4-UAS targeted expression system in combination with RNA interference [15]. By crossing tissue and developmentally specific GAL4 driver fly lines with a fly line carrying the UAS-*Lsd1*IR (IR, inverted repeat), the *Lsd1* gene can be specifically knocked-down in any desired tissue or developmental stage. The knockdown experiments in this study revealed that *Lsd1* is necessary for the development of *Drosophila* wings, possibly through maintaining the function of mitochondria.

2. Materials and methods

Fly stocks

Fly stocks were maintained at 25°C on standard food (4% dry yeast, 9% cornmeal, 10% glucose, 0.8% agar, 0.5% propionic acid, and 0.05% ethyl parahydroxybenzoate). RNAi stock v30884 (FBgn0039114) with no off target effects, and carrying inverted repeats of the *Lsd1* gene (targeting the region from nucleotide number 437-782 of *Lsd1* mRNA) located on the second chromosome (*w*; UAS-*Lsd1*IR; +), was obtained from the Vienna *Drosophila* Resource Center (VDRC). The FB-GAL4 driver line (expressed in the fat body) was kindly supplied by Dr. Ronald P. Kuhnlein of the Max Planck Institute for Biophysical Chemistry (Germany) [16]. All other lines used in this study were obtained from the Bloomington Stock Center at Indiana University.

The USA-*Lsd*1I*R* fly line was crossed with several GAL4 drivers to knockdown *Lsd1* in specific tissues of the F1 generation. For dorsal wing disc-specific knockdown of *Lsd1,* virgin female MS1096-GAL4 flies, with two copies of the MS1096-GAL4 gene on the X chromosome (MS1096-GAL4; +; +), were crossed with male UAS-*Lsd1*IR flies with two copies of the UAS-*Lsd1*IR gene on the second chromosome (*w*; UAS-*Lsd1*IR; +). Yellow white (*yw*) and the *Drosophila* carrying inverted repeats of green fluorescent protein gene (*yw*; UAS-*GFP*IR; +) were crossed with the female MS1096-GAL4 driver as controls. *Lsd1* was knocked down in the posterior wing disc and embryo (using en-GAL4 driver), in eye discs (using GMR-GAL4 driver), in the fat body (using Fb-GAL4

driver), ubiquitously throughout tissues (using Act5C-GAL4 driver or Tubp-GAL4 driver). All knockdown experiments were performed by crossing UAS-*Lsd1*IR flies with the specific driver line.

Immunohistochemistry

Third instar larvae were dissected in phosphate buffered saline (PBS), and wing discs were fixed in 4% paraformaldehyde for 30 min at 25°C. After washing with 0.3% Triton X-100 in PBS (PBST), samples were blocked for 30 min at 25°C with 0.15% PBST containing 1% bovine serum albumin. Samples were then incubated with rabbit anti-Lsd1 antibody (kindly provided by Dr. Ronald P. Kuhnlein of the Max Planck Institute for Biophysical Chemistry, Germany) [2] at a 1:1000 dilution, at 4°C for 16 h. Following an extensive wash with PBST, the samples were incubated with goat anti-rabbit IgG Alexa Fluor™ 488 (Molecular Probes, Invitrogen) at a 1:400 dilution for 2 h at 25°C, further washed with PBST and PBS, and mounted in Vectashield mounting medium (Vector laboratories). Samples were inspected with a fluorescence BX-50 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K.K., Shizuoka, Japan).

In vivo ROS (reactive oxygen species) detection

Wing discs from the third instar larvae were dissected in PBS and then incubated with 10 μM CM-H2DCFDA (5-(and- 6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester) (Molecular Probes, Invitrogen) for 5 min to detect ROS. After washing with

PBS, samples were fixed in 1% paraformaldehyde for 5 min, washed three times with PBS, and then mounted in Vectashield mounting medium. Preparations were examined under a fluorescence BX-50 microscope equipped with a cooled CCD camera. ROS-positive cells were counted using MetaMorph software (version 7.7.7.0; Molecular Devices, Sunnyvale, CA, USA).

Apoptotic detection

Third instar larvae were dissected in PBS, and wing discs were fixed in 4% (w/v) paraformaldehyde in PBS for 30 min at 25°C. After washing with PBST, samples were blocked for 30 min at 25°C with 0.15% (v/v) Triton X-100 in PBS containing 1% (w/v) bovine serum albumin. Samples were then incubated with rabbit anti-cleaved caspase-3 IgG (Cell Signaling Technology) at a 1:100 dilution for 16 h at 4°C. After extensive washing with PBST, the samples were incubated with goat anti-rabbit IgG Alexa Fluor™ 488 (Molecular Probes, Invitrogen) at a 1:400 dilution for 2 h at 25°C, washed with PBST and PBS, and then mounted in Vectashield mounting medium. Preparations were examined under a fluorescence BX-50 microscope equipped with a cooled CCD camera. Apoptotic cells were counted using MetaMorph software.

Autophagy assay

Larval tissues dissected in PBS were subjected to the process of acidic organelle staining, including autolysosome. Samples were incubated with 100 nM LysoTracker Blue (Invitrogen) in PBS for 2 min, washed twice in PBS, and mounted in 50% glycerol in

PBS. Preparations were immediately examined by a fluorescence BX-50 microscope to obtain images.

ATP assay

We used CellTiter-Glo® luminescent cell viability assay kit (Promega) to quantify ATP. Assay kit buffer (100 μL) was used for homogenizing each adult fly, and the homogenate was centrifuged at 12,000 × *g* for 10 min. The supernatant was collected, and 10 μL of the supernatant was mixed with 100 μL of measure buffer. Luminescent signals were maintained by incubating the compound at 25°C for 10 min before being read on a Lumat LB 9507 luminometer (Berthold).

FFA level measurement

Free fatty acid (FFA) levels were measured using a free fatty acid quantification kit from Sigma (catalog number MAK044-1KT) with the procedure as described in instruction manual. Briefly, 5 third instar larvae were homogenized in 200 μL of 1% Triton X-100-chloroform, and the debris was removed by centrifugation. The organic phase (50 μL) was collected, dried by N_2 gas flush, 200 μL of fatty acid assay buffer was added (from the assay Kit,) and then dissolved by extensive vortex mixing for 5 min. For FFA measurement, 50 μL of extracted sample was used. The relative FFA level of flies was calculated against those of control flies. All measurements were performed at least three times.

3. Results

Effect of Lsd1 knockdown in various tissues and entire Drosophila

We knocked down *Lsd1* by crossing the USA-*Lsd*1I*R* fly line with several GAL4 driver lines. As summarized in Table 1, *Lsd1* knockdown in the whole fly by Act5C-GAL4 or Tubp-GAL4 resulted in a lethal phenotype at the embryonic stage. *Lsd1* knockdown by En-Gal4 also caused lethality, probably because of the reported leaky expression of GAL4 during embryogenesis [17,18]. These results indicate an essential role of the *Lsd1* gene for viability and/or development of *Drosophila. Lsd1* knockdown in the fat body caused a delay in growth at 25°C and lethality at 28°C. These results are consistent with previous studies of *Lsd1* mutants and indicate that *Lsd1* plays an important role in lipid metabolism [8]. Eye disc-specific knockdown of the *Lsd1* gene by GMR-GAL4 (at 28°C) exhibited no detectable phenotype, suggesting that *Lsd1* plays no apparent role during eye development. These observations suggest the tissue-specific role of *Lsd1* in the development, although the possibility of low level expression of GAL4 protein leading to insufficient knockdown of *Lsd1* in eye disc can not be excluded.

Knockdown of Lsd1 disrupted normal wing development

Although the function of *Lsd1* in lipid metabolism is well known, its potential function in wing development has not been explored. We, therefore, focused on the analyses of the wing phenotype induced by *Lsd1* knockdown. Flies carrying a single copy of the MS1096-GAL4 driver and UAS-*Lsd1*IR (MS1096-GAL4>UAS-*Lsd1*IR) exhibited

severe atrophied wing phenotypes, compared to control flies carrying one copy of MS1096-GAL4 only or flies carrying both MS1096-GAL4 and UAS-*GFP*IR (MS1096-GAL4>UAS-*GFP*IR) (Fig.1). This effect may be caused by the disruption of the developmental processes of the wings.

To confirm the effective knockdown of *Lsd1* in the dorsal wing disc, we performed immunostaining of wing imaginal discs from third instar larvae using anti-Lsd1 antibody. The specificity of the anti-Lsd1 antibody we used has been fully characterized [2]. *Lsd1*-knockdown flies (MS1096-GAL4>UAS-*Lsd1*IR) showed extensive decrease of Lsd1 signals in the wing pouch of the wing imaginal disc (circled in Fig. 2B). Control flies carrying MS1096-GAL4 alone showed strong Lsd1 signals in the wing pouch as well as other regions of the wing disc (Fig. 2A). Immunostaining of wing imaginal discs of both MS1096-GAL4 and MS1096-GAL4>UAS-*Lsd1*IR flies using only the secondary antibody showed no detectable signal in wing discs (Figs. 2C and 2D). These results confirmed the effective knockdown of *Lsd1* in the wing disc, which very likely resulted in the atrophied wing phenotype.

Knockdown of Lsd1 lead to increased cell death

The atrophied wings of *Lsd1*-knockdown flies suggested the possible involvement of apoptotic and/or autophagy processes. In *Drosophila*, baculoviral P35 and DIAP1 (Death-associated inhibitor of apoptosis 1) have been shown to effectively inhibit apoptosis when ectopically expressed [19]. *Lsd1*-knockdown flies were crossed with the

flies carrying UAS-P35 or UAS-DIAP1. The atrophied wing phenotype induced by *Lsd1*-knockdown (Figs. 3A and 3A') was effectively suppressed by wing specific expression of P35 (Figs. 3B and 3B') or DIAP1 (Figs. 3C and 3C'). These data suggest that *Lsd1*-knockdown induced atrophied wing phenotype is at least partially due to apoptosis in wing imaginal discs. Therefore, wing discs from third instar larvae of *Lsd1*-knockdown and control flies were immunostained with anti-cleaved caspase-3 IgG to detect caspase-dependent apoptotic cells. The wing pouch compartment of the wing imaginal disc of *Lsd1* knockdown flies (MS1096-GAL4>UAS-*Lsd1*IR) showed an increase in apoptotic cells compared with the control (Figs. 3D-F). As shown in Fig. 3G, the difference in the number of apoptotic cells in the wing pouches of *Lsd1*-knockdown flies and control flies was statistically significant ($p < 0.05$, Student's t test). These results indicate that knockdown of *Lsd1* in the wing disc induces apoptosis.

We also examined autophagy in *Lsd1*-knockdown flies using LysoTracker Blue, which showed that, compared with the control, *Lsd1*-knockdown induced autophagy (Figs. 3H and 3I). Since it is reported that over production of ROS causes both apoptosis [20] and autophagy [21], these data suggest that ROS generation may accumulate in dorsal wing discs of *Lsd1*-knockdown flies and cause cell death via apoptotic and autophagy pathways.

Knockdown of Lsd1 increased ROS generation

To detect intracellular ROS in *Lsd1*-knockdown flies, we used the non-fluorescent substrate CM-H2DCFDA, which can be oxidized by ROS to an intracellular green fluorescent product. Faint signals were detected in wing imaginal discs of MS1096-GAL4 and MS1096-GAL4>UAS-*GFP*IR control flies as shown in Figs. 4A and 4B, respectively. In contrast, much stronger fluorescent signals were detected in the wing pouch region of imaginal discs from *Lsd1*-knockdown flies (Fig. 4C). ROS signal detected outside of wing pouch in Fig. 4C could be explained by non-cell autonomous effect. The quantified data of fluorescent signals (Fig. 4D) confirmed that ROS generation was induced by the knockdown of Lsd1 in wing discs.

Knockdown of Lsd1 caused stress in mitochondria and defects in ATP production

The results described above suggest the primary effect of *Lsd1* knockdown during *Drosophila* wing development is ROS production, followed by induction of apoptosis and autophagy. A number of studies have demonstrated that mitochondria are an important source of ROS within cells [22-24]. Therefore, we examined whether the *Lsd1*-knockdown could affect mitochondrial function in *Drosophila*. First, we examined the morphology of mitochondria in *Lsd1*-knockdown flies by simultaneously expressing UAS-MitoGFP. MitoGFP has been widely used to mark mitochondria in both *Drosophila* and mammalian cells by expressing GFP fusion protein containing the mitochondrial-targeting sequence of citrate synthase [25-27]. Compared to control flies (Fig. 5A), the mitochondrial morphology in wing imaginal discs appeared to be expanded in *Lsd1*-knockdown flies expressing MitoGFP (MS1096-GAL4>UAS-

*Lsd1*IR/UAS-MitoGFP) (Fig. 5B). These data suggest that mitochondria are under stress in the *Lsd1*-knockdown flies.

Mitochondria are responsible for the production of ATP, a major cellular energy source [28,29]. Since fat body in *Drosophila* is known for its importance in not only for energy storage but also lipid metabolism, similarly to the mammalian liver [30], we measured the amount of ATP in entire third instar larvae in which *Lsd1* was knocked down in the fat body by the Fb-GAL4 driver. Compared to control flies carrying Fb-GAL4 alone, the ATP level in *Lsd1*-knockdown flies (Fb-GAL4>UAS-*Lsd1*IR*)* was decreased (Fig. 5C). Moreover, accumulation of free fatty acids may result in increased fatty acid uptake and oxidation and may ultimately increase ROS production [31]. We then examined total FFA levels, and found that FFA levels were significantly elevated in *Lsd1*-knockdown flies compared with the control (Fig. 5D). These data strongly suggest a defect in mitochondrial function caused by *Lsd1*-knockdown.

4. Discussion

Lsd1 has been reported to act as a conserved surface-associated module of lipid droplets that promotes stimulated lipolysis by response to cAMP/PKA signaling [2]. PKA-dependent perilipin phosphorylation and recruitment of HSL to LDs, which are distinctive features of stimulated lipolysis in mammalian adipocytes, lead to the discovery of a conserved function of the TG mobilization module between flies and mammals [32]. However, to date, there has been no report of other possible *Lsd1*

functions during development. This study demonstrates that knockdown of the *Lsd1* gene in the entire *Drosophila melanogaster* body caused lethality, indicating that *Lsd1* is essential function for viability. When *Lsd1* was knocked down in wing discs using the MS1096-GAL4 driver, severely atrophied wings were observed. Cell death in the wing pouch of *Lsd1* knockdown flies was demonstrated to be due to an increase in apoptosis and autophagy. Previous reports have shown that the increase in ROS production is accompanied by apoptosis in eukaryotic cells. Our observations demonstrated that knockdown of Lsd1 expressed a causal signal of increasing ROS generation, which might be related to apoptosis and autophagy in wing imaginal discs. Further analysis is necessary to clarify this point. Moreover, the atrophied phenotype of *Lsd1*-knockdown flies in the present study was rescued by the expression of anti-apoptotic genes DAIP1 and P35 [19]. Overall, these results indicated that increased ROS generation might cause apoptosis and autophagy and result in the atrophied wing phenotype in adults.

Perilipin 1 has a central role in modulating adipocyte lipid metabolism. Under normal conditions, perilipin 1 suppresses maximal lipolysis by forming a complex with CGI-58/abhydrolase domain-containing protein 5 (ABHD5), which is an activator of BMM in *Drosophila* (corresponding to ATGL) [2]. Interestingly, the interaction between CGI-58 and perilipin 1 is sensitive to hormonal stimulation. The lipolytic activation of fat cells disrupts this interaction and leads to the dissociation of CGI-58 from the complex, resulting in the binding of CGI-58 and ATGL for maximal lipolysis [33]. We hypothesize that absence of *Lsd1* may lead to the release CGI-58 and enhance the activity

of BMM. Enhanced BMM activity can promote the increase of free fatty acids, which enter mitochondria and are responsible for ATP synthesis. Specifically, the overproduction of free fatty acids can cause mitochondrial stress, increase ROS production, and lead to cell death [34]. Mitochondrial stress can also cause decrease of ATP levels and affect the mitochondrial morphology [35]. In the present study, we observed that the morphology of mitochondria became extensively expanded in wing imaginal discs, and the ATP level decreased in *Lsd1*-knockdown flies. In addition, the FFA level in knockdown flies was also significantly elevated. Taken together, these observations at least partially support our hypothesis that increased free fatty acids in *Lsd1* knockdown flies cause mitochondrial stress and consequently induces cell death by the apoptotic and autophagy pathways, ultimately affecting wing phenotype.

5. References

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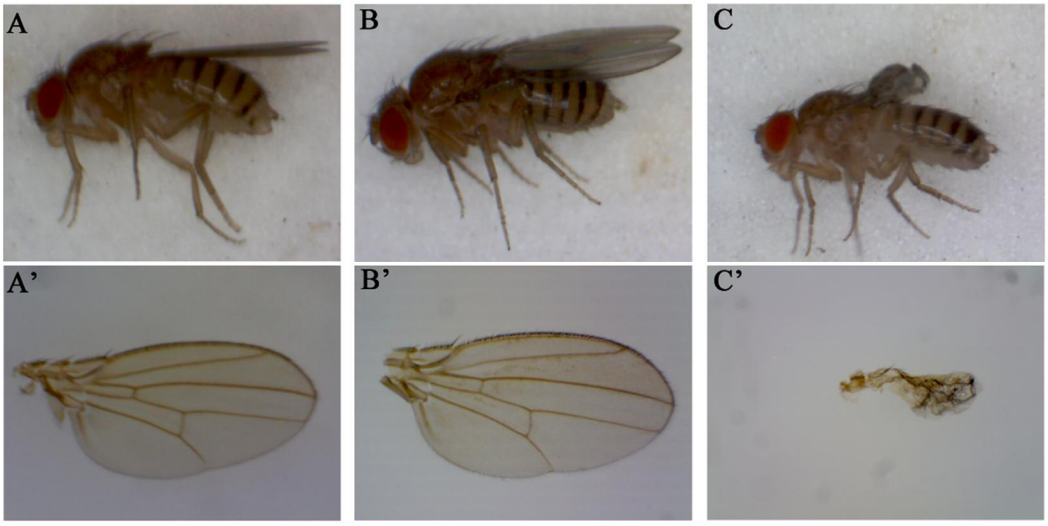

Figure 1. Morphological aberrations in the adult wing of *Lsd1* knockdown flies by MS1096-GAL4 driver. A-C, whole body of adult flies. A'-C', enlarged images of adult wings. A and A', control fly (MS1096-GAL4/+; +; + [MS1096-GAL4]). B and B', control fly carrying *GFP*IR gene (MS1096-GAL4/+; UAS-*GFP*IR/+; + [MS1096-GAL4>UAS-*GFP*IR]]. C and C', *Lsd1*-knockdown fly (MS1096-GAL4/+; UAS-*Lsd1*IR/+; + [MS1096-GAL4>UAS-*Lsd1*IR]). Flies were reared at 25°C. Control flies, MS1096-GAL4 (A and A') and MS1096-GAL4>UAS-*GFP*IR (B and B') showed normal wing phenotypes, while *Lsd1*-knockdown fly had atrophied wing (C and C').

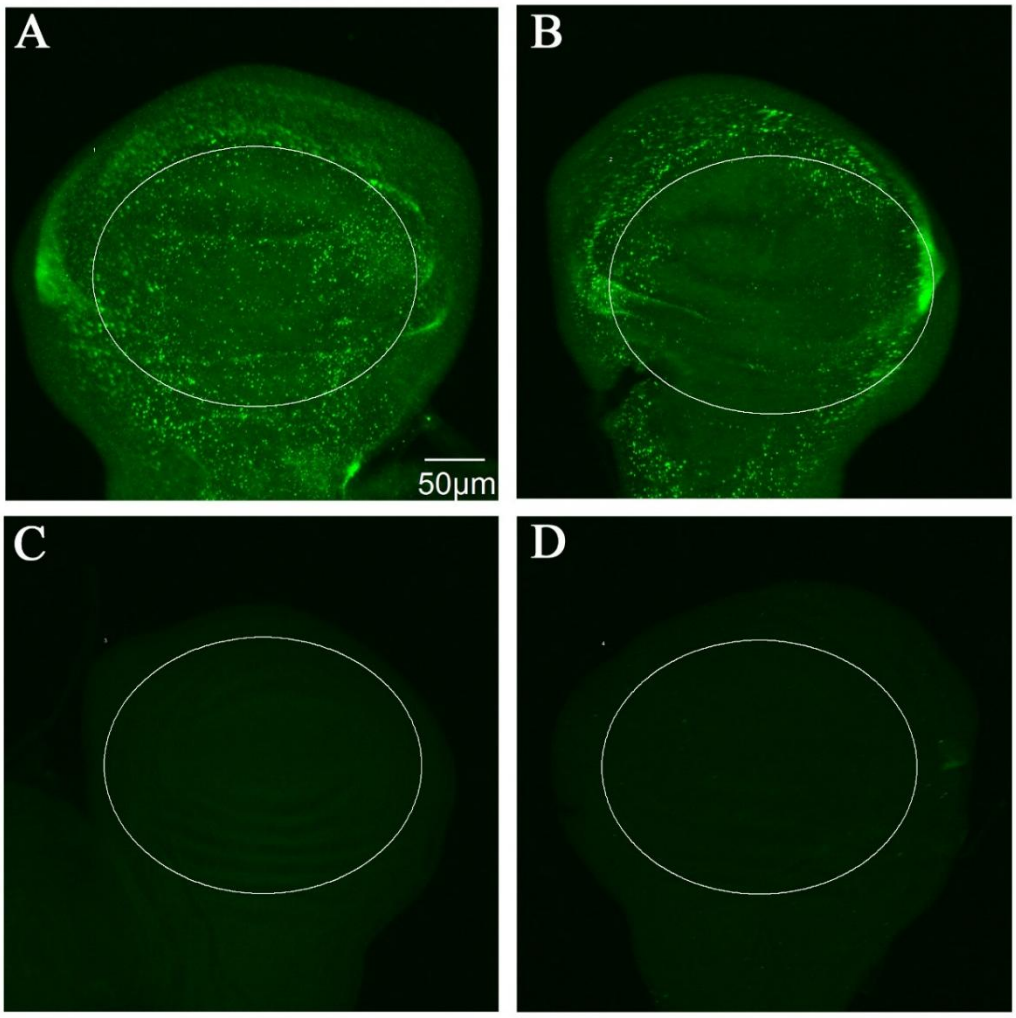

Figure 2. Immunostaining of wing imaginal discs with anti-Lsd1 antibody. A and B, wing imaginal discs were reacted with rabbit anti-Lsd1 antibody followed by anti-rabbit IgG Alexa Fluor™ 488 antibody. MS1096-GAL4 flies (A) showed signal clearly and MS1096-GAL4>UAS-*Lsd1*IR flies (B) showed decreased Lsd1 signal in the wing of wing imaginal discs. C and D, immunostaining of wing imaginal discs with only the anti-rabbit IgG Alexa Fluor™ 488 antibody showed no detectable signal. C, MS1096-GAL4; D, MS1096-GAL4>UAS-*Lsd1*IR. Flies were reared at 25°C. The circles indicate wing pouch of wing discs where *Lsd1* was knocked down.

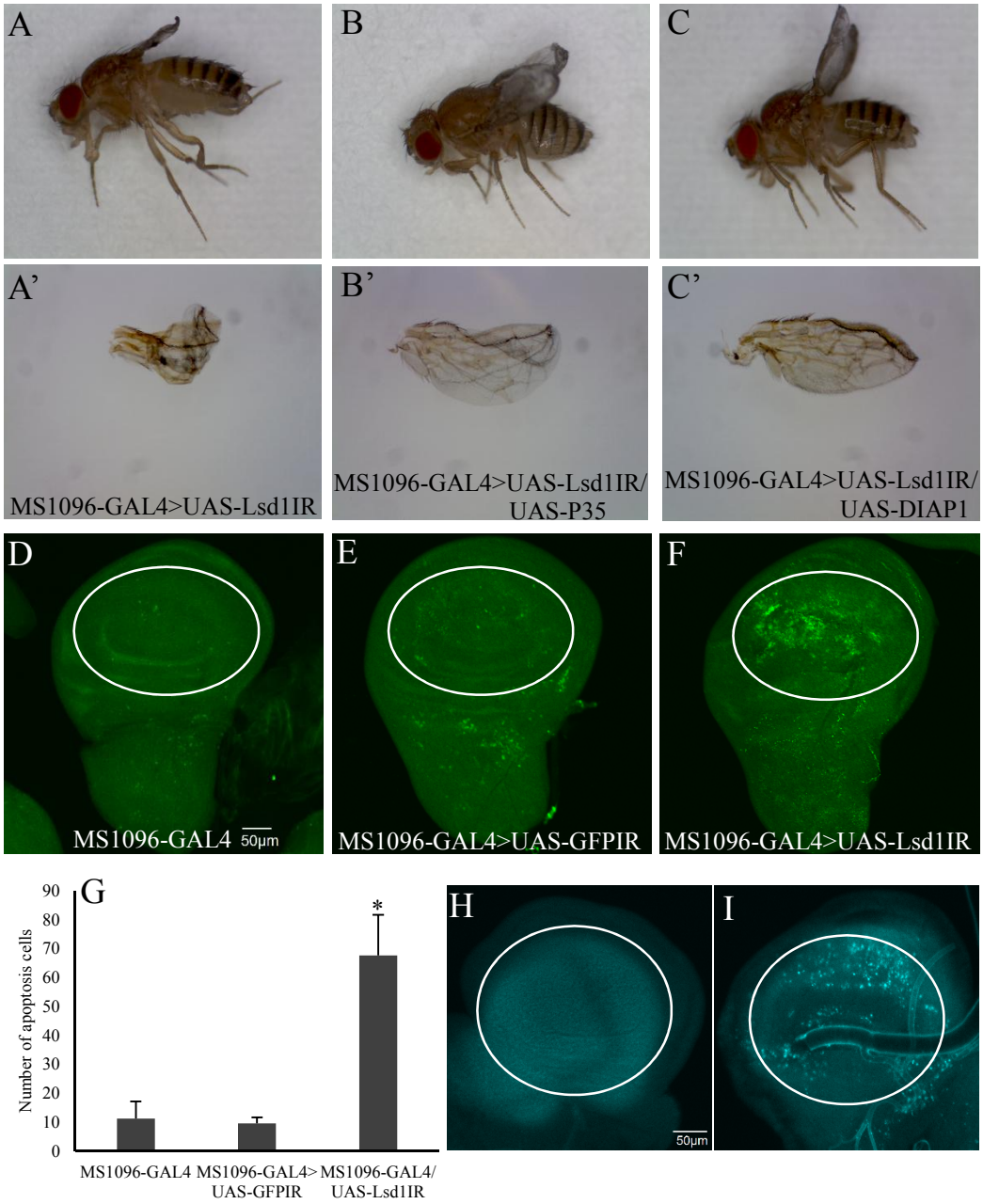

Figure 3. Knockdown of *Lsd1* induces cell death in wing imaginal discs. The atrophied wing phenotype of *Lsd1*-knockdown flies was rescued by P35 (B and B') and DIAP1 (C and C'). A-A', MS1096-GAL4>UAS-*Lsd1*IR. B-B', MS1096-GAL4/+; UAS-*Lsd1*IR/+; UAS-P35/+ (MS1096-GAL4>UAS-*Lsd1*IR/UAS-P35). C-C', MS1096-GAL4/+; UAS-*Lsd1*IR/+; UAS-DIAP1/+ (MS1096-GAL4>UAS-*Lsd1*IR/UAS-DIAP1). D-F, immunostaining of wing imaginal discs with anti-active caspase-3 antibody. Control

lines, MS1096-GAL4 (D) and MS1096-GAL4>UAS-*GFP*IR (E), showed few cell death signals, while *Lsd1*-knockdown line MS1096-GAL4>UAS-*Lsd1*IR (F) showed increased cell death signals via apoptosis. G, average number of apoptotic cells in the wing pouch (n = 10); *, $p < 0.05$. Data are expressed as the mean ± S.D. H-I, autophagy was determined by LysoTracker staining. H, the control fly MS1096-GAL4. I, *Lsd1*-knockdown fly MS1096-GAL4>UAS-*Lsd1*IR. The circle indicates the wing pouch of the wing disc. The flies were reared at 25°C.

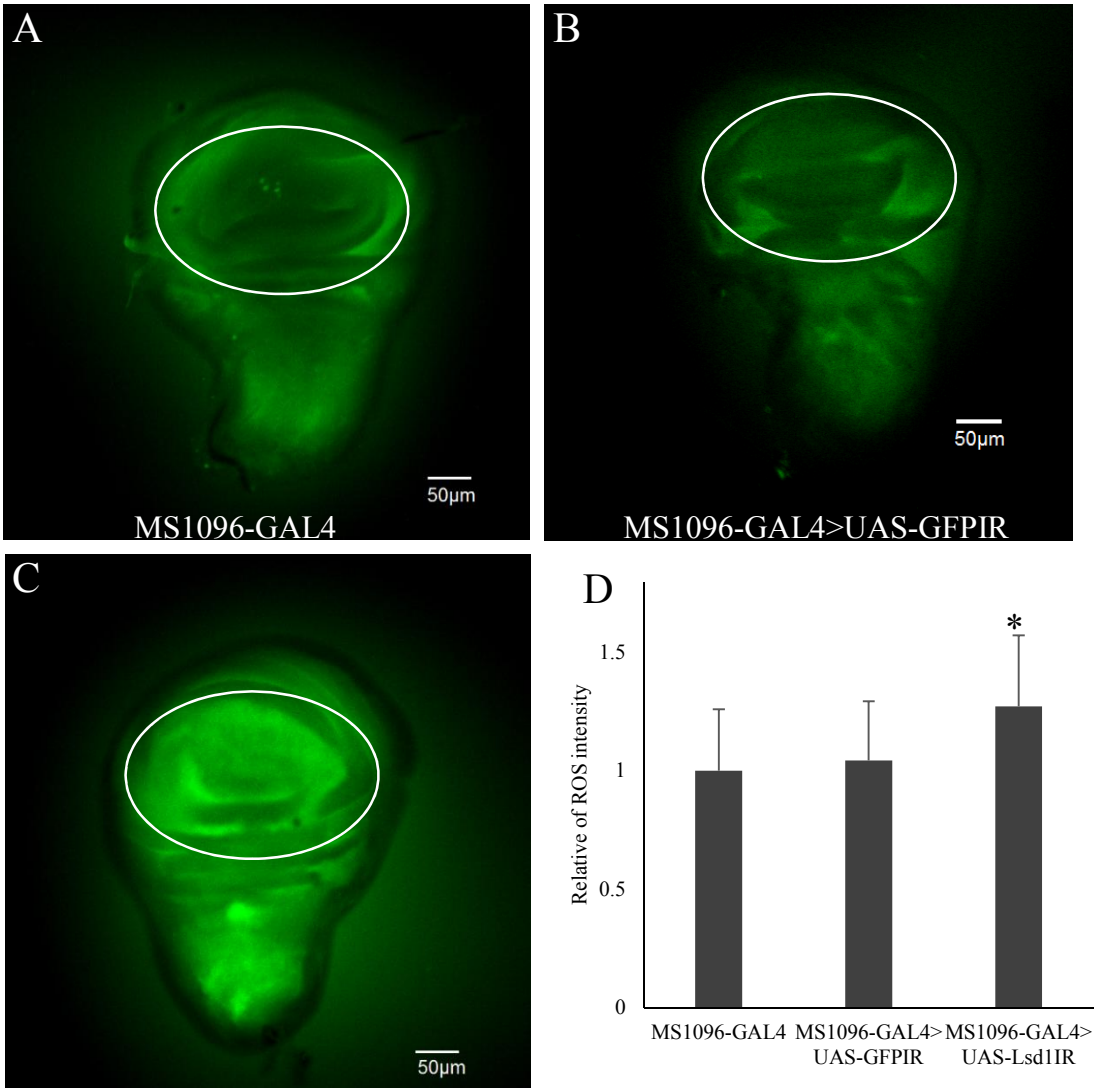

Figure 4. Knockdown of *Lsd1* induces ectopic ROS in wing imaginal discs. Wing discs of third-instar larvae were incubated with substrate CM-H2DCFDA. The control lines MS1096-GAL4 (A) and MS1096-GAL4>UAS-*GFP*IR (B) showed unclear ROS signal in the wing disc (marked by the circle), while the *Lsd1*-knockdown line (MS1096-GAL4>UAS-*Lsd1*IR) showed an increased ROS signal (C). D, Quantification of fluorescent ROS signals in the wing pouch region. Mean intensities with standard deviation from 6 imaginal discs are shown; *, $p < 0.05$, Student's *t* test.

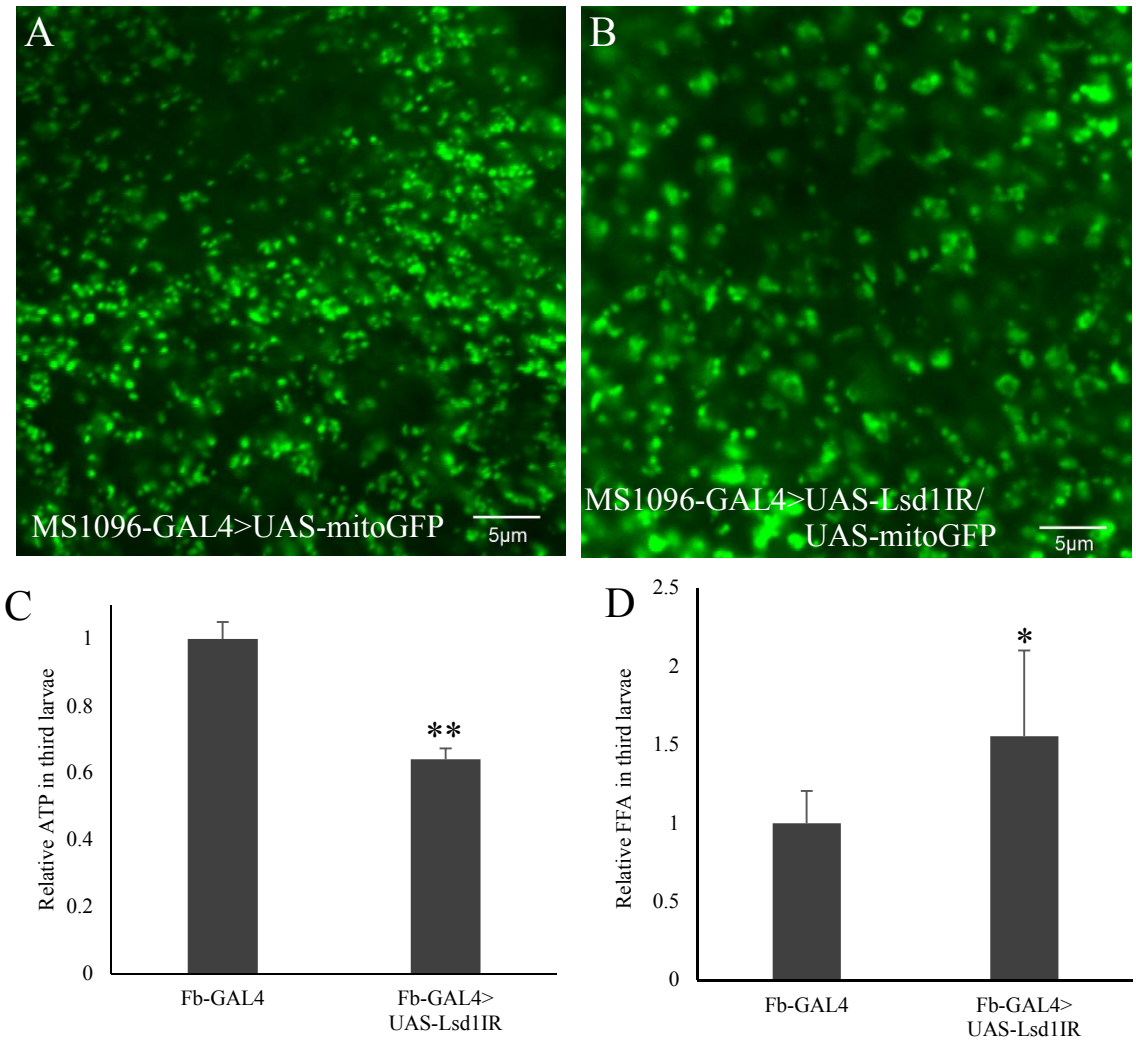

Figure 5. Knockdown of *Lsd1* induces extensive mitochondrial expansion and defects in ATP and FFA production. A and B, the GFP signal expressed in mitochondria of wing disc cells were observed under BX-50 fluorescence microscopy. The control flies MS1096-GAL4/+; +; UAS-MitoGFP/+ (MS1096-GAL4>UAS-MitoGFP) showed normal mitochondrial matrix (A), while the *Lsd1*-knockdown flies MS1096-GAL4/+; UAS-*Lsd1*IR/+; UAS-MitoGFP/+ (MS1096-GAL4>UAS-*Lsd1*IR/UAS-MitoGFP) exhibited extensive mitochondrial expansion (B). Relative amounts of ATP (C) and free fatty acids (FFA) (D) of *Lsd1*-knockdown flies *yw*; Fb-GAL4/UAS-*Lsd1*IR; + (Fb-

GAL4>UAS-*Lsd1*IR). ATP and FFA amounts in whole bodies of third instar larvae were normalized to body weight. The experiments were repeated at least 3 times. *, $p < 0.05$ **, $p < 0.001$, Student's *t* test. The flies were reared at 25°C.

Table 1. Summary of phenotypes induced by knockdown of *Lsd1* with various GAL4 driver lines.

GAL4 Line	Expression Pattern	Phenotype with UAS-*Lsd1*IR
MS1096	Dorsal wing disc	Atrophied wing at 25 and 28 °C
En	Posterior wing disc and embryo	Lethal at 25 °C
GMR	Eye disc	No detectable phenotype
Fb	Fat body	Delay in growth at 25 °C, lethal at 28 °C
Act5C	All tissues	Lethal at 25 °C
Tubp	All tissues	Lethal at 25 °C

Conclusions

In my thesis study, *Drosophila* is used as a model to establish the transgenic *bmm* promoter-GFP *Drosophila* for screening anti-obesity agents (Chapter 1), and discover novel *Lsd1* gene function in *Drosophila* wing development (Chapter 2).

Chapter 1

Triacylglycerol is the major component for lipid storage, it is essential for normal physiology, its excessive accumulation causes obesity in adipose tissue and is associated with organ dysfunction in non-adipose tissue. Here, we focused on the *Drosophila* model to develop therapeutics for preventing obesity. The brummer (*bmm*) gene in *Drosophila melanogaster* is known to be homologous with human adipocyte triglyceride lipase, which is related to the regulation of lipid storage. We established a *Drosophila* model for monitoring *bmm* expression by introducing the green fluorescent protein (*GFP*) gene as a downstream reporter of the *bmm* promoter. The third instar larvae of *Drosophila* showed the GFP signal in all tissues observed and specifically in the salivary gland nucleus. To confirm the relationship between *bmm* expression and obesity, the effect of oral administration of glucose diets on *bmm* promoter activity was analyzed. The *Drosophila* flies administered with high-glucose diets showed higher lipid contents, indicating the obesity phenotype; this was suggested by a weaker intensity of the GFP signal as well as reduced *bmm* mRNA expression. These results demonstrated that the transgenic *Drosophila* model established in this study is useful for screening anti-obesity

agents. We also report the effects of oral administration of histone deacetylase inhibitors and some vegetables on the *bmm* promoter activity.

We have developed a *Drosophila* model with GFP-indicating ability to be used as a lipid storage marker. Our model is recommended for the fast and simultaneous screening of a large number of samples. In addition, because of the similarities of the basic metabolic functions and analogous organs between *Drosophila* and vertebrates, this *Drosophila bmm*-expressing monitor constructed in our study is a promising model for the screening of novel drugs for treating obesity.

Chapter 2

Perilipins are evolutionarily conserved from *Drosophila* to humans, the lipid storage droplet 1 (*Lsd1*) is a *Drosophila* homolog of human perilipin 1. The function of *Lsd1* as a regulator of lipolysis in *Drosophila* has been demonstrated, as the *Lsd1* mutant causes an increase of lipid droplet size. However, the functions of this gene during development are still under investigation. In order to determine the function of *Lsd1* during development, *Lsd1* was knocked down in *Drosophila* using the GAL4-UAS system. Selective knockdown of *Lsd1* in the dorsal wing disc caused an atrophied wing phenotype. The generation of reactive oxygen species in the wing pouch compartment of the *Lsd1*-knockdown flies was significantly higher than in the control. Immunostaining with caspase-3 antibody revealed a greater number of apoptotic cells in *Lsd1*-knockdown wing discs than in the control. Cell death by autophagy was also

induced in the knockdown flies. Moreover, cells deprived of *Lsd1* showed mitochondrial expansion and decreased ATP levels. These results strongly suggest that knockdown of *Lsd1* induces mitochondrial stress and the production of reactive oxygen species that result in cell death, via apoptosis and the autophagy pathway. These results highlight the roles of *Drosophila Lsd1* during wing development.

In this study, we present data which indicate that knockdown of *Lsd1* could induce mitochondrial stress and result in cell death via apoptotic and autophagy pathways. Therefore, we conclude that *Lsd1* is necessary for the development of *Drosophila* wings, possibly through maintaining the function of mitochondria. This is the first report stating the role of *Lsd1* during *Drosophila* development and will provide the starting point for further elucidation of the roles of *Lsd1* in *Drosophila* development.

List of publications

1. A Drosophila Model for Screening Antiobesity Agents

Tran Thanh Men, Duong Ngoc Van Thanh, Masamitsu Yamaguchi, Takayoshi Suzuki, Gen Hattori, Masayuki Arii, Nguyen Tien Huy, and Kaeko Kamei

BioMed Research International, Vol. 2016 (2016), 10 pages, 2016.

Doi: 10.1155/2016/6293163.

2. Function of Lipid Storage Droplet 1 (Lsd1) in Wing Development of *Drosophila melanogaster*

Tran Thanh Men, Tran Duy Binh, Masamitsu Yamaguchi, Nguyen Tien Huy and Kaeko Kamei

Int. J. Mol. Sci. 2016, 17(5), 648;

Doi: 10.3390/ijms17050648.

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