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Chapter 7

Autophagy Regulates Lipid Droplet Formation and Adipogenesis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/51447

1. Introduction

Proteolysis in eukaryotic cells can be separated into two major pathways: one is mediated by the ubiquitin-proteasome system and by the autophagy-lysosome system. Substrates of lysosomes may be taken through heterophagocytosis, endocytosis, or autophagy into heterophagosomes, early endosomes, or autophagosomes, which receive lysosomal enzymes via transporting vesicles from the trans-Golgi network or lysosomes and become heterophagolysosomes, late endosomes, or autolysosomes [1-4]. Different from endocytosis and heterophagocytosis, substrates of autophagy are limited to intracellular constituents and include various membranous organelles together with a part of the cytoplasm [3]. Until recently, 18 autophagy-related proteins (Atgs) have been shown to be involved in autophagosome formation, although more than 30 Atgs have been uncovered to regulate autophagy [2, 5]. Such autophagy contributes to the maintenance of cellular homeostasis [6]. Impairment of autophagy, therefore, causes severe degenerative alterations in various tissue cells [1, 4, 7]. In addition to the maintenance of basal cellular metabolism, autophagy is induced in response to various stresses such as starvation and diseases [8-13].

Since one of the major sources in living organisms is lipid, the metabolism of lipid is finely regulated. Besides adipose tissues, neutral lipids, most of which are mainly triacylglycerol (TAG) and cholesterol ester (CE) in cells, are stored in a sort of inclusion body, called the lipid droplet or lipid body [14-16]. Under nutrient-rich situations, excess fatty acids may be converted to TAG through lipogenesis and stored in lipid droplets, whereas lipid droplets under starvation conditions may be a source to produce lipids by lipolysis for cell usage [16]. Under stress or starvation states, proteins are also degraded to produce an amino acid pool that is used in part for energy metabolism through glyconeogenesis, while free fatty acids (FFAs) from adipocytes are delivered to hepatocytes and cardiac myocytes where they are used as an energy source via β-oxidation [15, 16]. At the initial stage during fasting,

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FFAs are also converted to triacylglycerol (TAG) and stored as lipid droplets (LDs) that are used for an energy source when starvation continues.

It has been shown that loss of Atg7 largely suppresses LD formations in hepatocytes and cardiac myocytes 24 hours after the start of starvation, although numerous LDs accumulate in normal hepatocytes and cardiac myocytes under the same conditions [11]. Moreover, a mouse model with a targeted deletion of *atg7* in adipose tissue has been generated; the mutant mice were slim and contained only 20% of the mass of white adipose tissue (WAT) found in wild-type mice [17]. These mutant mice exhibit a high sensitivity to insulin that reduces low fed plasma concentrations of FFAs, and also exhibit a marked decrease in plasma concentrations of leptin but not adiponectin, and lower plasma concentrations of TAG. LDs, initially considered inert lipid deposits, have gained the classification of cytosolic organelles during the last decade due to their defined composition and the multiplicity of specific cellular functions in which they are involved [18]. At present, it remains largely unknown how autophagy is involved in LD metabolism, although lipophagy may occur in cells.

One thing that we have found is that a lipidated form of LC3, representing the Atg8 family of proteins, is localized on the surface of LDs and also in LD fractions, in addition to ADRP and perilipin, representing the PAT family of proteins that cover the surface of LDs. In this review we will introduce the LC3 conjugation system that is involved in lipid metabolism via LD formation..

2. Microtubule-associated protein 1A/1B light chain 3 (LC3) localizes not only to autophagosomal membranes but also on the surface membrane of LDs in hepatic and cardiac tissues under starvation conditions

It is well known that hepatocytes are morphologically and functionally different between the periportal and perivenous regions; periportal hepatocytes are glycogenic and lipolytic, while perivenous hepatocytes are glycolytic and lipogenic [19]. Lysosomes in hepatocytes are also more abundant in the perivenous region than in the periportal region [19, 20].

Such a morphological difference is largely altered when mice are starved for 24 or 48 hours; numerous autophagosomes that contain part of the cytoplasm and possess the cisternal or double isolation membranes and autolysosomes appear near bile canaliculi in hepatocytes 24 hours after the commencement of starvation and those that in some cases contain mitochondria are seen 48 hours later [4, 9]. In addition to autophagosomes and autolysosomes, LDs accumulate abundantly in the cytoplasm of hepatocytes. When livers of conditional Atg7-deficient mice ($Atg7^{Flox/Flox}$: Albumin-Cre) at 22 days, or 6 or 8 weeks of age were examined, positive staining for LDs is sparsely detected in the hepatocytes under starvation conditions for 12 or 24 hours, although they are abundant in the cytoplasm of the hepatocytes of the control littermate mouse liver (Fig. 1). Size and amount of stained LDs were much smaller in hepatocytes deficient in Atg7 than in the control hepatocytes. When examined by electron microscopy, the diameter of LDs in the control hepatocytes is various

and ranged up to 2.61 μm (the mean diameter (± SD)) is 1.12 ± 0.17 μm). Different from the control, mutant hepatocytes mainly possess smaller LDs whose diameter ranged up to 0.83 μm (the average diameter is 0.19 ± 0.17 μm). Moreover, small LD-like bodies were observed in the luminal space of rough surfaced endoplasmic reticulum (rER), Golgi cisternal ends, and vesicles near the Golgi apparatus of the cells, indicating that the lumenally-sorted LDs are normally produced in the rER of both mutant and control hepatocytes. Importantly, the total TAG amount in Atg7-deficient liver and control livers is half of that in the control liver. These data indicate that the conjugation system of LC3 by Atg7 is required for the formation of LDs.

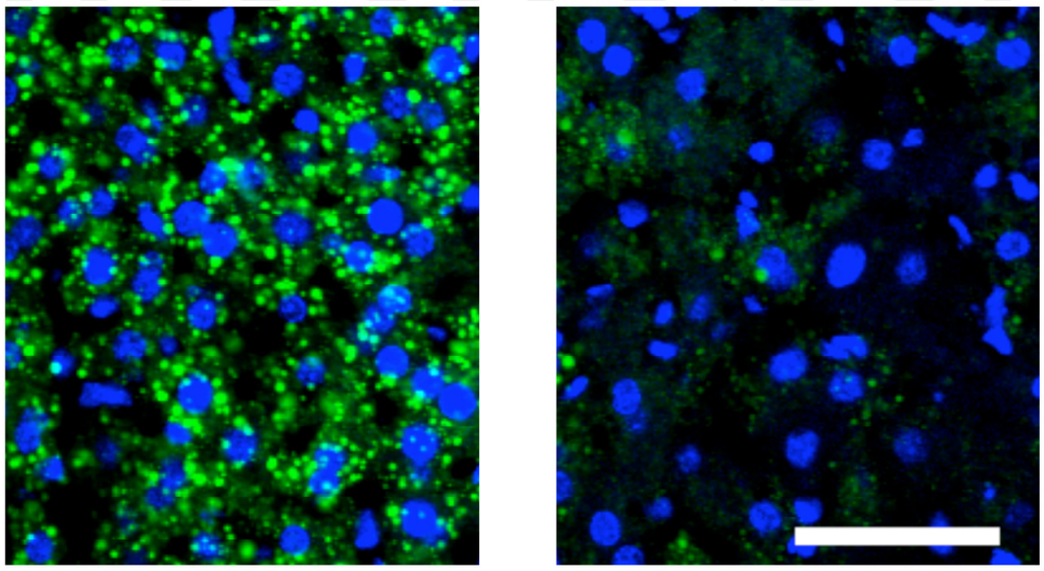

Figure 1. BODIPY staining (green) of hepatocytes obtained from control littermate (left) and Atg7-deficient (right) mice at the age of 6 weeks. Mice were housed under starvation conditions for 24 hours. BODIPY-positive LDs are abundant in hepatocytes from control littermate mouse, while positive LDs largely disappear from the Atg7-deficient hepatocytes. Bar= 50 μm

3. Inhibition of LD formation in hepatocytes deficient in Atg7 after deprivation

It has been shown that autophagy may play an important role in normal adipogenesis and that inhibition of autophagy by disrupting the *atg7* gene has a unique anti-obesity and insulin sensitization effect [17]. LDs are ubiquitous in eukaryotic cells, while excess free fatty acids and glucose in plasma are converted to TAG and stored as LDs (Fig. 1). However, the mechanism for the generation and growth of LDs in cells is largely unknown. As stated above, Atg7 that mediates LC3 lipidation and is essential for autophagy is involved in LD formation [4, 9]. LD formation accompanied by accumulation of TAG induced by starvation is largely suppressed in hepatocytes that cannot execute autophagy.

It is well known that LC3, microtubule-associated protein A/B light chain 3, is localized on the surface of the isolation membrane when starvation is induced [9]. Using GFP-LC3 transgenic mice, GFP-LC3 becomes dot-shaped, cap-shaped and ring-shaped in

hepatocytes and cardiac myocytes under starvation conditions [21]. When immunostained for LC3 under starvation conditions, positive staining is, as shown by GFP-LC3 in TG mice, dot-shaped, cap-shaped, and ring-shaped in hepatocytes and cardiac myocytes (Figs. 2 and 3, Tables 1 and 2). Interestingly, positive staining of LC3 in cardiac myocytes is longitudinally arrayed in parallel to myofilaments. In both hepatocytes and cardiac myocytes, LDs are abundant 24 hours after the onset of starvation [9]. In particular, electron microscopic observations show that no clear-cut autophagosomes are detected in cardiac myocytes, although many large LDs are arranged longitudinally in parallel to the array of myofilaments together with mitochondria. This arrangement of LDs in cardiac myocytes is very similar to that of LC3-positive granules. To examine the relationship between staining patterns of LC3 and LDs in both hepatocytes and cardiac myocytes, double staining for perilipin with LC3 was performed. The results indicate that perilipin-positive LDs are also immunopositive for LC3 on the surface of LDs in both hepatocytes and cardiac myocytes [9]. In hepatocytes, there is also dotted staining of LC3 that is free of LD staining, whereas most LD-positive staining is co-localized with LC3 in cardiac myocytes (Figs. 2 and 3, Tables 1 and 2).

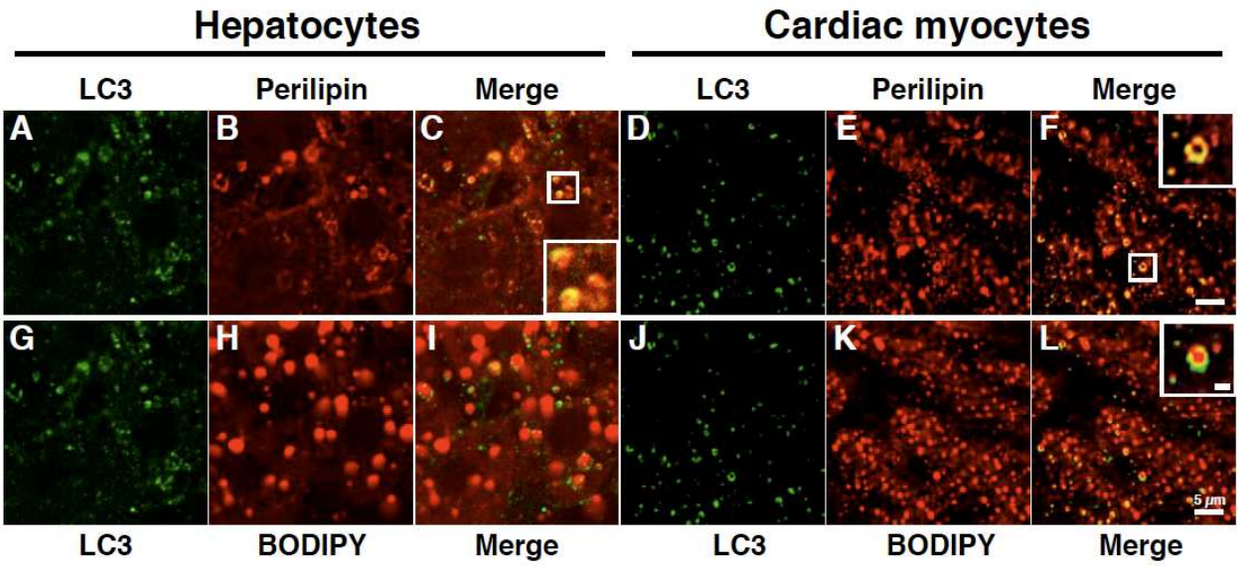

Figure 2. LC3 localizes not only to autophagosomes but also LDs in liver and heart tissues of mice under starvation conditions for 24 hours. (A-L) Double staining of LC3 (green) (A, D, G, J) and perilipin (B, E) or BODIPY (H, K) (red) in liver (A-C, G-I) and heart (D-F, J-L) tissues. Ring-shaped structures that were costained for LC3 and perilipin in boxed areas (C, F) are enlarged in insets. One of the ring-shaped structures that were costained for LC3 and BODIPY in a boxed area (L) was enlarged in an inset. Bar indicates 5 μm (1 μm in inset). This figure is referred from Biochemical and Biophysical Research Communications (Shibata et al., 382 (2009) 419–423).

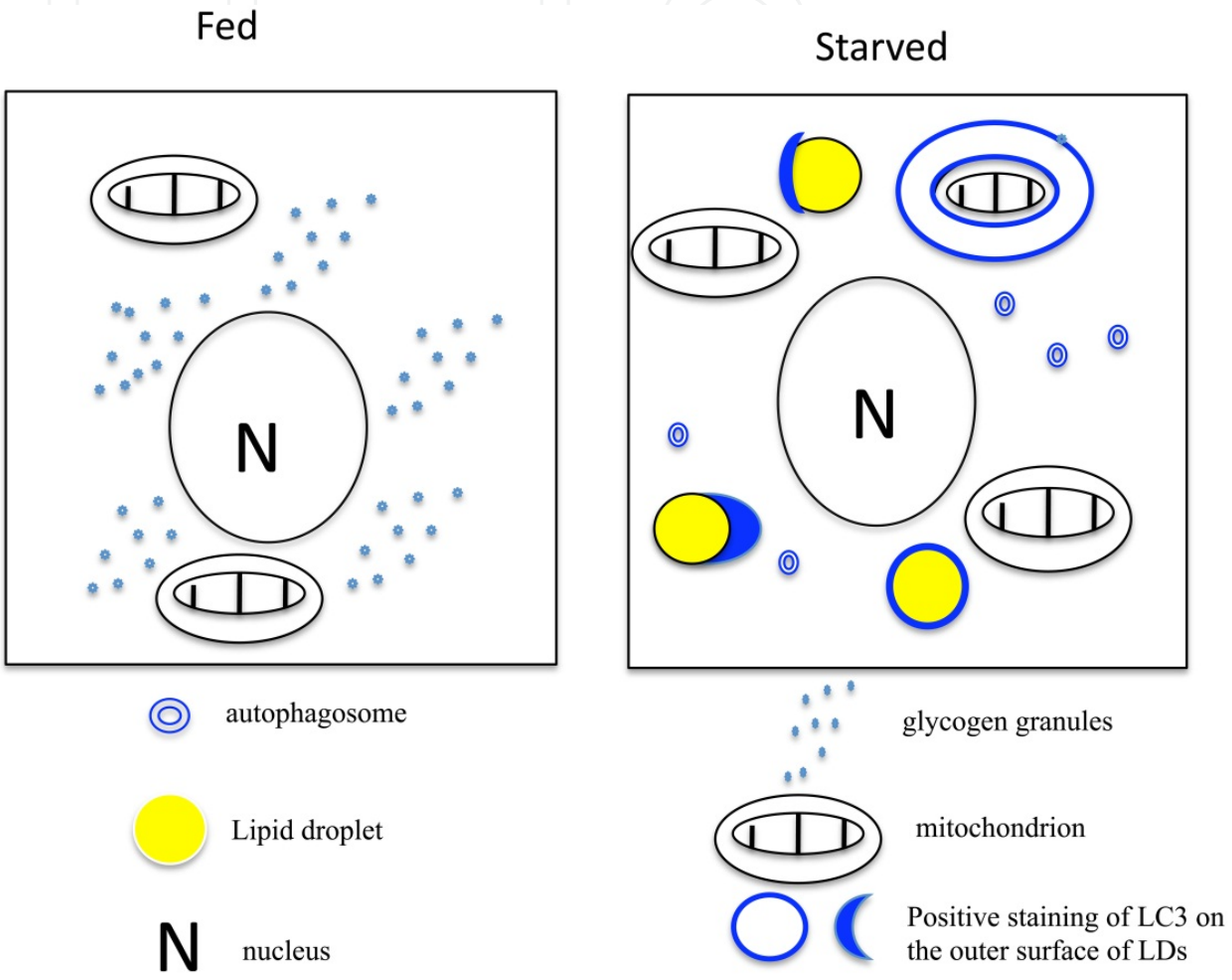

Figure 3. Schematic demonstrations of LC3 staining in cells from mice under fed (left panel) and starved (right panel) conditions. When mice are fed, cells start to produce and store glycogen granules, whereas under starved conditions, lipid droplets are increased and many autophagic granules with double membranes increase in the cytoplasm. In this situation, the lipidated form of LC3 is attached to the isolation membrane of autophagosomes (blue color) and the surface membrane of LDs (yellow). Starvation is further continued, and some mitochondria are enwrapped by the isolation membrane (mitophagy).

	hepatocytes				cardiac myocytes			
Starvation	Apg		LD		Apg		LD	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
	++	++	++	++	+/-	+/-	++	+
LC3 (ICH)	++	++	++	++	+/-	-	+	+/-
LC3-II (WB)	++	++	++	++	++	+/-	+	+/-
Mitophagy (EM)	+/-	+	not detected					

Apg, autophagosome; EM, electron microscopy; h, hours; ICH, immunocytochemistry; ++, highly positive; +, positive; +/-, rare; WB, Western blot

Table 1. Morphological changes detected in hepatocytes and cardiac myocytes after starvation

	Liver	heart
lipid fraction		
ADRP	++	++
LC3-II	+	+
Staining of LD in LD fraction from livers with anti-LC3	++	

++, highly positive; +, positive

Table 2. Proteins detected in lipid fractions

In fact, cytosolic LC3 is converted to membrane-bound LC3 (LC3-II) in both hepatocytes and cardiac myocytes 24 hours after the start of starvation. Electron microscopic morphometry reveals that the volume densities of autophagosomes/autolysosomes and LDs increase in hepatocytes 24 and 48 hours after the onset of starvation, whereas autophagosomes and autolysosomes are rarely found in cardiac myocytes and the volume density of LDs is only counted and significantly increased in them [9]. The amounts of TAG in hepatocytes and cardiac myocytes significantly increase after the onset of starvation, whereas the increase in TAG amount is much lower in cardiac myocytes than in hepatocytes and continues until 24 hours. Moreover, LC3 is localized on the surface of LDs and LC3-II (lipidated form) is fractionated into a perilipin and ADRP (LD marker)-positive lipid fraction from the starved liver and cardiac myocytes, respectively. In fact, the surface of such LDs obtained from the LD fraction is labeled by gold particles showing the antigenicity of LC3. Taken together, these results indicate that the LC3 conjugation system is critically involved in lipid metabolism via LD formation.

4. Generation of LDs is inhibited in various LC3 mRNA-knockdown cultured cells

The cytoplasmic LD is an organelle that has a neutral lipid core with a single phospholipid layer. LDs are believed to be generated between the two leaflets of the endoplasmic reticulum (ER) membrane and to play various roles, such as high efficiency energy storage [16]. However, it remains largely unknown how LDs are generated or grow in the cytoplasm. As has been shown previously, the Atg conjugation system that is essential for autophagosome

formation is involved in LD formation in hepatocytes and cardiac myocytes. This tendency has also been confirmed in white adipose tissue of conditional Atg7-knockout mice that show less production fat bodies in the tissue [17].

It has been shown that LDs temporally accumulate in the cultured cell lines during proliferation [22]. We have confirmed that LDs are produced in cultured cells seeded at a density of 70% confluency [11]. Accordingly, it has been shown that LDs that are stained with BODIPY are significantly augmented in PC12 cells 12 hours after the start of cultures, while immunosignals for LC3 are colocalized with BODIPY-positive LDs [11]. By immunoelectron microscopy, gold particles indicating LC3 are found on the surface of LDs in PC12 cells. Moreover, by cell fractionation the membrane type of LC3 is demonstrated in the perilipin-positive LD fraction. It still remains unknown whether LC3 itself is involved in LD formation. Since LC3 is a substrate of Atg7, cultured cell lines such as HeLa cells, PC12 cells, HepG2 cells, and Cos-1 cells, were examined to check the relationship of LC3 and LD formation. Expression of LC3 was suppressed by the method of RNA interference (RNAi), and it was found that LD formation is largely inhibited in these cells. TAG, a major component of LDs, is synthesized and degraded in LC3 mRNA-knockdown cells as well as in control cells. Interestingly, the potential for bulk protein degradation in the knockdown-cells is also evident in the control cells.

3T3 L1 cells, a progenitor cell line of adipocytes, accumulate LDs 12 hours after the start of cultures and LD formation is suppressed in the cells when mRNA of LC3 is knocked down [11]. Differentiation of L1 cells into adipocytes is confirmed by the mRNA expression of sterol regulatory element binding factor 1 (SREBF1) and peroxisome proliferator activated receptor γ (PPARγ), adipose specific proteins. It takes 6days until the L1 cells differentiate, and as the cells differentiate, it is found that the amount of LC3 mRNA also increases. In this differentiated situations, the surface of LDs in L1 cells is covered with perilipin and LC3. In LC3 mRNA-knockdown L1 cells, however, BODIPY-positive LDs largely disappear.

These findings indicate that LC3 is involved in the LD formation regardless of the bulk degradation, and that LC3 has two pivotal roles in cellular homeostasis mediated by autophagy and lipid metabolism.

5. Connection between autophagy and lipid metabolism

Recent studies provide supporting evidence for a connection between autophagy and lipid metabolism, both lipid storage and lipolysis. The involvement of autophagy in lipolysis of LDs in hepatocytes has been reported by the groups of Czaja and Cuervo, who showed that loss of Atg7 ($Atg7^{Flox/Flox}$:albumin-Cre mice) results in accumulation of LDs in hepatocytes [18, 23-25]. Lipophagy, which is a form of autophagy that enwraps LDs by the isolation membrane has recently been considered important for the production of FFAs by degrading TAGs under acidic milieu and the FFAs produced fuel cellular rates of mitochondrial β-oxidation [18, 23-25]. This process, called lipophagy has recently been

thought to function to regulate intracellular lipid stores, cellular levels of free lipids such as fatty acids and energy homeostasis [25]. On the contrary, as described in this chapter, it has been shown that loss of Atg7 ($Atg7^{Flox/Flox}$:albumin-Cre mice) largely suppresses LD formation in hepatocytes and cardiac myocytes 24 hours after the start of starvation, although numerous LDs accumulate in normal hepatocytes and cardiac myocytes under the same conditions [9] (Fig. 1).

Electron microscopic analysis of wild-type mice by the former group shows LD-containing autophagosomes (lipophagosomes) under starvation conditions, although the latter group indicates that it is hard to see the presence of LDs enwrapped by double membranes even under starvation conditions, and that different from lipophagy, mitophagy can easily be found in hepatocytes if the mice are starved for 48 hours.

Two groups used different experimental approaches in their studies, which may underlie different conclusions. One critical point is to consider that $Atg7^{Flox/Flox}$: albumin-Cre mice cause hepatomegaly and hepatitis with accumulation of abnormal organelles in hepatic cells [26].

Recently, metabolic contributions of amino acids released from liver by starvation-induced autophagy in adult animals using liver-specific Atg7-deficient mice have also been studied systematically [8]. That is, liver specific conditional Atg7-knockout mice ($Atg7^{Flox/Flox}$:Mx1 mice) are generated by the different method from $Atg7^{Flox/Flox}$:albumin-Cre mice [25]. To delete Atg7 from the liver, Cre expression in the liver was induced by intraperitoneal injection of polyinosinic acid-polycytidilic acid (pIpC), while complete deletion of the Atg7 protein in the liver was verified using immunoblotting analyses. Atg7-knockout mice are used 10 days after the injection of pIpC. No sign of hepatomegaly and hepatitis is observed within 2 weeks after the injection of pIpC. For synchronous induction of autophagy in the liver, mice previously fasted for 24 hours and preserve numerous LDs in hepatocytes, are fed by a pelleted laboratory diet for 2 hour (20:00–22:00) in the dark to suppress autophagic activity to a minimum [8]. The diet is then withdrawn and the mice are again starved. In separate experiments, it has been confirmed that the stomach and intestine are filled with a digested diet at the end of the 2 hour-feeding period. In this situation, hepatocytes become to change from the stage of lipogenesis and glycolysis to that of lipolysis and glycogenesis in hepatocytes. Electron microscopic examinations clearly show that in the control mice numerous LDs are continuously present in hepatocytes after 2 hour-feeding, and decrease during starvation [8]. Most LDs disappear in the liver after 24 hours of starvation, while glycogen granules increase in hepatocytes. In contrast, the number of autophagic vacuoles in the liver after 24 hour of starvation is increased dramatically. Such vacuoles and LDs are not observed in liver-specific Atg7-deficient mice [8].

These findings seem to indicate that LDs that accumulate in the liver during 24 hours fasting decrease in parallel with the following activation of autophagy after a 2 hour-feeding , but that loss of autophagy does not inhibit the disappearance of LDs in the liver. Thus another mechanism may operate in the removal of LDs from the liver that accumulated by starvation.

In adipose tissue, knock out of either Atg7 [9] or Atg5 [17] leads to reduced accumulation of lipid and impaired differentiation of adipocytes. The mutant white adipocytes are smaller with multiple LDs. It has been suggested that Atg5 deletion causes adipogenesis arrest at the later stages of mouse embryos. The possibility that a defect of Atg5-dependent Atg8 (LC3) lipidation and translocation to LDs of adipocytes may result in inefficient droplet fusion, which contributes to the defect of adipogenesis cannot be ruled out. The same authors observed reduced differentiation in Atg 5-deficient mouse embryonic fibroblasts (MEFs) model. An essential role of autophagy in lipid storage is also suggested in fat body cells of Drosophila. Knockdown of Atg1 or Atg6 led to small lipid droplets [27]. The paper has also indicated that Rab32 may regulate lipid storage by affecting autophagy [27]. Rab32 and several other Rabs have been found to affect the size of lipid droplets [27] and the mechanism of droplet fusion with atg8 (LC3) and Rab families awaits further studies. Thus, in adipocytes autophagy may contribute to LD formation and not significantly to lipolysis.

For hydrolysis of TAG in LDs of adipocytes, the molecular processes of lipolysis are becoming clear. Adipose tissue TAG lipase first acts on TAG to hydrolyze a fatty acyl chain [28]. Hormone sensitive lipase mediates the second step of lipolysis, diacylglycerol cleavage to monoacylglycerol. Finally monoacylglycerol lipase hydrolyzes the last side chain.

6. Concluding remarks

Lipid droplets (LDs) are key cellular organelles involved in lipid storage and mobilization. In non-adipocytes, LDs are small, mobile and interact with other cellular compartments. In contrast, adipocytes primarily contain very large, immotile LDs. The marked morphological differences between LDs in adipocytes and non-adipocytes suggest that key differences must exist in the manner in which LDs in different cell types interact with other organelles and undergo fusion and fission with other droplets. It has suggested that droplet fusion is dependent on microtubules, the motor protein dynein [29], Rab proteins [27] and the SNARE fusion machinery [31].

PAT proteins also target to the surface of LDs likely through different mechanisms. In adipocytes, TIP47 is found on smaller droplets and perilipin is found on larger LDs [31]. They may bind to different droplets associated proteins. Cleaved (and lipidated) LC3 demonstrated on isolated LDs from non-adipocytes [9] may be involved in LDs biology as well as PAT proteins (Figs. 2 and 3). Nakatogawa et al. [32] have shown that lipidated ATG8 (yeast homologue of LC3) mediates tethering between adjacent membranes of liposomes and stimulates membrane hemifusion, an event that may mimic expansion of the autophagosomal membrane during autophagy (Fig. 4). In LDs, homotropic interactions in adipocytes and non-adipocytes may involve a hemifusion type mechanism to facilitate lipid transfer (Fig.4). In adipocytes lipidated LC3 may also involved in growth of LDs (Fig. 4), as shown by morphological evidence that the mutant white adipocytes are smaller with multiple LDs [17].

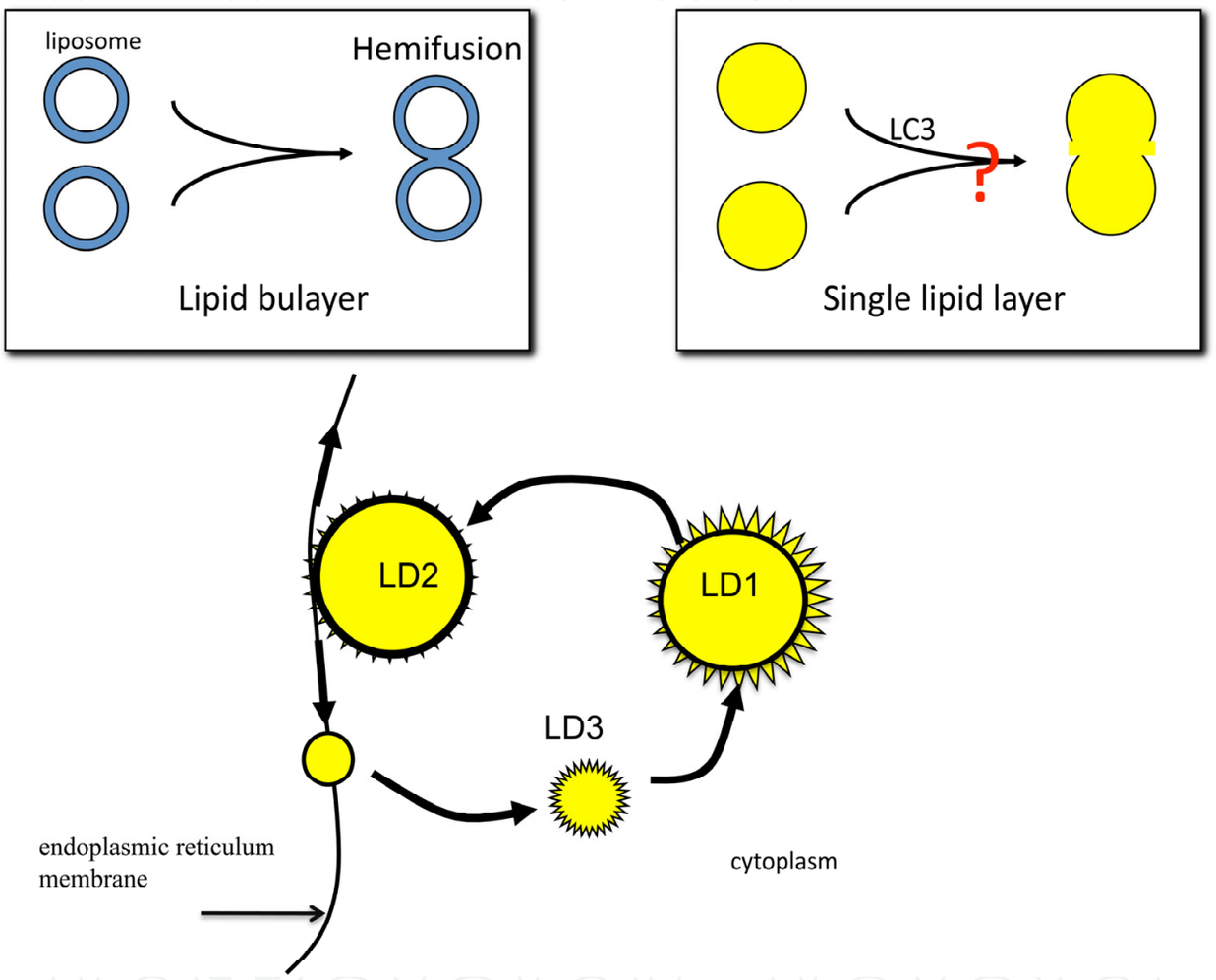

Figure 4. As shown by Murphy [16], a large LD3 that is attached to the endoplasmic reticulum may be degraded by ER-associated lipases and TAG may be re-synthesized. Newly formed LD1 would re-grow as LD2 in the cytoplasm. As has been shown by Nakatogawa et al. [32], bilayered liposomes with Atg8 undergo hemifusion (left upper), since Atg8 molecules are oligomerized with each other. If LC3 molecules are oligomelized on the surface of LDs (right upper), LDs would undergo fusion and grow in size.

It must be readdressed whether autophagy may affect lipid metabolism in a tissue- or cell-specific manner. If autophagy functions and regulates lipogenesis or lipolysis in a different way in non-adipocytes, it must be answered what molecular mechanisms work by which the autophagic machinery recognize LDs to enwrap for degradation or fuse with each other, and how they are regulated.

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Acknowledgement

This paper was supported by Grants-in-Aid for Scientific Research (B), (23390041) and partly for Scientific Research on Innovative Areas (23111004), and by MEXT-supported Program for the Strategic Research Foundation at Private Universities.

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