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# Lipophagy is essential for lipid metabolism in fish

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Lipophagy is a form of autophagy in mammals. In this cellular process, lipid droplets (LDs) are degraded through the lysosomal degradative pathway [1]. During lipophagy, LDs sequestrated in autophagosomes are delivered to lysosomes, which fuse to form autophagosomes and then develop into autophagolysosomes, leading to LDs degradation of [1]. A number of studies have demonstrated that lipophagy play important roles in lipid metabolism and energy homeostasis in mammals [1–3]. However, studies about the existence and biological functions of lipophagy in other organisms have been very limited until now. For example, no evidence has been provided for lipophagy in fish, which is the largest population of vertebrates. Currently, excess lipid accumulation and related metabolic disorders, such as fatty liver and obesity, occur widely in farmed fish, resulting in decreased survival rate, growth performance, disease resistance, and so on [4,5]. Therefore, in order to reduce lipid deposition in fish, fish physiologists are seeking key genes, proteins or metabolic pathways in lipid catabolism in fish, and these could be used as potential modulatory targets in nutritional regulation or genome editing for directional breeding in aquaculture [6,7]. In view of the key regulatory roles of lipophagy in lipid metabolism in mammals, it is essential to determine whether lipophagy exists and plays important roles in lipid metabolism of fish.

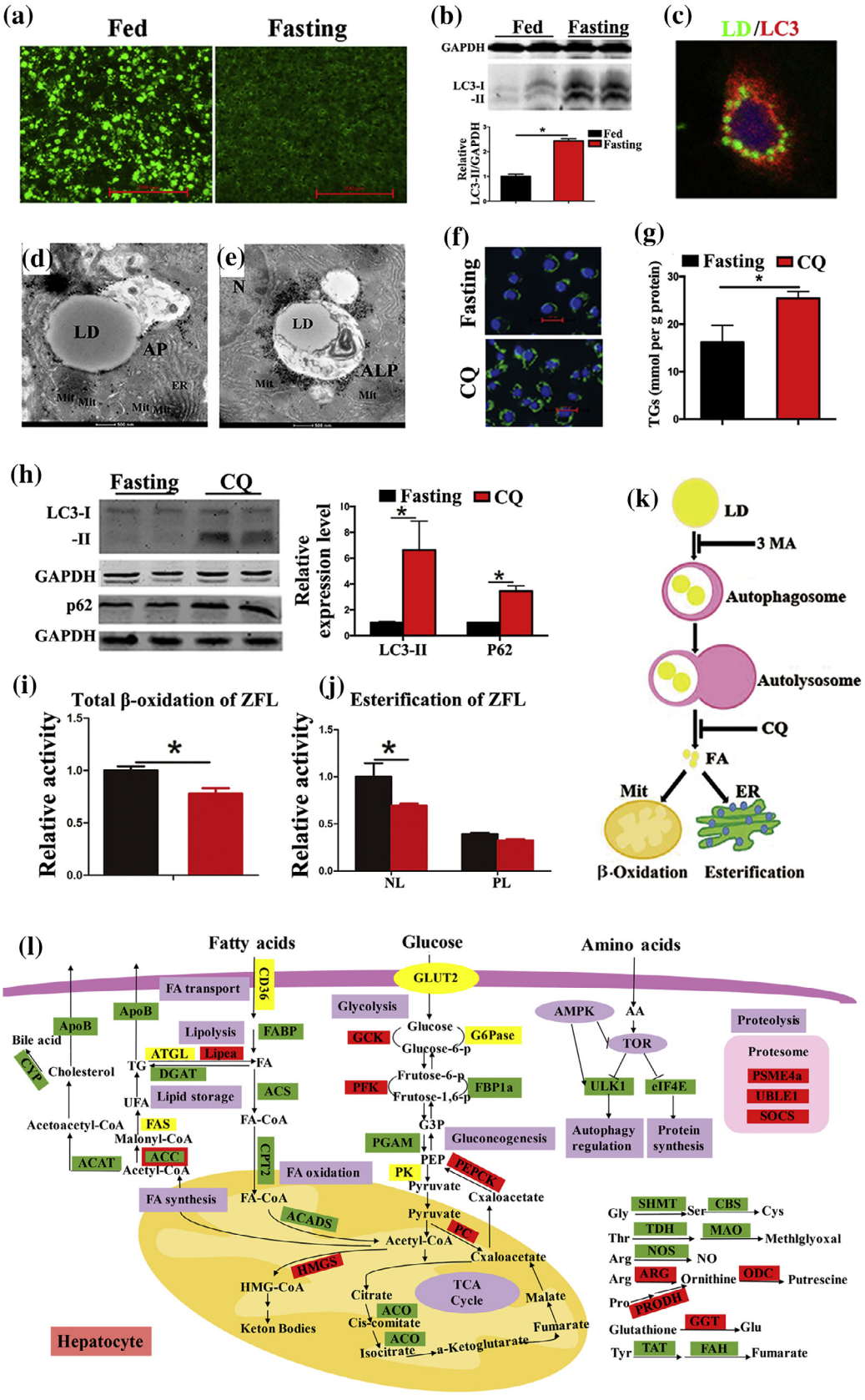
In this study, we first investigated whether lipophagy could be induced by fasting in zebrafish. We found that, the number of LDs stained by BODIPY 493/503 decreased significantly in the zebrafish liver after 36 h of starvation (Fig. 1a), suggesting that LDs were consumed as an energy source during starvation. During this process, the amount of microtubule-associated protein 1 light chain 3 (LC3-II), which is an autophagosomal marker, increased significantly in fasted fish compared with fed state (Fig. 1b), indicating that autophagy is induced by fasting in zebrafish. Furthermore, double immunofluorescence studies revealed co-localization of LDs with LC3 in ZFL cells in the fasting state, indicating a direct association between LDs and autophagosomes (Fig. 1c). The electron microscopy (EM) is a commonly used method as the gold standard to detect cellular lipophagy during different stages of LD sequestration (phagophore, autophagosome, and autolipophagosome) [1,8]. We assessed fasting liver samples by EM and found different subcellular structures associated with lipophagy in zebrafish, such as the structure of autophagosome (AP, Fig. 1d) containing a portion of a larger LD or whole small LDs; the structure of autolipophagosome (ALP) (Fig. 1e) containing big or small LDs, which were degraded by lysosome lipase in the ALP. These findings suggest that lipophagy exists in zebrafish and contributes to the fasting-induced decrease in the number of LDs.

To further determine whether lipophagy is involved in lipid metabolism, the effects of inhibiting autophagy on LD accumulation and biochemical processes of fatty acid metabolism were examined in ZFL cells (Figs. 1 and S1 (online)). Pharmacologically inhibiting autophagy with chloroquine (CQ) and 3-methyladenine (3-MA), which have been widely used as biochemical inhibitors of autophagy [8,9], increased significantly the accumulation of LDs and cellular TG content (Figs. 1f, j and S1a, b (online)). As CQ-induced dysfunction of lysosomes mainly inhibits degradation of LC3-II [10], and 3-MA-induced inhibition of autophagosome formation suppresses the process of degrading substance engulfment [11], we found LC3-II protein or cytosol LC3-I protein highly accumulated in CQ- or 3-MA-treated ZFL cells, respectively (Figs. 1h and S1c (online)). The protein levels of p62, which is a marker of blocked autophagy [12], were also increased in both CQ- and 3-MA-treated cells (Figs. 1h and S1c (online)). These results indicate that CQ and 3-MA caused accumulation of LDs in ZFL cells by suppressing autophagy. One of the main functions of lipophagy in mammals is to release FFAs from TGs as substrates for further metabolic reactions, such as β-oxidation for energy production [13]. In our present experiments, the use of CQ and 3-MA not only decreased significantly fatty acid (FA) β-oxidation, but also inhibited significantly esterification of FA into neutral lipids (mostly triglycerides [TGs]) in the ZFL cells (Figs. 1i, j and S1d, e (online)). This suggests the dysfunction of autophagy downregulated lipid metabolic activities, including catabolism or anabolism, which was caused by a decrease in the LD-sourced free FAs as metabolic substrates (Fig. 1k).

To further verify the roles of lipophagy in lipid metabolism in vivo, the effects of inhibiting autophagy with CQ were examined in zebrafish. Our preliminary experiment indicated that 100 μmol/L CQ bathing would affect autophagy-related gene expression, but not induce higher expression of the genes in apoptosis, inflammation, and detoxification, (Fig. S2a online). Therefore, 100 μmol/L CQ bathing treatment was used in the zebrafish experiment. The fish were fed a high-fat diet (HFD) containing 13% lipid for one week.

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(a)
Fed
Fasting
(b)
Fed
Fasting
GAPDH
LC3-I
-II
Relative LC3-II/GAPDH
Fed
Fasting
(c)
LD/LC3
(d)
LD
AP
ER
Mit
(e)
N
LD
ALP
Mit
(f)
Fasting
CQ
(g)
Fasting
CQ
TGs (mmol per g protein)
(h)
Fasting
CQ
LC3-I
-II
GAPDH
p62
GAPDH
Relative expression level
LC3-II
P62
(i)
Total β-oxidation of ZFL
Relative activity
(j)
Esterification of ZFL
Relative activity
NL
PL
(k)
LD
3 MA
Autophagosome
Autolysosome
CQ
FA
Mit
ER
β-Oxidation
Esterification
(l)
Fatty acids
Glucose
Amino acids
FA transport
CD36
GLUT2
Glycolysis
AMPK
AA
TOR
Proteolysis
Protesome
PSME4a
UBLE1
SOCS
ApoB
Bile acid
CYP
Cholesterol
ApoB
Lipolysis
FABP
ATGL
Lipea
TG
FA
DGAT
Lipid storage
ACS
UFA
FA-CoA
Acetoacetyl-CoA
FAS
Malonyl-CoA
ACC
ACAT
Acetyl-CoA
CPT2
FA oxidation
FA synthesis
FA-CoA
ACADS
Acetyl-CoA
HMGS
HMG-CoA
Keton Bodies
Hepatocyte
GCK
Glucose
G6Pase
Glucose-6-p
PFK
Frutose-6-p
FBP1a
Frutose-1,6-p
G3P
PGAM
Gluconeogenesis
PEP
PK
PEPCK
Pyruvate
Cxaloacetate
Pyruvate
PC
Cxaloacetate
Citrate
TCA Cycle
Malate
ACO
Cis-comitate
ACO
Isocitrate
a-Ketoglutarate
Fumarate
ULK1
eIF4E
Autophagy regulation
Protein synthesis
Gly
SHMT
Ser
CBS
Cys
Thr
TDH
MAO
Methlglyoxal
Arg
NOS
NO
Arg
ARG
Ornithine
ODC
Putrescine
Pro
PRODH
Glutathione
GGT
Glu
Tyr
TAT
FAH
Fumarate

Afterwards, the fish were bathed in 100 μmol/L CQ and fasted for 24 h (Fig. S2b online). The use of CQ caused more LD accumulation and TG concentration in the liver than those in the control (Fig. S2e, f online). The protein level of LC3-II was higher in CQ-treated fish, showing the blocked autophagy in fish (Fig. S2g online). These are consistent with our previous results in ZFL cells. Moreover, the exposure to CQ decreased significantly the oxygen consumption rate in fish and total FA β-oxidation in the fish liver (Fig. S2c,d online), indicating a reduced metabolic state. The mRNA expression of carnitine palmitoyltransferase (CPT)-1, a key gene of FA β-oxidation, was downregulated in CQ treatment, but CQ did not change the protein amount of PPARα between both groups (Fig. S2g online). Therefore, if inhibiting autophagy leads to a reduction in TG-sourced FFAs, the FFA breakdown-related genes would not be activated and β-oxidation activity would decrease. Of note, previous mammalian studies indicated that lipolysis cooperates with lipophagy in TG breakdown [14,15], however, in the present study, the inhibited autophagy did not significantly affect the lipolysis-related enzyme proteins (ATGL and $^{p-}$HSL) (Fig. S2g online). This might be caused by species difference, but also indicated that the reduced TG-sourced FFA caused by the impaired lipophagy could not be recovered by lipolysis, at least in the present experimental conditions. The gene expression assay in zebrafish liver also showed that CQ lowered mRNA levels of apolipoprotein B (Apo B) and fatty acid synthase (FAS) which are key genes involved in lipid transport and lipogenesis, respectively (Fig. S2g online). These results indicate that inhibiting lipophagy affected most genes related to lipid metabolism, revealing that lipophagy plays essential roles in regulating lipid metabolism in fish.

A transcriptomic assay of inhibited autophagy in zebrafish liver was performed to investigate the systemic metabolic changes in zebrafish with dysfunctional lipophagy. There were 1002 differentially expressed genes (DEGs) compared with the control (Fig. S3a online). These DGEs were further analysed by KEGG pathway enrichment to identify the compendium of participating biological processes. The distributions of DEGs in three main biological processes are shown in Fig. S3b (online). Among them, the most predominantly affected process was "metabolism" (74%) and the contributions of amino acid, carbohydrate, and lipid metabolism were 20%, 10%, and 13%, respectively. We generated a schematic illustration of the significantly changed genes related to amino acid, carbohydrate, and lipid metabolism (Figs. 1l and S3c (online)). Apparently, the use of CQ inhibited most genes related to lipid metabolism, such as FA transport (FABP and ApoB), FA oxidation (CPT2 and short-chain acyl-CoA dehydrogenases), lipogenesis synthesis (ACC and DGAT), and cholesterol synthesis (acetyl-CoA acetyltransferase 1). However, significantly upregulated changes were detected in the glycolytic genes (glucokinase and phosphofructokinase), gluconeogenesis (pyruvate carboxylase and phosphoenolpyruvate carboxykinase), and proteolysis (proteasome activator subunit 4a, SUMO1 activating enzyme subunit 1, and suppressor of cytokine signalling). Our transcriptomic data indicate that inhibiting autophagy not only reduced lipid metabolism, but also affected the glucose and protein metabolic pathways in the zebrafish liver. Considering that lipids, carbohydrates, and proteins all contribute to energy supply in animals, the decreased energy portion sourced from lipid metabolism could be supplemented by increased energy production from glucose and protein catabolism.

The present study demonstrated that lipophagy exists in fish and plays essential roles in lipid metabolism by changing TG-released FFAs as substrates. Lipophagy also affected glucose and protein metabolism, indicating that lipophagy is an important process in maintaining energy homeostasis in vertebrates. This is the first study to show the metabolic roles of lipophagy in fish.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

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## Appendix A. Supplementary data

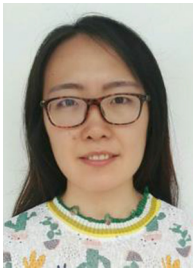
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.scib.2018.05.026.

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**Fig. 1.** Lipophagy exists and involves in lipid metabolism in fish. (a) LDs stained with BODIPY 493/503 in zebrafish liver in fasting condition. (b) The immunoblot and densitometric analysis of LC3 in the samples from the fasting zebrafish liver. ($^*P < 0.05$, $n = 6$). Error bars, S.E.M. (c) Co-localization of LD (green) with LC3 (red) in ZFL cell line in fasting condition. Nuclei are highlighted with 4,6-diamidino-2-phenylindole (DAPI). (d, e) Electron micrographs of zebrafish liver. The structure of an autophagosome (AP) containing a lipid droplet (d). The structure of an autolipophagosome (ALP) containing a lipid droplet (e). N: nucleus; ER: endoplasmic reticulum; Mit: mitochondria. (f, g) LDs stained with BODIPY 493/503 and TG content in the ZFL cells in fasting condition. (h) Immunoblot and densitometric analysis of LC3 and p62 in samples from F. ($^*P < 0.05$, $n = 6$). Error bars, S.E.M. (i, j) The β-oxidation and esterification activities of [1-$^{14}$C] oleic acid with CQ in the ZFL cells in fasting condition. ($^*P < 0.05$, $n = 4$). Error bars, S.E.M. (k) Hypothetical model showing the function of lipophagy in FA metabolism. (l) Global regulatory effects of the inhibited autophagy on zebrafish hepatic metabolism by transcriptomic assay. Red indicates that the up-regulated genes in the CQ-treated fish compared to those in the control. Green indicates the down-regulated genes in the CQ-treated fish compared to the control, and yellow indicates no difference. *Abbreviations:* ACADS: short chain specific acyl-CoA dehydrogenase; ACAT: acetyl-CoA acetyltransferase; ACC: acetyl-CoA carboxylase; ACO: aconitase; ACS: acetyl-CoA synthetase; ApoB: apoliporotein B; ARG: arginase; ATGL: adipose triglyceride lipase; CBS: cystathionine-beta-synthase; CD36: fatty acid translocase; CPT1: carnitine palmitoyltransferase 1; CPT2: carnitine palmitoyltransferase 2; CYP: cytochrome P450; DGAT: diacylglycerol o-acyltransferase; eIF4E: eukaryotic translation initiation factor 4E; FABP: free fatty acid binding protein; FAH: fumarylacetoacetate hydrolase; FAS: fatty acid synthase; FBP: fructose-1,6-bisphosphatase; GCK: glucokinase; GGT: glutamyltransferase; Glut2: glucose transporter 2; G6Pase: glucose-6-phosphate dehydrogenase; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; Lipea: hormone-sensitive lipase; MAO: monoamine oxidase; NOS: nitric oxide synthase; ODC: ornithine decarboxylase; PC: pyruvate carboxylase; PEPCK: phosphoenolpyruvate carboxykinase; PFK: 6-phosphofructokinase; PK: pyruvate kinase; PRODH: proline hydroxylase; PSME4a: proteasome activator subunit 4a; SAE1: SUMO1 activating enzyme subunit 1; SHMT: serine hydroxymethyltransferase; SOCS: suppressor of cytokine signaling; TAT: tyrosine aminotransferase; TDH: tyrosine hydroxylase; ULK1: unc-51 like autophagy activating kinase 1.

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Jing Wang is a Ph.D. candidate in the School of Life Sciences at East China Normal University. Her present study focuses on the cellular metabolism of lipid droplets, particularly the progress and molecular regulation of lipophagy in fish and cell models.



Zhen-Yu Du obtained two Ph.D. degrees from Sun Yat-sen University, China (Hydrobiology) and University of Burgundy, France (Nutrient Physiology), respectively, in 2005. Later, after 7 years' research career in France and Norway as post-doc and senior scientist/PI, he joined School of Life Sciences, East China Normal University as a full professor in 2012. His present research interests include nutrient metabolism, stress physiology and environmental health, by using fish and cell models.