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REVIEW

Dysfunction of autophagy in high-fat diet-induced non-alcoholic fatty liver disease

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases with a global rising prevalence, which is closely associated with a high-fat diet (HFD) intake. Macroautophagy/autophagy is an evolutionarily conserved degradation process for cytosolic macromolecules and damaged organelles. The potential role of autophagy in hepatic lipid metabolism has been recognized, while dysfunction of hepatic autophagy has been found to contribute to NAFLD. Herein, we provide an overview of the autophagy phases with the regulatory machinery, and the current understanding of hepatic autophagy in its protective role in HFD-induced NAFLD. We also discuss the genetic and pharmacological interventions that may help elucidate the molecular mechanisms of autophagy and influence the future therapeutic direction in NAFLD.

Abbreviations: ACOX1: acyl-CoA oxidase 1; ADH5: alcohol dehydrogenase 5 (class III), chi polypeptide; ADIPOQ: adiponectin, C1Q and collagen domain containing; ATG: autophagy related; BECN1: beclin 1; CRTC2: CREB regulated transcription coactivator 2; ER: endoplasmic reticulum; F2RL1: F2R like trypsin receptor 1; FA: fatty acid; FOXO1: forkhead box O1; GLP1R: glucagon like peptide 1 receptor; GRK2: G protein-coupled receptor kinase 2; GTPase: guanosine triphosphatase; HFD: high-fat diet; HSCs: hepatic stellate cells; HTRA2: HtrA serine peptidase 2; IRGM: immunity related GTPase M; KD: knockdown; KDM6B: lysine demethylase 6B; KO: knockout; LAMP2: lysosomal associated membrane protein 2; LAP: LC3-associated phagocytosis; LDs: lipid droplets; Li KO: liver-specific knockout; LSECs: liver sinusoidal endothelial cells; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; MAP3K5: mitogen-activated protein kinase kinase kinase 5; MED1: mediator complex subunit 1; MTOR: mechanistic target of rapamycin kinase; MTORC1: mechanistic target of rapamycin complex 1; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NFE2L2: NFE2 like bZIP transcription factor 2; NOS3: nitric oxide synthase 3; NR1H3: nuclear receptor subfamily 1 group H member 3; OA: oleic acid; OE: overexpression; OSBPL8: oxysterol binding protein like 8; PA: palmitic acid; RUBCNL: rubicon like autophagy enhancer; PLIN2: perilipin 2; PLIN3: perilipin 3; PPARA: peroxisome proliferator activated receptor alpha; PRKAA2/AMPK: protein kinase AMP-activated catalytic subunit alpha 2; RAB: member RAS oncogene family; RPTOR: regulatory associated protein of MTOR complex 1; SCD: stearoyl-CoA desaturase; SIRT1: sirtuin 1; SIRT3: sirtuin 3; SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SQSTM1/p62: sequestosome 1; SREBF1: sterol regulatory element binding transcription factor 1;SREBF2: sterol regulatory element binding transcription factor 2; STING1: stimulator of interferon response cGAMP interactor 1; STX17: syntaxin 17; TAGs: triacylglycerols; TFEB: transcription factor EB; TP53/p53: tumor protein p53; ULK1: unc-51 like autophagy activating kinase 1; VMP1: vacuole membrane protein 1.

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Introduction

Non-alcoholic fatty liver disease (NAFLD), an umbrella term used to define different conditions where triacylglycerols (TAGs) accumulation is present in more than 5% of hepatocytes in the absence of excessive alcohol consumption [1,2]. Nowadays, NAFLD is the most prevalent chronic liver disease globally and is strongly associated with metabolic syndrome features, such as obesity, hypertension, hypertriglyceridemia and type 2 diabetes [3–5]. Histologically, the livers of patients with NAFLD exhibit obvious steatosis, and the patients may further experience nonalcoholic steatohepatitis (NASH) with increased inflammation, hepatocyte death and ballooning as well as fibrosis, cirrhosis and even hepatocellular carcinoma [6–8]. Recently, an international expert consensus panel has proposed that NAFLD should be renamed to metabolic-associated fatty liver disease [9], and this updated definition could better encapsulate the central role of metabolic syndrome as main pathogenesis of NAFLD and classify the variants of the disease [10]. While the classification of the liver metabolic disorders remains to be defined by the international consensus panel, we considered literatures on the NAFLD rather than the new nomenclature.

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NAFLD has imposed a very large healthcare and economic burden worldwide with an overall estimated prevalence of 25% approximately [11–13], and the population with NAFLD will increase to 590 million by 2035, including 300 million in China alone [12,14]. Of note, NAFLD occurs in 70–80% of individuals with obesity and diabetes [15]. Epidemiological data on children is still scarce, and a pooled analysis of published studies established the global prevalence of NAFLD at 7.6% in the general pediatric population and 34.2% in children who were obese [16]. A diet containing more than 30% of energy from fat is considered as a high-fat diet (HFD) and a model of diet-induced obesity and liver steatosis [17]. It has been shown from the review [18] and experimental studies [19–24] that NAFLD is closely associated with the overconsumption of high-fat food. For example, a recent article found that HFDs feeding could promote hepatic steatosis in numerous experimental studies, most of which were performed in male C57BL/6 mice fed 60% energy from fat [25]. Therefore, this review focuses on the HFD condition, which is believed to be an important risk factor for the pathogenesis of NAFLD in the general population.

Indeed, the mechanisms responsible for the progression of the NAFLD are still being elucidated. The liver is a large solid organ of the human body which plays a critical role in many physiological processes such as plasma protein synthesis, gluconeogenesis and glycogen storage, lipid and fatty acid (FA) metabolism, bile acid synthesis as well as purification of toxic chemicals [26,27]. Autophagy, an intracellular lysosomal degradative pathway, can rewire cellular metabolism linking catabolic to anabolic processes and thus sustain homeostasis which is especially relevant in liver physiology and pathology [28–31]. As such, autophagy has been proved to promote cellular health in the face of lipid overload [32]. With the considerable strides in the understanding of molecular mechanisms of autophagy, there is growing interest to understand the contributions of autophagy to metabolic and energetic homeostasis.

In the present review, we briefly outline the mechanisms and functions of autophagy, as well as the role of hepatic autophagy in the context of NAFLD. We further describe the inter-relation among nutrient-rich conditions, impaired autophagy, and the pathogenesis of NAFLD. We also discuss the autophagic genetic and pharmacological interventions in HFD-induced animal models of NAFLD. Our aim is to provide some new research ideas for targeting hepatic autophagy in the future therapy of NAFLD.

Mechanisms and functions of autophagy

Autophagy, a term acquired from the Greek words “auto (self)” and “phagein (to eat)”, literally translating “self-eating”, refers to an evolutionarily conserved lysosome-mediated intracellular degradation pathway for surplus or abnormal organelles, excess lipids and protein aggregates [28]. Overall, autophagy comes in three main forms: macroautophagy, microautophagy and chaperone-mediated autophagy, which differ in how the cargos are delivered to lysosomes [33]. The autophagic classification has

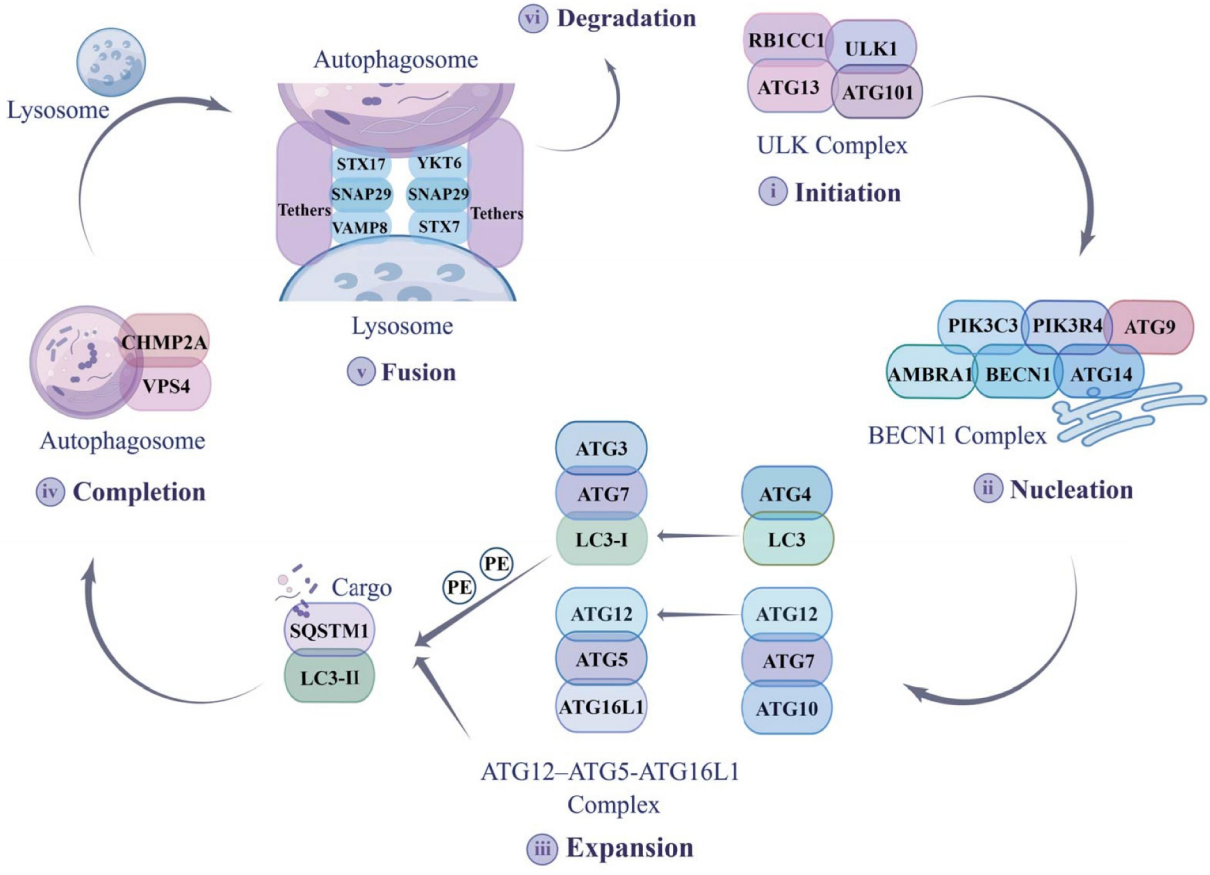

Figure 1. The steps of autophagy. See text for details.

been well established in a series of articles and out of the scope of this review [34–37].

Macroautophagy (hereinafter referred to as autophagy) represents the canonical autophagy which uses a special cytoplasmic vesicle to capture cargos. Upon induction, a precursor structure named phagophore will elongate and engulf some materials in the cell. Previous studies have shown that the de novo biosynthesis of autophagosomes requires membrane materials from multiple sources, including endoplasmic reticulum (ER) [38,39], Golgi network [40–43], mitochondria [44], the plasma membrane [45], the endosomes [46–48] and ER-Golgi intermediate [49–52]. As the phagophore extends, the edges of the membrane fuse to generate a double-membrane structure named autophagosome [53], which fuses with lysosomes. Subsequently, the lysosomal hydrolases degrade the inner leaflet of the autophagosome to form a monolayer membrane structure named autolysosome [54]. Lastly, the engulfed cytosolic materials contained in the autophagosome are degraded by lysosomal enzymes [55].

Autophagy, a tightly regulated process, has been executed by the *ATG* (autophagy related) genes which are evolutionarily conserved from yeast to more complex eukaryotes [56–58]. Canonically, the specific autophagy process can usually take place in six sequential steps: initiation, nucleation, expansion, closure, fusion and degradation [59] (Figure 1), and the different steps involve the spatiotemporal coordinated recruitment of specialized ATG proteins [60]. (i) Initiation: In mammalian cells, the initiation of autophagy mainly requires the action of the ULK complex to allow the recruitment and activation of other ATG proteins [61]. The ULK complex is composed of ULK1 (unc-51 like autophagy activating kinase 1), RB1CC1/FIP200 (RB1 inducible coiled-coil 1), ATG101 and ATG13 [62], and the activity of the complex is negatively regulated by MTOR (mechanistic target of rapamycin kinase) complex 1 (MTORC1) and positively regulated by AMP-activated protein kinase (AMPK). (ii) Nucleation: Once activated, ULK1 further phosphorylates BECN1 (beclin 1) [63]. The pre-phagophore formation occurs through the activation of the BECN1 complex which contains BECN1, ATG14, AMBRA1 (autophagy and beclin 1 regulator 1), PIK3C3/VPS34 (phosphatidylinositol 3-kinase catalytic subunit type 3) and PIK3R4/VPS15 (phosphoinositide-3-kinase regulatory subunit 4) [56,64]. The only transmembrane ATG protein, ATG9, works together with the BECN1 complex to function in the phagophore membrane expansion and retrieval process [65]. (iii) Expansion: After the activation of BECN1 complex, the phagophore expansion is accomplished by ubiquitin-like conjugation system [66]. ATG7, an E1-like enzyme, catalyzes the formation of the ATG12–ATG5-ATG16L1 complex (in concert with ATG10) [27]. MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) is first processed by the ATG4 to form LC3-I which resides in the cytosol. LC3-I is then activated by ATG7 and ATG3, conjugating with phosphatidyl ethanolamine to form a membrane associated LC3-II with the ATG12–ATG5-ATG16L1 complex participating [67]. SQSTM1/p62 (sequestosome 1) is most known as a receptor that links ubiquitinated proteins to LC3 and transfers it to autophagosomes [68]. Thus, the evaluation of LC3-II and SQSTM1 protein levels are widely used as monitors of autophagic flux in the literatures [25,69]. (iv) Closure: Once the cargo is bound to the phagophore membrane, the expanding membrane closes around its cargo to form a complete autophagosome [70]. A set of endosomal sorting complexes required for transport including CHMP2A (charged multivesicular body protein 2A) and VPS4 (vacuolar protein sorting 4 homolog), regulate autophagosome closure in mammalian cells [71,72]. (v) Fusion: The autophagosome fuses with the lysosomes, producing the autolysosome. This process requires the concerted actions of multiple regulators of membrane dynamics, including SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes, tethering factors and RAB (member RAS oncogene family) GTPases [73]. Membrane fusion is driven by the assembly of SNARE complexes formed by autophagosomal-localized STX17 (syntaxin 17), SNAP29 (synaptosome associated protein 29) and lysosomal-localized VAMP8 (vesicle associated membrane protein 8), or by the autophagosomal-localized YKT6 (YKT6 v-SNARE homolog), SNAP29 and lysosomal-localized STX7 (syntaxin 7) [74,75]. Tethering factors, recruited by small RAB GTPases, phosphoinositides and SNARE proteins, aid in capturing vesicles and linking the transported vesicles to target membranes [76]. (vi) Degradation: The autolysosomal cargo is digested within the acidic lysosomal environment.

When performed at the baseline level, autophagy fulfills housekeeping duties and plays a paramount role in regulating cellular homeostasis and survival, which is essential for maintaining the healthy state of the organism, conferring upon it the ability to adapt to endogenous or exogenous insults [77–79]. In addition, recent findings have demonstrated that autophagy modulates recycling of key circadian regulators by selectively degrading circadian proteins such as cryptochrome circadian regulator 1, resulting in gluconeogenesis and increased blood glucose levels [80].

It has been established that autophagy exhibits constitutive activity in the healthy liver by eliminating damaged organelles or proteins in the liver-related diseases [81,82]. Hepatocytes, the hepatic parenchymal cells that occupy approximately 80% of liver volume, have carried out the majority of these physiological tasks [27]. Hepatocytic autophagy is an essential cellular process, which is not only essential for replenishing the free amino acids through proteolysis, but also contributes to mobilization and hydrolysis of lipid and glycogen, thereby significantly contributing to the cellular energetic flux through different metabolic pathways [83]. Subsequent studies have indicated that dysfunction of hepatocytic autophagy intrudes hepatic homeostasis. For example, in hepatocyte-specific *Atg7* deletion murine model, autophagy impairment has been shown to promote liver size, fibrosis, progenitor cell expansion and hepatocarcinogenesis [84]. Autophagy may also inhibit the progression of steatosis and fatty hepatitis by preventing hepatocyte injury. As a result, impaired

hepatocytic autophagy will lead to abnormal hepatic lipid metabolism, thereby leading increased development of NAFLD.

Dysregulation of hepatocytic autophagy in HFD-induced NAFLD

As we known, autophagy plays an important role not only in normal liver physiology but also in the pathogenesis of many metabolic liver diseases. Dysregulated autophagy due to genetic variants or polymorphisms in *ATG* genes has been increasingly identified in NAFLD [78,79]. *IRGM* (immunity related GTPase M) is essential for autophagy activation upon infections [85]. Studies have demonstrated that genetic variants in *IRGM* conferred risk of human NAFLD, as the *IRGM*-rs10065172 genotype independently increased the odds ratio of NAFLD when compared to the control genotype [86,87]. Another line of research has already identified rare and low-frequency *ATG7* loss-of-function variants as modifiers of NAFLD progression by impairing autophagy in patients with severe NAFLD [88]. Changes in autophagic flux are also observed in humans diagnosed with NAFLD [89] and prediabetic obese patients [90]. In the context of NAFLD, accumulation of autophagic substrate SQSTM1 in hepatocytes was found in 68% or 88% of patients with NAFLD according to two clinical studies, while absence of SQSTM1 was identified in the control groups consisting of patients with normal liver function [91,92]. Importantly, these studies also demonstrated that lysosomal function was impaired in the livers from patients with NAFLD [89,91]. TFEB (transcription factor EB) is a major transcription factor, which is critical in the regulation of a gene network that regulates lysosomal functions [93,94]. There is a significant inverse correlation between TFEB activity and liver steatosis severity in the patients with NAFLD, supporting the clinical relevance of autophagy-regulated events [22]. In line with this, patients with NAFLD were shown to have slightly elevated levels of RUBCN (rubicon autophagy regulator), a protein with known anti-autophagic properties [95]. Autophagy has been generally found reduced in human NAFLD, while lifestyle-based approaches including fasting, dietary changes and exercise may be very potent inducers of beneficial autophagy-related changes in NAFLD [96–99]. Indeed, a recent study suggests that exercise may improve NAFLD through widespread stimulation of autophagy during physical activity [100].

The dysfunction of autophagy in NAFLD animal models is well reported in the literatures and is thought to be an important hallmark of steatosis worsening [18,101,102]. It has already been shown that a HFD feeding or a long-term accumulation of lipids may reduce hepatocytic autophagy activity and aggravate hepatocellular steatosis in mice [25,101,103,104], and this phenomenon can be more significant in mice deficient in *Atg* genes (Table 1). In HFD-induced NAFLD model, autophagy has been found to be downregulated in the liver, in combination with increased steatosis, particularly in autophagy-defective *Atg7*-downregulated mice [105]. This is in line with the research that hepatocyte-specific *sqstm1* knockout mice had decreased autophagy and increased cytoplasmic vacuolation in hepatocytes during the nutrient-rich condition [108]. Conversely, Adenoviral-*Atg14* overexpression in mice prevented accumulation of lipids in hepatocytes through the stimulation of autophagy [107]. Furthermore, a long-term feeding with HFD was sufficient to enhance hepatic steatosis in the hepatocyte-specific *tfeb* deletion murine model, while adenoviral-*Tfeb* overexpression restores autophagic flux and improved liver steatosis in a similar animal model [22,109]. The ULK1 is suppressed under nutrient-rich conditions, and it can be activated and translocate to the ER under autophagy-inducing conditions [111]. Under HFD feeding conditions, the presence of lipid droplets (LDs) and the inflammatory infiltrate were significantly increased in *ulk1* knockout mice when compared with wild-type mice [110]. Of note, liver-specific knockout of *Atg7* in mice resulted in the development of hepatic steatosis even under regular chow diet, suggesting that autophagy has a crucial role in preventing the accumulation of fat in hepatocytes [84,101].

Notably, the absence of autophagy in the liver also leads to elevated liver injury and inflammation which

Table 1. The ATG protein genetic interventions on hepatocytic autophagy in NAFLD models.

Genetic intervention	Animal models	Fat (% energy from fat) and duration (weeks)	Hepatocytic autophagy (up or down)	Hepatic steatosis outcome (up or down)	Ref.
Atg7	C57BL/6J mice with adenoviral-*Atg7* shRNA	60%, 8	Down	Up	[105]
Atg4	*atg4* KO mice	42%, 8	Down	Up	[106]
Atg14	C57BL/6J mice with adenoviral-*Atg14* overexpression	60%, 12	Up	Down	[107]
Sqstm1	*Sqstm1* f/f; *Alb-Cre* mice (*Sqstm1*-Li KO)	60%, 12	Down	Up	[108]
Tfeb	*Tfeb* f/f; *Alb*-Cre mice (*Tfeb*-Li KO)	42%, 12	Down	Up	[109]
Tfeb	C57BL/6J mice with adenoviral-*Tfeb* overexpression	60%, 16	Up	Down	[22]
Ulk1	*ulk1* KO mice	45%, 12	Down	Up	[110]
Atg7	*Atg7* f/f; *Alb*-Cre mice (*Atg7*-Li KO)	regular chow diet	Down	Up	[84]
Atg7	*Atg7* f/f; *Alb*-Cre mice (*Atg7*-Li KO)	regular chow diet	Down	Up	[101]

Articles using ATG protein genetic interventions and high-fat diets feeding involved hepatocytic autophagy in male mice. LC3-II and SQSTM1 were measured by western blots and described semi-quantitatively to detect hepatocytic autophagic activity (up or down).

may have a negative impact on adaptive lipid metabolism pathways, such as the NR1H3 (nuclear receptor subfamily 1 group H member 3)-mediated lipogenesis and the PPARA (peroxisome proliferator activated receptor alpha)-mediated lipid oxidation [112,113]. RUBCN, the beclin1-interacting negative regulator for autophagosome-lysosome fusion, has been found to be overexpressed in the liver tissue of mice fed with HFD and to promote NAFLD progression [95,114,115].

The "two hits" hypothesis is a classical hypothesis to explain the pathogenesis of NAFLD [116]. According to the hypothesis, hepatic steatosis is the first hit, and the lipotoxicity triggers a series of second hits including lysosome dysfunction, excessive oxidative stress, ER stress, mitochondrial dysfunction and inflammation, predisposing the liver to high-risk conditions [117]. Why NAFLD usually related to dysregulation of hepatocytic autophagy is still under exploration, and several mechanisms involving impaired cellular organelles such as lysosomal, ER, mitochondrial and LDs have been proposed in recent years. It has been found that the expression of TFEB is reduced in hepatic tissue with steatosis [118], and the reduction not only results in decreased lysosome biogenesis, but also affects overall activation of autophagy. Additionally, obesogenic diets lead to the localization of inducible NOS2 (nitric oxide synthase 2) at the surface of lysosomes which caused nitric oxide stress and impaired lysosomal function [119]. Consistent with this finding, the HFD feeding was shown to alter the intracellular ionic balance in hepatocytes, leading to the hindrance of autophagosome-lysosome fusion [89,120]. ER stress is caused by the accumulation of misfolded proteins and calcium depletion, often leading to catastrophic cell death [121]. ER stress, and unfolded protein response by which cells control ER protein homeostasis, are commonly found to be activated in the fatty liver of rodents and humans [122]. A recent study showed that ER stress activated a cellular response that increased asparagine levels and decreased lysosome acidification in mice fed with a HFD [102]. In vitro studies also have demonstrated that palmitate-induced ER stress impaired autophagosome-lysosome fusion in hepatocytes [123]. In addition, altered lipid availability is thought to contribute to impaired autophagy during HFD. It has been demonstrated that lipid overload can impair autophagosome-lysosome fusion due to altered lipid composition in membranes [124]. Phospholipid availability is known to modulate autophagosome formation, further contributing to decreased autophagy in HFD-induced NAFLD [125,126].

Damaged cellular organelles due to HFD treatment can impair autophagy, while dysregulation of hepatocytic autophagy can further lead to damage of cellular components and induce hepatic steatosis, thereby forming a "vicious circle". For example, *Atg7* deficiency which led to suppression of autophagy, resulted in ER stress induction [104]. Moreover, there is an interplay between ER stress and autophagy, as impaired autophagy can stimulate ER stress so that it acts deleteriously in NAFLD [127]. Autophagy is also necessary for mitochondrial quality control, and proper mitochondrial function is important for FA oxidation [112,128]. Lysosomal health has been maintained by autophagy through lysosomal membrane turnover [32], and the removal of damaged mitochondria and lysosomes through autophagy is widely regarded as a protective mechanism in long-term NAFLD development [129]. In addition, dysregulation of hepatocytic autophagy results in a decline in the degradation of LDs which further exacerbates the condition of steatosis, and we discussed this section in more detail below. Overall, there is a negative correlation between autophagy and hepatic steatosis, and dysregulation of hepatocytic autophagy has been linked to pathogenic steatosis in the context of NAFLD.

Lipophagy and regulation of hepatic lipid metabolism

Liver steatosis refers to the accumulation of lipids within LDs located in the hepatocyte cytoplasm, which is associated with a pathological state that resembles the occurrence of NAFLD [130]. LDs are dynamic cellular organelles that consist of a neutral lipid core surrounded by a phospholipid monolayer and LD-associated proteins, particularly of the perilipin protein family, serving as energy reservoirs and playing a key role in lipid homeostasis [131]. In lipid metabolism, autophagy has been found to regulate both biosynthesis and degradation of LDs, and this process is referred to as "lipophagy" [132]. Lipophagy, is a subtype of autophagy that plays its role using lysosomal acidic lipases [133], in which autophagosomes form and pinch off part of LDs then fuse with lysosomes to provide the liver with the ability of TAGs turnover and FA β-oxidation, preventing lipotoxicity and further progression of the metabolic liver disease [134,135]. Under physiological conditions, a balance is maintained between TAGs and FAs in the liver, while FAs can be anabolized to TAGs by esterification reactions and TAGs can be catabolized to FAs by lipophagy and lipolysis [133,136]. Lipolysis, a conventional lipase-driven process to regulate LDs turnover, targets larger-sized LDs to generate smaller-sized LDs which can be catabolized by lipophagy [137]. In particular, it has been demonstrated that lysosomal inhibition results in a buildup of small LDs within autophagosomes [137].

Since the regulation of lipid metabolism by lipophagy is crucial for cellular homeostasis, deficient lipophagy in hepatocytes has recently been identified as a potential pathophysiological mechanism of NAFLD [138,139]. Patients with NAFLD were considered to present symptoms of lipid accumulation as a result of a failure of lipophagy, therefore leading to increased TAGs storage within LDs in the liver [140,141]. The research which first confirmed that autophagy is critical for hepatic lipid homeostasis in mice has demonstrated that inhibition of autophagy heightens the hepatic TAGs and LDs content, resulting in a concomitant decrease in hepatic FA oxidation [101]. Recent data from HFD-fed mice models have suggested that lipophagy is dysregulated in NAFLD, and is associated with a failed conversion of LDs to FFAs [135]. ATG proteins such as ATG2A, ATG14 and LC3 have been involved in regulating LDs volume and distribution by decorating them [103,142,143]. Other proteins related to cellular

organelle function such as PLIN3 (perilipin 3) and LAMP2 (lysosomal associated membrane protein 2) have also been found to be associated with lipophagy, since hepatocyte-specific *plin3* or *lamp2* knockout mice both have decreased recruitment of autophagy machinery to LDs during the HFD feeding [144,145].

The RAB proteins are small GTPases that belong to the Ras-like GTPase superfamily and regulate the vesicle traffic process, and several RAB proteins have been implicated in the autophagy [146]. It has been demonstrated that RAB7 is a fundamental component of LDs [147] and is a key player in the regulation of targeting and fusion of "primed" autophagic LDs to late endocytic compartments [148]. Importantly, the activation of RAB7 is required for the trafficking of multivesicular bodies and lysosomes to the LDs surface during lipophagy, resulting in the formation of a lipophagic "synapse" [149]. Membrane-bound RAB7 facilitates LDs breakdown by interacting with its downstream effector, while the depletion of RAB7 results in significant morphological alterations in multivesicular bodies, lysosomes and autophagosomes, consequently leading to a reduction in hepatocellular lipophagy [149]. A more in-depth study has suggested that OSBPL8 (oxysterol binding protein like 8) which is a known ER lipid transfer protein, directly interacts with phagophore-anchored LC3 in lipophagy, and deletion of OSBPL8 or interruption of OSBPL8-LC3 interaction resulted in accumulation of LDs and increased intracellular TAGs [150]. It has become apparent that the inhibition of lipophagy in the liver results in massive lipid accumulation, and deficient lipophagy due to lipid accumulation alters lipid composition of autophagosomes and lysosomes which in turn affects the fusion process [124], thereby forming a "vicious circle" between lipid accumulation and lipophagy impairment.

Nowadays, restoring failed lipophagy is considered as a highly efficient therapeutic strategy for NAFLD. In a study using mice fed a HFD, overexpression of TFEB significantly improves liver function, likely due to increased activity of lipophagy which could involve the breakdown and removal of excess lipids [22]. Studies have demonstrated that some small molecules are able to prevent liver lipid accumulation by improving lipophagy, accomplished by impacting the activity of enzymes related to lipid metabolism or altering the expression of lipid transport proteins [151]. SCD (stearoyl-CoA desaturase) is a key enzyme controlling lipid metabolism and a link between its activity and NAFLD has been proposed [152]. CAY10566, an SCD-specific inhibitor, was highly effective in removing LDs in both in vitro and in vivo HFD models by activating lipophagy, identifying the SCD-lipophagy pathway a potential therapeutic target in NAFLD [153]. In addition, the fibroblast growth factor family members such as FGF21 (fibroblast growth factor 21) and the forkhead box class O family members such as FOXO1 (forkhead box O1), have been considered as regulators of lipophagy which may also serve as possible targets for combating NAFLD [105,154]. Theoretically, these studies all point to defects in lipophagy as a critical factor for the onset of NAFLD, and many candidates improving lipophagy in hepatocytes have been investigated and shown therapeutic potential in HFD-induced NAFLD models [155]. Therefore, the targeted upregulation of lipophagy may represent a viable therapeutic opportunity to promote the resolution of NAFLD.

Autophagy in hepatic non-parenchymal cells in NAFLD and liver fibrosis

Autophagy not only plays an important role in removing LDs from hepatocytes, but also impacts on hepatic non-parenchymal cells, including hepatic stellate cells (HSCs), hepatic macrophages and liver sinusoidal endothelial cells (LSECs), thereby affecting pro-inflammatory and fibrotic responses in NAFLD progression [156]. Due to the central role of autophagy in hepatocytes and relatively less data focused on the role of autophagy in non-parenchymal cells during NAFLD, we also mentioned functions of autophagy in hepatic non-parenchymal cells in the progression to liver fibrosis. The liver responds to chronic tissue injury by organ scarring, termed liver fibrosis [157]. The activation of quiescent HSCs is central for liver fibrogenesis, because these cells transdifferentiate into myofibroblasts which release a large amount of extracellular matrix [158–160]. In landmark studies, autophagy was proved to facilitate HSCs activation by promoting digestion of LDs in quiescent HSCs, while the link between autophagy and the loss of LDs was confirmed in vitro with pharmacological inhibitors of autophagy and small interfering RNAs against *Atg5* or *Atg7* [161–163]. Consistent with this, HSCs with *atg2a* knockout fail to undergo spontaneous transdifferentiation in cell culture [164]. In vivo studies further showed that mice bearing a specific *atg5* or *atg7* deletion in HSCs are resistant to fibrosis, and display decreased HSC activation and liver fibrosis in response to chronic carbon tetrachloride or thioacetamide challenge [161,163]. Deeper mechanistic studies demonstrated that ER stress [165] and oxidative stress [166] promote HSC activation by enhancing autophagy, resulting in enhanced fibrosis. Carvedilol, a recommended drug for treating portal hypertension, can alleviate liver fibrosis by inhibiting autophagic flux and subsequently inducing apoptosis in HSCs [167].

Nevertheless, those results are controversial since some of the studies demonstrated the beneficial role of autophagy in HSCs. It has been found that induction of autophagy is associated with inhibition of HSC activation and antifibrotic effects. For example, the study showed that the anti-fibrosis effect of methyl helicterate might depend on its apoptosis and autophagy-inducing mechanisms in HSCs [168]. A similar effect of autophagy of enhancing caffeine-induced apoptosis in HSCs is observed [169]. Autophagy also inhibits fibrogenic extracellular vesicles release in HSCs, which attenuates liver fibrosis in carbon tetrachloride treatment [170]. The TRIB3 (tribbles pseudokinase 3)-mediated autophagy caused migration, proliferation and activation of HSCs, while exerting

potent protective effects against hepatic fibrosis by restoring autophagic flux in HSCs [171]. Additionally, natural compounds used in primary cell lines of HSCs, exerted their antifibrotic role as autophagy inducers, while they had been also associated with inhibition of HSCs activation [172,173]. More studies are needed to further elucidate the complex roles of autophagy in HSCs in the future.

Macrophages are integral components of the innate immune system, and they primarily consist of resident Kupffer cells as well as infiltrating monocytes in the liver [174]. FAs are capable of activating hepatic macrophages via the transcription factor HIF1A (hypoxia inducible factor 1 subunit alpha), leading to impaired autophagy and a more inflammatory macrophage phenotype, thus aggravating inflammation in fatty liver disease [175]. It was demonstrated that hepatic macrophage autophagy was decreased in HFD-induced obesity, and mice with a myeloid cell-specific knockout of *Atg5* developed increased hepatic inflammation. The mechanism of increased inflammation was that hepatic macrophages with decreased autophagy, increased polarization into pro-inflammatory M1 macrophages and decreased polarization into anti-inflammatory M2 macrophages [176]. The specific disruption of autophagy in myeloid cells exacerbated liver fibrosis, hepatocellular injury and inflammation by exacerbating IL1A (interleukin 1 alpha) and IL1B (interleukin 1 beta) production [177]. In addition, chronic inflammation by M1-poralization of macrophages contributed to the disease progression from simple steatosis to NASH in concert with autophagic dysfunction in NAFLD patients [92]. Consequently, targeting macrophage autophagy may be a valued avenue for attenuating liver inflammation related diseases.

Another hepatic non-parenchymal cell type which is demonstrated an important function for autophagy in liver diseases is LSEC. LSECs are highly specialized endothelial cells that represent the first defense barrier of the liver between blood cells and hepatocytes or HSCs [178]. Decreased autophagy has been observed in the LSECs of patients with NASH [179] or in mice with selective disruption of *Atg7* when exposed to carbon tetrachloride [180]. Deficiency in autophagy in LSECs leads to cellular dysfunction, reduction in intrahepatic nitric oxide, impairment to handle oxidative stress and aggravates fibrosis [180]. Conflicting results were obtained regarding the effects of autophagy on LSECs. Increased autophagy was reported in LSECs in human fibrotic livers [181]. In a mouse model of fibrosis, the downregulation of the nitric oxide-dependent pathway occurs in LSECs during carbon tetrachloride treatment, and the autophagy inhibitor 3-methyladenine can attenuate this effect to improve liver fibrosis [181]. Differences in experimental design (i.e. the use of a chemical inhibitor in rats versus genetic deletion in mice) may explain this discrepancy [182]. Thus, there are arguments both for and against autophagy inhibition as a therapeutic strategy when considering only the effects on LSECs.

Taken together, these observations suggest that autophagy has a context-dependent impact on NAFLD and

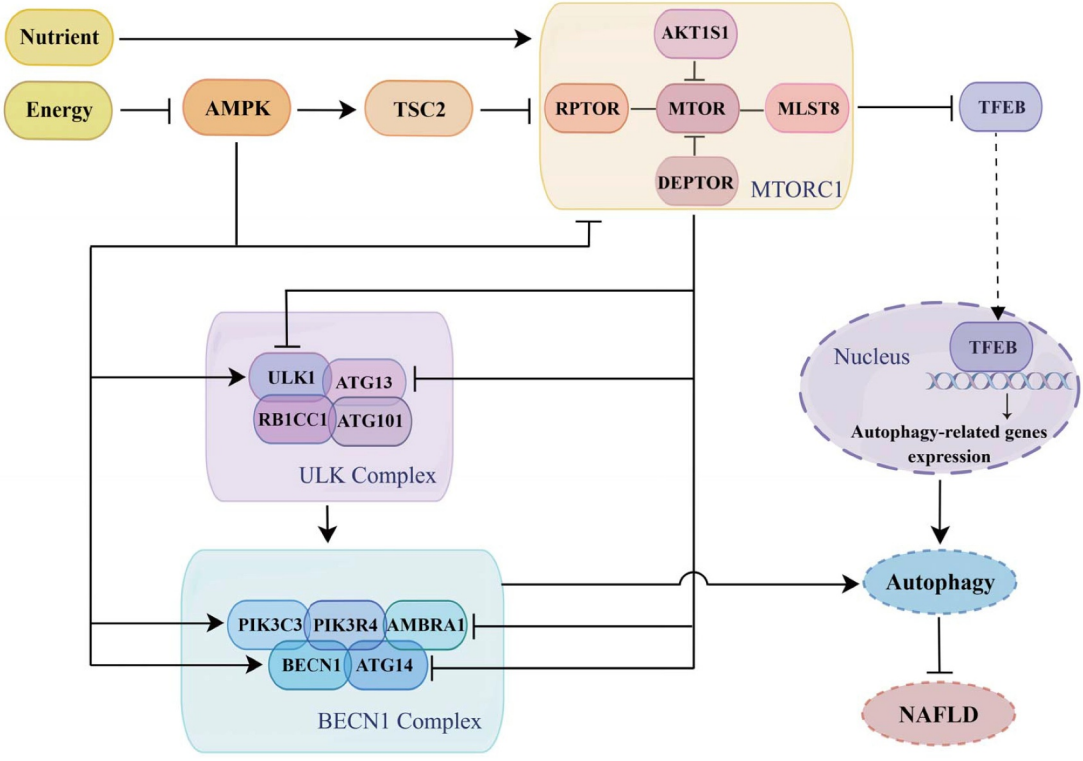

Figure 2. The AMPK-MTORC1 pathway-mediated autophagy under nutrient-rich conditions. The arrows represent promotion and the blunt arrows represent inhibition. See text for details.

liver fibrosis, and autophagy displays different functions depending on the cell type, which complicates the development of therapeutic paradigms based on autophagy modulation.

Regulation of autophagy by the AMPK-MTORC1 pathway

MTOR (mechanistic target of rapamycin kinase), a highly conserved serine/threonine protein kinase [183], has multiple functions including the regulation of protein synthesis, apoptosis and autophagy [184]. It has been shown that MTOR is a sensitive sensor for regulation of metabolism in hepatocytes, and over-activation of MTOR is closely associated with FA synthesis, hepatic insulin resistance and metabolic liver diseases [184,185]. Of note, MTOR can recruit several chaperones through the relatively independent domains to form two different complexes: MTOR complex 1 (MTORC1) and MTORC2 [184]. Because MTORC2 is relatively insensitive to rapamycin and its functional characterization is less advanced, MTORC1 is the focus of this review.

MTORC1 consists of three core components: MTOR, RPTOR (regulatory associated protein of MTOR complex 1), MLST8 (MTOR associated protein, LST8 homolog), and two inhibitory subunits: AKT1S1 (AKT1 substrate 1), DEPTOR (DEP domain containing MTOR interacting protein) [186–188]. Among them, MTOR is the catalytic subunit of the entire complex [189]. MTORC1 is the core hub negatively regulating autophagy which can be activated by nutrient, energy and growth factors [184]. Another crucial regulator of autophagy is AMPK, and it can be downregulated by increased ATP: AMP or ATP: ADP ratios due to excess energy as an energy-sensing kinase [190]. It has also been suggested that AMPK regulates TSC2 (TSC complex subunit 2) [191] and RPTOR [192] to inhibit the expression of MTORC1 on cellular metabolism. Since AMPK and MTORC1 are subject to different signaling pathways by regulating ATG proteins and lysosome biosynthesis in mammalian cells, we briefly listed some classical pathways associated with AMPK-MTORC1 mediated autophagy (Figure 2). First, when nutrient is sufficient, MTORC1 is activated and negatively regulates autophagy by directly phosphorylating ULK1 [193,194], and this phosphorylation also disturbs the interaction between ULK1 and AMPK to inhibit autophagy [195]. Moreover, MTORC1 also phosphorylates ATG13 to inhibit the activity of the ULK complex, whereas the MTORC1 phosphorylation site in ATG13 remains to be determined [196,197]. Second, under nutrient-rich conditions, MTORC1 directly phosphorylates AMBRA1 in the BECN1 complex, thereby suppressing autophagy [198]. Also, MTORC1 inhibits autophagy by directly phosphorylating ATG14 and thus inhibits the formation of autophagosomes [199,200]. Third, MTORC1 indirectly inhibit autophagy by regulating the lysosomal biogenesis. Upon entry into the nucleus, TFEB promotes transcription of genes encoding proteins required for lysosomal biogenesis and autophagy, thereby indirectly promoting autophagy [201]. MTORC1 can phosphorylate TFEB at several serine/threonine residues which traps TFEB in the cytosol, thereby impairing autophagy [201,202]. In addition, TFEB-regulated genes affect autophagosome biogenesis, the fusion between autophagosomes and lysosomes, and the degradation of the autophagic content in the lysosomes [203]. Fourth, the inhibition of AMPK can increase the activity of MTOR by stimulating the major subunit of MTORC1, RPTOR [192]. By the action of the above, the inhibition of AMPK can directly inactivate ULK1 [204] or autophagy core components such as PIK3C3/VPS34 and BECN1, to reduce autophagy [205,206].

In note, the MTORC1 expression level was significantly higher and liver steatosis was more severe in the rats fed a HFD when compared to the group fed a regular chow diet, suggesting that MTORC1 contributes to NAFLD progression [207]. A study was recently performed to understand how the regulatory signaling of MTORC1-TFEB responded to the stimulation of the HFD feeding in vivo, and it has found that enhancing TFEB results in a resumption of autophagic activity and improves hepatic steatosis, whereas sustaining MTORC1 exacerbates the pathology of NAFLD [22]. In line with this, AMPK is also necessary for regulation of autophagy, and it is well documented that increasing AMPK activity is one of the viable treatment strategies for NAFLD [208]. Hepatocyte-specific AMPK activation counteracted the accumulation of fat in the liver during obesity by autophagy activation [145]. Since AMPK and MTORC1 are both notable modulators of autophagy and MTORC1 is an important downstream target of AMPK, there is increasing evidence that AMPK-MTORC1-mediated autophagy plays an important role in the pathological condition of liver, especially in HFD-induced NAFLD [209]. For instance, the studies have confirmed that bariatric surgery in rodents improves liver steatosis while increasing AMPK and decreasing MTORC1 activation [210,211]. On the contrary, therapeutic dosages of acetaminophen aggravate fat accumulation in HFD-induced NAFLD, and the potential mechanism might be related to the inhibition of autophagy via the AMPK-MTORC1 pathway, which highlights the importance of using a lower dose of acetaminophen for patients with NAFLD [212]. Recently, atractyloside, a diterpenoid glycoside, has been considered to promote the activation of AMPK and decrease the MTORC1 activity, thus promoting autophagosomes formation and accelerating the degradation of HFD-induced accumulated lipids in the mice livers [213]. Consistent with this finding, the main mechanism of empagliflozin function is also to activate AMPK-MTORC1-mediated autophagy to improve HFD-induced NAFLD [214]. A recent study has revealed that AMPK-MTORC1-mediated autophagy was significantly decreased in HFD-fed mice. When MBOAT4/ghrelin o-acyltransferase is inhibited, there is a significant increase

Table 2. The genetic interventions on hepatocytic autophagy in NAFLD models.

Genetic intervention	Animal models	Fat (% energy from fat) and duration (weeks)	Cell lines and treatment	Hepatocytic autophagy (up or down)	Hepatic steatosis outcome (up or down)	Mechanisms	Ref.
Acox1	*acox1* f/f; *Alb-Cre* mice (*acox1*-Li KO)	60%, 16	/	Up	Down	Decreasing total cytosolic acetyl-CoA levels; Impairing RPTOR acetylation, lysosomal localization of MTOR and activation of MTORC1	[223]
Adh5	*adh5* KO mice; C57BL/6 with AAV8-TBG-*Adh5* injection (*adh5*-Li conditional KO)	60%, 16	/	Down	Up	Increasing lysosomal nitrosative stress and impairing autophagy in hepatocytes; Leading to hepatic insulin resistance	[224]
Adipoq	*adipoq* KO mice	45%, 20	*Adipoq* KD HepG2 cell; PA (0.5 mM) for 6 h	Up	Down	Suppressing AMPK phosphorylation; Promoting phosphorylation of the autophagy makers	[225]
Crtc2	*crtc2* f/f; *Alb*-Cre mice (*crtc2*-Li KO)	60%, 8	*Crtc2* KD AML12 cell; PA (0.15 mM) for 4 h	Up	Down	Regulating the SIRT1-MTORC1 pathway; Enhancing lipophagy and Limiting lipogenesis	[226]
F2rl1	*f2rl1* KO mice	60%, 16	/	Up	Down	Decreasing interaction between CAMKK2 (calmodulin dependent protein kinase kinase 2) and ARRB2 (arrestin beta 2); Enhancing AMPK activation and AMPK-mediated autophagy	[227]
Grk2	*grk2* KO mice	45%, 12	*Grk2* KD Huh7 cell; PA (0.5 mM) for 24 h	Up	Down	Reducing a readout of the MTORC1 autophagy-inhibitory pathway; Preserving autophagy, mitochondrial dynamics and better lipid handling	[228]
Htra2	C57BL/6 mice with AAV8-TBG-mNeongreen-*Htra2* injection (*Htra2*-Li conditional OE)	60%, 12	*Htra2* OE L02 cell; OA (0.66 mM) and PA (0.33 mM) for 24 h	Up	Down	Enhancing mitochondrial FA β-oxidation and autophagy-network genes expression; Improving mitochondrial functions and restoring autophagic flux	[229]
Kdm6b	*Kdm6b* f/f mice with AAV-TBG-Cre injection (*kdm6b*-Li conditional KO)	60%, 4	/	Down	Up	Inhibiting demethylation of histone and interaction with PPARA; Downregulating autophagy-network genes and autophagy-mediated lipid degradation	[105]
Lamp2	*lamp2* f/f; *Alb*-Cre mice (*lamp2*-Li KO)	60%, 12	*Lamp2* KD HepG2 cell; OA (0.1 mM) for 8 h	Down	Up	Destroying autophagy and the degradation of the perilipin protein; Hindering LDs breakdown but not increasing LDs formation	[144]
Map3k5	*map3k5* f/f; *Alb*-Cre mice (*map3k5*-Li KO)	58%, 20	*Map3k5* KD HepG2 cell; PA (0.4 mM) for 24 h	Down	Up	Blunting and impairing autophagy; Enhancing LDs accumulation and liver fibrosis	[230]
Med1	*med1* f/f; *Alb*-Cre mice (*med1*-Li KO)	40%, 16	*Med1* KD HepG2 cell; OA (0.2 mM) and PA (0.1 mM) for 24 h	Down	Up	Affecting transcriptional activity of nuclear receptors and transcription factors including PPARA and FOXO1; Impairing lipophagy and FA β-oxidation	[231]
Nfe2l2	*nfe2l2* KO mice	60%, 12	*Nfe2l2* KD HepG2 cell; OA (0.66 mM) and PA (0.33 mM) for 24 h	Down	Up	Regulating lysosomal associated membrane protein 1 transcriptional activity; Inhibiting the fusion of autophagosomes and lysosomes; Reducing autophagic flux and lipolysis	[232]
Nos3	*nos3* KO mice	45%, 18	*nos3* KO primary hepatocytes; OA (0.25 mM) and PA (0.25 mM) for 16 h	Down	Up	Suppressing the hepatic activation of NFE2L2 and pro-autophagy effect through its transcriptional target SQSTM1	[233]
Nr1h3	*nr1h3* KO mice	60%, 12	/	Up	Down	Increasing autophagy through upregulating ATG4B and RAB8B; Enhancing mitochondrial biogenesis and fuel consumption	[234]
Plin2	*plin2* f/f; *Alb*-Cre mice (*plin2*-Li KO)	45%, 12	*plin2* KO primary hepatocytes; OA (0.5 mM) for 2 h	Up	Down	Improving lipophagy and adipose triglyceride lipase-mediated lipolysis; Increasing cellular FA oxidation in an autophagic-dependent manner	[235]

(*Continued*)

Table 2. (Continued).

Genetic intervention	Animal models	Fat (% energy from fat) and duration (weeks)	Cell lines and treatment	Hepatocytic autophagy (up or down)	Hepatic steatosis outcome (up or down)	Mechanisms	Ref.
Plin3	C57BL/6 mice with AAV8-GFP-U6-m-M6PRBP1-Plin3 shRNA injection (*plin3*-Li conditional KO)	45%, 4	*Plin3* KD NIH-3T3 cell; OA (0.25 mM) for 6 h	Down	Up	Inhibiting recruitment of autophagy machinery to LD and lipophagy	[145]
Rubcnl	*rubcnl* f/f; *Alb*-Cre mice (*rubcnl*-Li KO)	42%, 16	*Rubcnl* KD L02 cell; OA for 4 h	Down	Up	Disrupting association with STX17 and the homotypic fusion and protein sorting complex; Abolishing RUBCNL-mediated autophagosome maturation	[236]
Sirt3	C57BL/6 mice with AAV8-TBG-*Sirt3* injection (*sirt3*-Li conditional OE)	45%, 8	*Sirt3* OE HepG2 cell; PA (0.5 mM) for 12 h	Down	Up	Enhancing manganese superoxide dismutase deacetylation and activation; Leading to AMPK inhibition, MTORC1 activation and autophagy suppression	[237]
Sirt3	*Sirt3* OE mice	60%, 12	*Sirt3* OE primary hepatocytes; PA (0.075 mM) for 24 h	Up	Down	Promoting BCL2 interacting protein 3-required autophagy	[238]
Srebf1	*srebf1* KO mice	60%, 12	*srebf1* KO primary hepatocytes; OA (0.5 mM) for 16 h	Up	Down	Promoting the cystathionine gamma-lyase and sulfhydration-dependent activation of ULK1; Increasing autolysosome formation	[239]
Srebf2	C57BL/6 mice with shRNA injection (*srebf2*-Li conditional KO)	60%, 19	*Srebf2* KD AML12 cell; PA (0.4 mM) for 24 h	Up	Down	Reducing lipid deposition and ER stress via an autophagy-dependent pathway	[240]
Sting1	/	/	*sting1* KO primary hepatocytes; PA (0.5 mM) for 24 h	Up	Down	Involving in MTORC1 activation which dependent on SQSTM1; Enhancing lipophagy and LDs degradation	[241]
Tp53	*trp53* KO mice	60%, 8	*Tp53* KD HepG2 and Huh7 cell; PA (0.4 mM) for 24 h	Up	Down	Promoting the HMGB1-mediated induction of autophagy	[242]
Vmp1	*vmp1* f/f; *Alb*-Cre mice (*vmp1*-Li KO)	45%, 6	/	Down	Up	Impairing hepatic autophagy; Altering the fatty acyl chain compositions of phospholipids; Decreasing FA β-oxidation	[243]

Articles using genetic interventions and high-fat diets feeding involved hepatocytic autophagy in male rodents. LC3-II and SQSTM1 were measured by western blots and described semi-quantitatively to detect hepatocytic autophagic activity (up or down). ACOX1: acyl-CoA oxidase 1; ADH5: alcohol dehydrogenase 5 (class III), chi polypeptide; ADIPOQ: adiponectin, C1Q and collagen domain containing; CRTC2: CREB regulated transcription coactivator 2; F2RL1: F2R like trypsin receptor 1; GRK2: G protein-coupled receptor kinase 2; HTRA2: HtrA serine peptidase 2; KD: knockdown; KDM6B: lysine demethylase 6B; KO: knockout; LAMP2: lysosomal associated membrane protein 2; Li KO: liver-specific knockout; MAP3K5: mitogen-activated protein kinase kinase kinase 5; MED1: mediator complex subunit 1; NFE2L2: NFE2 like bZIP transcription factor 2; NOS3: nitric oxide synthase 3; NR1H3: nuclear receptor subfamily 1 group H member 3; OA: oleic acid; OE: overexpression; PA: palmitic acid; RUBCNL: rubicon like autophagy enhancer; PLIN2: perilipin 2; PLIN3: perilipin 3; SIRT3: sirtuin 3; SREBF1: sterol regulatory element binding transcription factor 1; SREBF2: sterol regulatory element binding transcription factor 2; STING1: stimulator of interferon response cGAMP interactor 1; TP53/p53: tumor protein p53; VMP1: vacuole membrane protein 1.

in AMPK-MTORC1-mediated autophagy, which resulted in the alleviation of hepatic toxicity [215]. Furthermore, the therapeutic potential of hydrogen sulfide in treating NAFLD has been reported, and the primary mechanism of action of hydrogen sulfide is to activate AMPK-MTORC1-mediated autophagy by targeting AMPK [216].

Intriguingly, deacetylation of LC3 by SIRT1 (sirtuin 1) is essential for the redistribution of nuclear LC3 to the cytoplasm and its conjugation to autophagic membranes [217], suggesting a critical role of acetylation or deacetylation events in autophagy induction. During autophagy, SIRT1 could be activated by mechanisms that depend on AMPK, whereas MTORC1-dependent phosphorylation of acetyltransferase EP300 (E1A binding protein p300) suppresses autophagy [218]. Moreover, MTORC1 was found to play a pivotal role in the post-translational regulation of WIPI2 (WD repeat domain, phosphoinositide interacting 2) by which MTORC1 precisely controlled the intensity of induced autophagy [219]. Under nutrient-enriched conditions, MTORC1 has inhibited autophagy by phosphorylating UVRAG (UV radiation resistance associated) [220] which is known to regulate autophagosome maturation as well as early stages of autophagy [221,222].

To summarize this section, the mechanisms of studies that have been proven to induce autophagy and improve HFD-induced NAFLD, is the inhibitory effect of activated the AMPK-MTORC1 pathway or the downstream of MTORC1. Clarifying the inter-relation among AMPK, MTORC1 and autophagy will allow the development of additional therapeutic options which can better target and reprogram the underlying pathophysiological pathways, aiming to attenuate NAFLD progression.

Genetic interventions of autophagy in HFD-induced NAFLD

Not surprisingly, autophagy is involved in disease-limiting functions in a broad range of hepatological disorders. To understand the underlying mechanisms, studies have been conducted and revealed complex regulatory networks at genetic levels to explore the function of autophagy in HFD-induced NAFLD. In this section, we analyzed the articles that used genetic interventions to either promote or inhibit hepatocytic autophagy and measured hepatic steatosis outcome in rodent models. The results are summarized in Table 2.

The HFD feeding has been found to promote hepatic steatosis in all studies, and more than half of studies were performed in mice fed 60% energy from fat. In addition, we drew a conclusion that impaired autophagy occurred in mice fed with HFDs in virtually all studies. Hepatocyte-specific knockout of *Acox1* which could catalyze the first step in peroxisomal β-oxidation, protected mice against hepatic steatosis caused by a HFD due to decreased RPTOR acetylation and reduced lysosomal localization of MTORC1, resulting in the stimulation of autophagic degradation of LDs [223]. Another study has shown that hepatic depletion of *Crtc2* protected mice from the progression of HFD-induced fatty liver phenotype via the SIRT1-MTORC1 pathway to enhance the lipophagy in hepatocytes [226]. In line with this, hepatocyte-specific knockout of *Srebf2* by shRNA interventions improved the impaired autophagic flux, and reduced lipid deposition and ER stress via an autophagy-dependent pathway in a mouse model of NAFLD [240]. Besides, it has been showed that liver ablation of *Plin2*, the most abundant LD-associated protein in steatosis liver, protected mice from HFD-induced liver steatosis by increasing cellular FA oxidation in an autophagic-dependent manner [235]. In contrast, mice with adenoviral-mediated liver-deletion of *Plin3* presented increased hepatic LDs due to inhibited recruitment of autophagy machinery to LDs when exposed to the nutrient-rich condition [145]. The two experiments with completely opposite results have demonstrated that different genes may have different effects on autophagy in HFD-induced NAFLD even though they are members of the same protein family. Also, more LDs accumulated in liver-specific *lamp2* knockout mice under high-fat challenge due to dysregulation of hepatocytic autophagy and LD breakdown, clearly indicating a close relationship between lysosomal functional integrity in autophagy and NAFLD development [144]. Under nutrient-rich conditions, liver deletion of *Rubcnl* disrupts the association of RUBCNL with STX17 and the transmembrane and ubiquitin like domain containing 1 complex, and thus abolished RUBCNL-mediated autophagosome maturation, resulting in lipid accumulation and liver fibrosis in a mouse model of NAFLD [236]. Moreover, liver-specific knockout of *Kdm6b* increased hepatic steatosis induced by HFD in a mechanism dependent on downregulating global autophagy-network genes and autophagy-mediated lipid degradation. This effect can be abolished by administration of FGF21 which improves defective autophagy in a KDM6B-dependent manner, further indicating that autophagic activity counteracts lipid accumulation in HFD-induced NAFLD [105]. Notably, hepatocyte-specific *Htra2* overexpression in both in vitro and in vivo models of NAFLD improves hepatic steatosis by enhancing mitochondrial FA β-oxidation and restoring autophagic flux [229]. These studies all have corroborated the idea that regulation of gene expression in hepatocytes to reactivate autophagy in NAFLD may be a strategy to improve hepatic steatosis.

Within nutrient-overload steatosis mice models, the knockout of some genes systemically also has a significant impact on hepatocytic autophagy. An experiment using *adipoq* knockout mice elicited protective effect against hepatic steatosis, possibly associated with autophagy regulation in persistent HFD intake [225]. The *nr1h3* knockout mice fed a HFD showed increased autophagy through upregulating ATG4B and enhancing mitochondrial oxygen consumption [234]. This highlights a new function of NH1R3, which culminates in the progression of NAFLD, and the identified targets may be applied for a therapeutic strategy in the future. During a HFD feeding, *f2rl1* knockout mice presented decreased lipid accumulation, accompanied by increasing AMPK-MTORC1-mediated autophagy when compared to wide-type mice, suggesting that restoring autophagy could prevent hepatic lipid accumulation in certain context [227]. In addition, the anti-NAFLD effect of functional *Tp53* silencing in HFD-fed mice was associated with the HMGB1 (high mobility group box 1)-mediated induction of autophagy [242], and *srebf1* knockout mice showed apparent lower accumulation of LDs in the liver through sulfhydration-dependent activation of ULK1 [239]. Conversely, the *nos3* or *nfe2l2* knockout mice fed with HFD showed more lipid accumulation in hepatocytes in comparison to wild-type mice, which was mainly due to suppressed fusion of autophagosomes and lysosomes and inhibited autophagic flux in the liver [232,233]. Overall, the autophagy appears to be a part of complex signaling networks that involve a range of important genes to adapt nutritional overload.

Consistently, in vitro studies also help understand how the participation of autophagy in hepatic lipid metabolism can be modulated. For instance, knockout of *Sting1* in primary hepatocytes treated with palmitic acid (PA) led to enhanced lipophagy and LDs degradation, involving in MTORC1 activation which dependent on SQSTM1 [241]. In line with this, in primary hepatocytes treated with oleic acid (OA), depletion of *Srebf1* or *Plin2* both present reduced lipid deposition via an autophagic-dependent manner [235,239]. At the cellular level, HepG2 cells with *Med1* knockdown shows decreased lipophagy and FA β-oxidation that is accompanied by a significant increase in lipid content during PA and OA treatment. When faced to the stimulation of a superfluous level of nutrients, knockdown of *Rubcnl* in L02 cells abolished RUBCNL-mediated autophagosome maturation, resulting in lipid accumulation [236].

Interestingly, the two experiments that overexpressed *Sirt3* in hepatocytes or in the whole body of mice fed with HFDs showed completely opposite results. Hepatocyte-

Table 3. The pharmacological interventions on hepatocytic autophagy in NAFLD models.

Supplement	Chemical properties	Concentration (per body weight) and duration (weeks)	Animal models	Fat (% energy from fat) and duration (weeks)	Hepatocytic autophagy (up or down)	Hepatic steatosis outcome (up or down)	Mechanisms	Ref.
Acetaminophen	P-aminophenol derivative	200 mg/kg, 12	C57BL/6J mice	42%, 8	Down	Up	Inhibiting autophagy associated with the AMPK-MTORC1 pathway	[212]
Ajugol	Active alkaloid	50 mg/kg, 12	C57BL/6 mice	60%, 12	Up	Down	Inactivating MTORC1 and inducing nuclear translocation of the TFEB; Enhancing lysosomal biogenesis and the fusion of autophagosome and lysosome	[244]
Atractyloside	Diterpenoid glycoside	2 or 4 mg/kg, 2	ICR WT mice	60%, 8	Up	Down	Increasing the activation of AMPK and RPTOR expression; Decreasing the MTORC1 activity; Promoting autophagosomes formation via the AMPK-MTORC1 signaling pathway	[213]
Aurantio-obtusin	Bioactive compound	5, 10 or 15 mg/kg, 4	C57BL/6J mice	42%, 8	Up	Down	Ameliorating hepatic steatosis via AMPK-autophagy- and AMPK-TFEB-mediated suppression of lipid accumulation	[245]
Berbamine	Natural bisbenzylisoquino-line alkaloid	50 or 150 mg/kg, 4	Wistar rats	40%, 12	Up	Down	Enhancing the SIRT1-AMPK pathway	[246]
Caffeine	Xanthine alkaloid compound	Drinking water containing .05% caffeine, 4	C57BL/6 mice	60%, 8	Up	Down	Reducing intra-hepatic lipid content and stimulating β-oxidation in hepatic cells and liver via an autophagy-lysosomal pathway	[247]
Carbamazepine	Tricyclic compound	25 mg/kg, 1	C57BL/6 mice	60%, 12	Up	Down	Acting MTOR independent autophagy inducer by reducing inositol levels	[248]
Catalpol	Bioactive compound	50 mg/kg, 12, 4	C57BL/6 mice	60%, 12	Up	Down	Stimulating AMPK and increasing nuclear translocation of TFEB	[249]
Eucommia ulmoides leaf extract	Natural plant extracts	200 mg/kg, 10	SD rats	60%, 10	Up	Down	Suppressing ER stress and enhancing lysosomal functions	[250]
Empagliflozin	Sodium-dependent glucose transporters 2 inhibitor	10 mg/kg, 5	C57BL/6J mice	45%, 5	Up	Down	Increasing AMPK phosphorylation and decreasing MTORC1	[214]
Fenofibrate	PPARA agonist	100 mg/kg, 4	C57BL/6J mice	60%, 9	Up	Down	Activating PPP3/calcineurin and the CAMKK2-AMPK-ULK1 pathway; Promoting TFEB dephosphorylation and nuclear translocation	[251]
Formononetin	Natural isoflavone	100 mg/kg, 14	C57BL/6J mice	60%, 16	Up	Down	Activating AMPK and promoting subsequent nuclear translocation of TFEB; Enhancing lysosome biogenesis and autophagosome-lysosome fusion	[252]
Liraglutide	Glucagon like peptide 1 receptor (GLP1R) agonist	400 μg/kg, 4	C57BL/6J mice	60%, 18	Up	Down	Restoring autophagic flux through the GLP1R-TFEB-mediated autophagy-lysosomal pathway	[253]
Liraglutide	GLP1R agonist	150 μg/kg, 4	C57BL/6J mice	60%, 16	Up	Down	Diminishing oxidative stress by enhancing mitochondrial architecture; Promoting autophagy through the SIRT1-FOXO3-LC3 pathway	[254]
Metformin	Biguanide	300 mg/kg, 9	C57BL/6J mice	60%, 14	Up	Down	Increasing the activity of TFEB	[255]
Naringenin	Flavanone and the aglycone of naringin	10, 30 or 90 mg/kg, 2	SD rats	60%, 12	Up	Down	Increasing AMPK activity at low concentrations	[256]
Nuciferine	Aromatic ring containing alkaloid	HFD containing .01 or .03% nuciferine, 4	C57BL/6 mice	60%, 16	Up	Down	Suppressing lysosomal localization and activity of MTORC1; Activating TFEB-mediated autophagy lysosomal pathway	[257]
Phillygenin	Lignan	25 mg/kg, 16	C57BL/6J mice	60%, 16	Up	Down	Activating PPP3/calcineurin and regulating TFEB dephosphorylation and nuclear translocation; Promoting lysosomal biogenesis	[258]
Rapamycin	Organic heterotricyclic compound	2 mg/kg, 14	C57BL/6J mice	60%, 14	Up	Down	Inhibiting the phosphorylation of MTORC1 and its downstream translational regulators; Inhibiting hepatic CD36 protein expression	[259]
Rapamycin	Organic heterotricyclic compound	5 mg/kg, 8	C57BL/6J mice	60%, 13	Up	Down	Reducing SREBF1-dependent de novo lipogenesis; Promoting PPARA-mediated FA oxidation	[260]
Resveratrol	Natural polyphenol compound	50 mg/kg, 3	C57BL/6J mice	45%, 12	Up	Down	Reducing lipogenesis while restoring lipolysis in the murine liver via ULK1-mediated autophagy pathway	[110]

Articles using pharmacological interventions and high-fat diets feeding involved hepatocytic autophagy in male rodents. LC3-II and SQSTM1 were measured by western blots and described semi-quantitatively to detect hepatocytic autophagic activity (up or down).

specific overexpression of *Sirt3* leads to AMPK inhibition, MTORC1 activation and autophagy suppression in mice fed 60% energy from fat during 12 weeks, suggesting that restraining SIRT3 overactivation can be a potential therapeutic choice for the treatment of NAFLD [237]. In contrast, mice with *Sirt3* overexpression promote autophagic activity and improve hepatic steatosis when fed 45% energy from fat during 8 weeks, and strategies for enhancing SIRT3 activity can be used to treat NAFLD based on this finding [238]. This phenomenon may be related to the type of genetic interventions as well as the percentage of energy from fat or the duration of HFD, and the molecular mechanisms are still under investigation. Even so, studies involved the genetic interventions have investigated nutritional overload liver steatosis covered a greater variety of strategies, such as genetic knockdown, knockout or overexpression of the vital gene both systemically or in a liver-specific manner, indicating the importance and potential of genetic modulation in HFD-induced NAFLD.

Pharmacological interventions of autophagy in HFD-induced NAFLD

The crucial role of autophagy in support of energy homeostasis and regulation of signaling pathways in hepatic metabolism makes it an attractive therapeutic target for NAFLD. In this section, we analyzed the articles that evaluated the effects of pharmacological interventions on hepatocytic autophagy and measured hepatic steatosis outcome in rodent models. The results are summarized in Table 3.

Pharmacological targeting of autophagy may ameliorate hepatic pathologies involved impaired autophagy. So far, a number of autophagy regulators have been discovered and applied in NAFLD models, including inhibitors of lysosomotropic autophagy such as chloroquine, as well as rapamycin [28]. Rapamycin, a macrolide antibiotic originally purified from *Streptomyces hygroscopicus* [261], can inhibit the activity of MTORC1 [262], thus stimulating autophagy. It is well documented that rapamycin obviously ameliorated hepatic steatosis and liver injury in HFD-fed mice through reducing SREBF1-dependent de novo lipogenesis and promoting PPARA-mediated FA oxidation [260]. Consistent with this finding, rapamycin inhibited hepatic CD36 translational efficiency through the MTOR signaling pathway, resulting in reduction of CD36 protein expression and alleviation of hepatic steatosis [259]. Chronic administration of rapamycin in mice fed HFDs in above experiments resulted in improved outcomes for steatosis, indicating that activating autophagy mitigated reduced lipid accumulation in hepatocytes. Nevertheless, it is worth noting that while autophagic activation improved hepatic steatosis in the majority of studies, the chronic use of rapamycin could be harmful to adipose tissue functions [263,264], making it a controversial therapy for NAFLD.

Another encouraging target of pharmacological therapy for NAFLD by manipulating autophagy is the AMPK-MTORC1 pathway. In this context, aurantio-obtusin, a main bioactive compound isolated from *Cassia semen*, was identified to ameliorate hepatic steatosis via AMPK-autophagy- and AMPK-TFEB-mediated suppression of lipid accumulation in mice fed a HFD [245]. Besides, the treatment of ajugol was showed to significantly relieve HFD-induced hepatic steatosis and inhibit PA-induced lipid accumulation in hepatocytes. Mechanistically, ajugol inactivated MTORC1, induced TFEB-mediated lysosome biogenesis to combat the pathogenesis of NAFLD [244]. Additionally, supplementation with formononetin exhibited significantly decreased hepatic steatosis and hepatic LDs accumulation by activating AMPK and promoting subsequent nuclear translocation of TFEB in mice during a HFD feeding [252]. A recent study has revealed that nuciferine suppressed lysosomal localization and activity of MTORC1, and activated TFEB-mediated autophagy lysosomal pathway in mice fed with a HFD, thereby preventing the progression of NAFLD [257]. Phillygenin, which is isolated from *Forsythia suspensa*, has been found to attenuate LD accumulation by activating autophagy via the regulation of TFEB dephosphorylation and nuclear translocation [258]. Furthermore, catalpol [249], metformin [255], naringenin [256], berbamine [246] and fenofibrate [251] have been reported to affect autophagy through mechanisms similar to above those in HFD-induced animal NAFLD models. In addition, carbamazepine played a protective role in reducing steatosis and improving insulin sensitivity in HFD-fed mice, while acting a MTOR-independent autophagy inducer by reducing inositol levels [249]. Interestingly, persistent HFD inducement resulted in severe NAFLD while liraglutide treatment significantly reversed the trend in the two experiments [253,254]. Although the mechanisms through which liraglutide worked differed between the two experiments, one by restoring autophagic flux through the GLP1R-TFEB-mediated autophagy-lysosomal pathway, and the other by promoting autophagy through the SIRT1-FOXO3 (forkhead box O3)-LC3 pathway, they both aimed to improve NAFLD by stimulating autophagy. These studies all have assigned that the use of supplements to induce autophagy as an emerging approach to the treatment of NAFLD.

Recently, polylactic acid particles have been prepared by nanoprecipitation without any surfactant, followed by surface peptide adsorption to design autophagy-inducing particles using the autophagy-inducing peptide. The nanoparticles are of particular interest as they are able to modulate hepatic autophagy and improve liver lipid metabolism [265]. In addition, studies using intermittent fasting and second-generation MTOR inhibitors are especially exciting [266], indicating that the targeted enhancement of autophagy may represent a viable chance to improve hepatic steatosis. In summary, increasing evidence supports an important role of autophagy in protecting against HFD-induced NAFLD, and pharmacological upregulation of autophagy may be a potential therapeutic option for treatment. Notably, while autophagy plays an active role in the beneficial actions of above-mentioned supplements in the prevention of NAFLD, it is important to note that these properties are often tied to their ability to intercept multiple

(rather than individual) cellular pathways which makes their use not advisable [267], so a better understanding of the mechanisms along with their complex interrelationships is needed.

Conclusions, controversies and future perspectives

Unhealthy lifestyle habits, especially the excessive intake of high-fat food, are the cornerstone for the pathogenesis of NAFLD. No treatment is currently available, but autophagy induction has been proposed as a promising therapeutic strategy while promoting autophagic activity (pharmacologically or genetically) has improved steatosis outcome by decreasing lipid accumulation in hepatocytes (Table 2 and 3). As numerous high-throughput screenings for autophagy inducers have been ongoing, more studies will be anticipated in the future to test these newly identified autophagy modulators for treating metabolic disease conditions.

However, the contribution of autophagy to liver lipid metabolism is still controversial. Although the majority of articles have demonstrated a protective role of autophagy in nutritional overload steatosis, there is still increasing evidence that genetic lack of hepatic autophagy leads to unchanged or decreased steatosis outcome in HFD-induced NAFLD [112,268]. The above divergence may be due to the different experimental designs in each study. Time and type of genetic intervention as well as animal genetic background may result in apparent differences, and duration of diet feeding may be another decisive factor [25]. Interestingly, several studies using autophagy-deficient mice have indicated an impaired capacity for lipid accumulation in response to fasting-induced hepatic steatosis [269–272]. It is possible that the role of autophagy in steatosis development may vary according to the source of stimulation, and more in-depth research is needed to improve our knowledge in this context.

At present, although canonical autophagy is the most prevalent form of autophagy and the focus of this review, the LC3-associated phagocytosis (LAP) which is a novel form of non-canonical autophagy also plays a critical role in NAFLD development. This phagocytic process results in the recruitment of some, but not all, members of the autophagic machinery to the stimulus-containing phagosome, facilitating degradation of engulfed pathogens and modulation of the immune response [273,274]. LAP has been found to protect against hepatic inflammation during chronic liver injury with beneficial antifibrogenic effects [275,276], suggesting that LAP may open therapeutic perspectives for patients with chronic liver disease in the future.

Additionally, some important issues remain to be resolved. For instance, the tissue-specific autophagy hampers the translation of autophagy induction therapeutic since autophagy decreased in the liver [89] but activated in the white adipose tissue [277] in patients with NAFLD. With the advent of single cell technologies in the preclinical routine, it will be of primary importance to clarify the mechanisms underlying how tissue-specific autophagy contributes to disease, and develop useful tools to selectively modulate autophagy in certain tissues. Finally, as there are various cell types that have distinctive functions in the liver, how to selectively target the autophagy process in a specific cell type without affecting the others remains to be another important issue.

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