University of Crete
Faculty of Medicine
Division of Basic Sciences

MSc Thesis

Regulation of hepatic apolipoprotein A-I levels by autophagy

Michalis D. Gounis

Supervisor: Prof. Aristides Eliopoulos

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Summary

Apolipoprotein A-I (ApoA-I), the major protein component of high-density lipoprotein (HDL), plays important roles in lipid metabolism through its known function in reverse cholesterol transport (RCT). Numerous epidemiological and clinical studies have demonstrated an inverse correlation between plasma high-density lipoprotein (HDL) cholesterol levels or circulating ApoA-I levels and the risk for coronary heart disease in humans. Despite these associations, ApoA-I cell biology (e.g. transport, degradation, recycling) has remained largely unexplored. Here, we describe a novel role for autophagy in regulating the degradation of ApoA-I in hepatocellular carcinoma cells (HepG2). After treatment of hepatocytes with inhibitors of autophagosome-lysosome fusion or lysosomal acidification (bafilomycin A1, chloroquine), intracellular ApoA-I accumulated within cells forming aggregate-like structures. In fact, these structures partially co-localized with LC3, an autophagosomal marker, while ApoA-I was also found to accumulate within lysosomes/late endosomes as detected by LAMP-1 staining. Inhibition of mTOR (Torin2) led to activation of autophagy and reduced ApoA-I levels, which were not entirely due to autophagic clearance. Genetic inhibition of autophagy (knockdown of autophagy-related genes) prevented degradation of ApoA-I in a *Beclin1*-dependent manner, though it seemed to be *Atg5*-independent. These data demonstrate that autophagy controls the degradation of an intracellular ApoA-I pool within hepatocytes. Our findings raise the interesting possibility that regulation of ApoA-I degradation via autophagy may affect its secretion, which could have an impact on HDL metabolism, atherosclerosis and hepatic lipid homeostasis.

Keywords

apolipoprotein A-I (ApoA-I), autophagy, *Beclin 1*, *Atg5*, liver

Περίληψη

Η απολιποπρωτεΐνη A-I (ApoA-I), που συνιστά το κύριο πρωτεϊνικό συστατικό των λιποπρωτεϊνών υψηλής πυκνότητας (HDL), κατέχει σημαντικό ρόλο στον μεταβολισμό των λιπιδίων μέσω της γνωστής λειτουργίας της στην αντίστροφη μεταφορά χοληστερόλης. Επιδημιολογικές και κλινικές μελέτες έχουν καταδείξει μια αντιστρόφως ανάλογη συσχέτιση μεταξύ των επίπεδων της HDL χοληστερόλης ή των επιπέδων της ApoA-I στην κυκλοφορία και του κινδύνου εμφάνισης στεφανιαίας νόσου στον άνθρωπο. Παρά αυτούς τους συσχετισμούς, η κυτταρική βιολογία της ApoA-I (π.χ. μεταφορά, αποικοδόμηση, ανακύκλωση) παραμένει ανεξερεύνητη σε μεγάλο βαθμό. Στην παρούσα μελέτη, περιγράφουμε έναν νέο ρόλο της αυτοφαγίας στην ρύθμιση της αποικοδόμησης της απολιποπρωτεΐνης A-I στην καρκινική σειρά ηπατοκυττάρων HepG2. Ύστερα από επώαση αυτών των κυττάρων με αναστολείς της σύντηξης των αυτοφαγοσωμάτων με λυσοσώματα ή του όξινου περιβάλλοντος των λυσοσωμάτων (bafilomycin A1, chloroquine), η ApoA-I συσσωρεύτηκε μέσα στα κύτταρα σχηματίζοντας δομές παρόμοιες με συσσωματώματα. Μάλιστα, αυτές οι δομές βρέθηκαν να συνεντοπίζονται μερικώς με τον αυτοφαγοσωμικό δείκτη LC3, ενώ φάνηκαν να συσσωρεύονται μέσα σε λυσοσώματα/όψιμα ενδοσώματα, όπως ανιχνεύτηκε μέσω LAMP-1 ανοσοφθορισμού. Επίσης, η αναστολή της λειτουργίας του mTOR (Torin2) οδήγησε σε ενεργοποίηση της αυτοφαγίας και μειωμένα επίπεδα της ApoA-I, τα οποία δεν οφείλονταν μόνο στην αποικοδόμησή της μέσω αυτοφαγίας. Γενετική παρεμπόδιση του μηχανισμου της αυτοφαγίας (σίγηση γονιδίων που σχετίζονται με την αυτοφαγία) απέτρεψε την αποικοδόμηση της ApoA-I μέσω ενός *Beclin1*-εξαρτώμενου, *Atg5*-ανεξάρτητου μονοπατιού. Τα εν λόγω δεδομένα καταδεικνύουν ότι η αυτοφαγία ρυθμίζει την αποικοδόμηση ενός μέρους του πρωτεϊνικού συνόλου της απολιποπρωτεΐνης A-I μέσα στα ηπατοκύτταρα. Τα ευρήματά μας εγείρουν την ενδιαφέρουσα πιθανότητα η ρύθμιση της αποκοδόμησης της ApoA-I μέσω αυτοφαγίας να επηρεάζει την έκκρισή της, γεγονός που θα μπορούσε να έχει επιπτώσεις στον HDL μεταβολισμό, αθηροσκλήρυνση και την ομοιόσταση των ηπατικών λιπιδίων.

Λέξεις- κλειδιά

απολιποπρωτεΐνη A-I (ApoA-I), αυτοφαγία, *Beclin 1, Atg5*, ήπαρ

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1 Introduction

1.1 Apolipoprotein A-I (ApoA-I) in HDL and lipid metabolism

1.1.1 ApoA-I as a major protein component of high-density lipoprotein (HDL)

Human apolipoprotein A-I (ApoA-I) is primarily expressed in the liver and small intestine as a 267 amino acid pre-proprotein, which is targeted to the endoplasmic reticulum for secretion. After the intracellular cleavage of an 18 amino acid signal peptide, proApoA-I is secreted into plasma, where a six amino acid propeptide is proteolytically removed forming mature ApoA-I[1]. Mature ApoA-I is a 243 amino acid nonglycosylated protein with a molecular mass of 28.1 kDa, mainly present in plasma as the major protein constituent of high-density lipoprotein (HDL). While intestinally derived ApoA-I is transferred to HDL through its association with chylomicrons that enter the circulation and get converted to chylomicron remnants by lipoprotein lipase[2], hepatic ApoA-I is secreted in the bloodstream in a lipid-free or lipid-poor form[3].

Secreted ApoA-I plays a crucial role in the *de novo* biogenesis of HDL[4]. In the early steps of the pathway, lipid-free (or minimally lipidated) ApoA-I is the initial acceptor of phospholipids and cholesterol via its interaction with the ATP-binding cassette A1 (ABCA1). Through not fully understood intermediate steps, the lipidated ApoA-I is gradually converted to discoidal particles enriched in unesterified cholesterol. The free cholesterol is subsequently converted to cholesteryl ester (CE) by the plasma enzyme lecithin:cholesterol acyl tranferase (LCAT) generating spherical (mature) HDL particles[5] and ApoA-I is a co-activator of LCAT in plasma. Both discoidal and spherical HDL particles can interact functionally with scavenger receptor-class B type I (SR-BI) and these interactions lead to selective lipid uptake and net efflux of cholesterol conferring some of the HDL atheroprotective functions[5].

Clinical and epidemiological studies have consistently shown that there is an inverse association of HDL cholesterol (HDL-C) levels with risk of coronary heart disease (CHD)[6] in humans. Although HDL protects against atherosclerosis through multiple mechanisms (inhibition of LDL oxidation, restoration of endothelial function, anti-inflammatory and anti-apoptotic actions[7]), the most popular hypothesis revolves around its role in macrophage reverse cholesterol transport[8] (RCT; Figure 1.1). In this instance, excess cholesterol is effluxed from artery-wall macrophages to HDL-based acceptors via ABCA1 and ABCG1 transporters. After efflux to HDL, free cholesterol is esterified in the plasma by LCAT and ultimately transported from mature HDL to the liver, either directly (via SR-BI) or indirectly (via CETP mediated transfer to ApoB-containing lipoproteins)[9]. Undoubtedly, RCT is necessary to maintain whole-body steady-state cholesterol metabolism, but whether it is the most important atheroprotection mechanism by HDL still remains to be established.

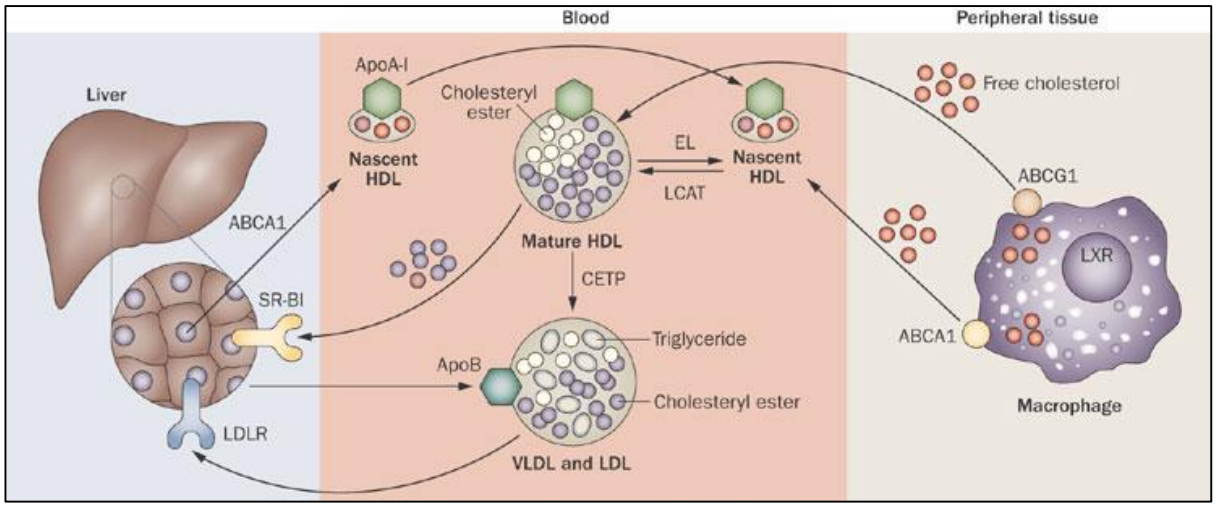

Figure 1.1 HDL metabolism and macrophage reverse cholesterol transport.

The liver synthesizes and secretes lipid-poor or lipid free ApoA-I, which quickly acquires cholesterol as well as phospholipids via the hepatocyte ABCA1 transporter. Lipid-poor ApoA-I also promotes the efflux of free cholesterol from macrophages via ABCA1. Esterification of free cholesterol to cholesteryl esters by LCAT leads to the formation of mature HDL particles, which promote cholesterol efflux from macrophages via the ABCG1 transporter, as well as from other peripheral tissues by not fully defined processes. Cholesterol transfer from mature HDL to the liver can be either achieved, directly via SR-BI or, indirectly via CETP-mediated transfer to ApoB-containing lipoproteins, which are subsequently taken up by the liver via the LDL receptor. Also, mature HDL can be remodeled to smaller HDL particles through the action of hepatic lipase (hydrolysis of HDL triglycerides or TGs) and endothelial lipase (hydrolysis of HDL phospholipids). Hepatic cholesterol can be excreted directly into the bile as cholesterol or after conversion to bile acids and, unless reabsorbed by the intestine, is ultimately excreted in the feces[9].

1.1.2 Beyond HDL metabolism: ApoA-I and hepatic lipid deposition

Despite the critical contribution of ApoA-I to lipoprotein metabolism, which affects the trafficking of plasma lipids, ApoA-I cell biology has remained largely unexplored. To date, the main focus of numerous studies has been whether ApoA-I expression affects plasma lipid levels and atherosclerosis, without investigating its possible involvement in lipid deposition to the liver. A recently reported function of ApoA-I is the modulation of processes associated with glucose intolerance and diet-induced hepatic accumulation of triglycerides[10]. In this study, it has been demonstrated that ApoA1-/- mice challenged with a high-fat diet display increased levels of steatosis, elevated number of lipid droplets and reduced hepatic cholesterol content. Interestingly, increased deposition of triglycerides in the livers of ApoA1-/- mice is not due to increased de novo biogenesis of triglycerides, while TGs secreted as VLDL by the liver, derived predominantly from the hydrolysis of TGs in lipid droplets[11], are significantly decreased in knockout mice[10]. Although researchers failed to provide a mechanistic interpretation to these findings, it is clear that ApoA-I deficiency severely affects hepatic triglyceride deposition, which could be either mediated by its functions as a modulator of lipoprotein metabolism in plasma or by intracellular functions that warrant further investigation.

1.2 The interplay between autophagy and lipid metabolism in liver

1.2.1 Autophagic pathways in the liver

Autophagy or 'self-eating' is an evolutionarily conserved process that mediates degradation of intracellular components in lysosomes. Recycling of macromolecular structures maintains a constant nutrient flow (amino acids, free fatty acids, carbohydrate moieties) and essential cellular functions during starvation, whereas constitutive autophagy participates in quality control by eliminating damaged proteins and organelles, aggregated and long-lived proteins, and plays a critical role in innate immunity and pathogen clearance[12]. Both degradation and recycling are particularly important in highly metabolically active organs such as the liver, in which three types of autophagy co-exist: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Figure 1.2).

In macroautophagy (hereafter, referred to as autophagy), cargo can be sequestered in *de novo* formed double-membrane vesicles (autophagosome) to be degraded by lysosomal hydrolases upon fusion of the autophagosome with lysosomes. This form of autophagy includes very different variants depending on whether the cargo is sequestered 'in bulk' (cytosol with any content present in that area) or selectively thanks to a subset of autophagy receptors (mitophagy, ribophagy, lipophagy, pexophagy, aggregophagy etc.)[13]. In endosomal microautophagy, cargo is sequestered 'in bulk' or selectively through a chaperone-cargo protein interaction and small vesicles forming on the surface of late endosomes or lysosomes are pinched off into the lumen to be degraded. By contrast, in CMA, the substrate proteins are recognized by a chaperone complex in the cytosol and delivered one-by-one to the lysosomal membrane, where they undergo internalization into the lumen through a membrane translocation complex instead of being delivered by vesicles[14].

1.2.2 Overview of autophagic process

Although autophagy was initially described as a non-selective bulk process induced in response to starvation, it is now clear that many of its functions are highly selective and tightly regulated by key components of the autophagy machinery[15,16]. In fact, following induction of autophagy, autophagosome formation requires more than 30 autophagy-related proteins (ATGs) that have been identified by molecular dissection of the autophagic process through yeast genetic screens[17,18]. The coordinated action of a cascade of ATGs that form functional complexes (with each other or with lipids[18]) oversees individual steps of autophagy; initiation or induction, nucleation, membrane elongation, cargo recognition, and the fusion of autophagosomes with lysosomes (many of these steps are shown in Figure 1.2).

The activation of the autophagy initiation complex (or the class III phosphatidylinositol-3-kinase/PI3K) is required for the *induction* of autophagy and is achieved through the release of Beclin-1 (ATG6 in the

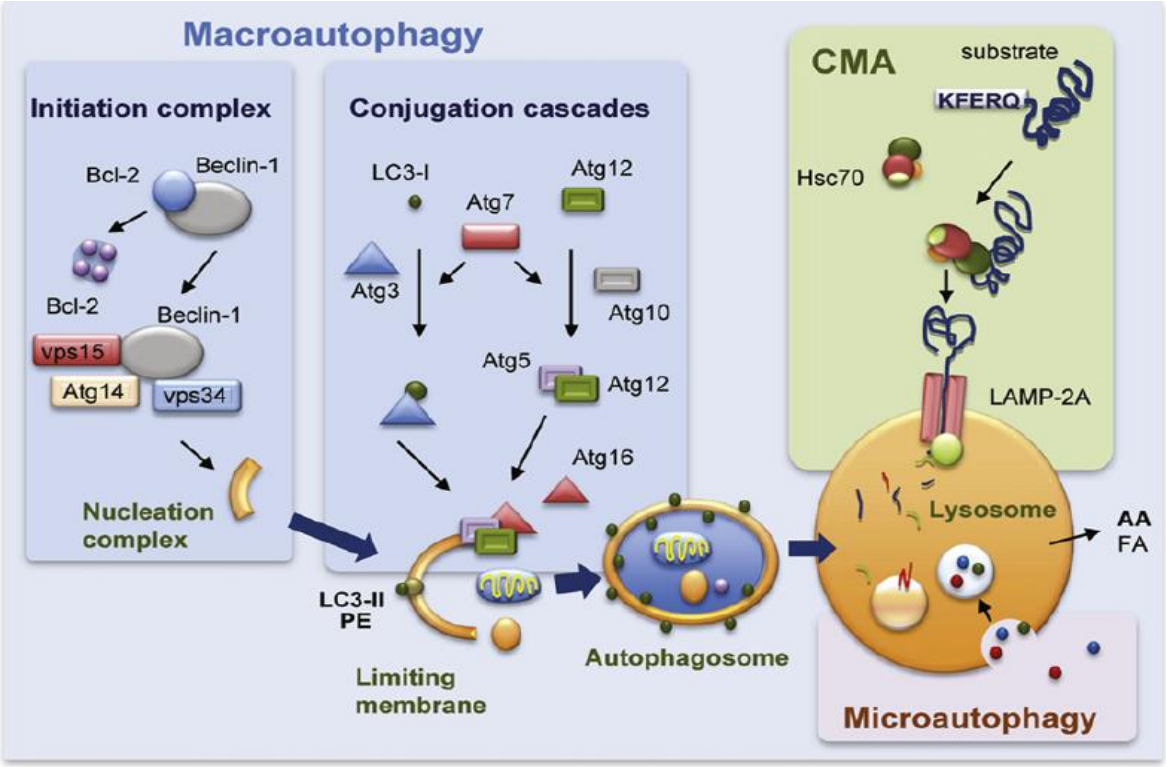

Figure 1.2 Molecular dissection of autophagic pathways in the liver.

Three types of autophagy that co-exist in liver are depicted. In **macroautophagy**, initiation requires assembly and activation of the class III PI3K complex that serves to recruit components of the two conjugation cascades, the LC3/PE and Atg5/12 cascades, to the limiting membrane. Cytosolic components are sequestered within a newly formed limiting membrane that seals to form an autophagosome, which then fuses with lysosomes for degradation. In **chaperone-mediated autophagy (CMA)**, substrate proteins bear a targeting motif (KFERQ) recognized by a cytosolic chaperone (hsc70), which then delivers them to the lysosomal membrane. Upon binding to the lysosome-associated protein type 2A (LAMP-2A), proteins unfold and cross the lysosomal membrane assisted by a lumenal chaperone. In **microautophagy**, cytosolic cargo is trapped within invaginations at the lysosomal membrane and is internalized after the vesicles pinch off into the lysosomal lumen[14]. AA: amino acids; FA: free fatty acids; Atg: autophagy-related protein; PE: phosphatidylethanolamine.

yeast) from the Bcl-2–Beclin-1 complex upon starvation or stress-induced phosphorylation of Bcl-2[19]. Beclin-1 is then recruited along with additional molecules (vps15, vps30, vps34, ATG14L, and UV radiation-resistance associated gene protein/UVRAG) to form the active class III PI3K complex[20,21]. Following activation of class III PI3K complex, a process called *nucleation*, involves the mobilization of this initiation complex to the site of the limiting membrane formation (nascent phagophore)[17]. This limiting membrane forms through the assembly of proteins and lipids from different cellular organelles such as the endoplasmic reticulum (ER), Golgi, mitochondria, endocytic system or plasma membrane. In addition, lipid phosphorylation (of PI to form PI3P) by active class III PI3K recruits to the surface of the limiting membrane two conjugation cascades, the microtubule-associated protein 1 light chain 3 (LC3 or ATG8)/PE and the ATG5–12 complexes. These conjugation cascades are crucial for membrane elongation and resemble to the ubiquitin conjugation system involving discrete ligases for activation

and enzymatic conjugation of substrates[17]. In particular, formation of the ATG5–12 complex is achieved through conjugation of ATG5 and ATG12 by the ubiquitin-like ligases ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme), after which Atg16 multimerizes to form the Atg12-Atg5-Atg16 tetrameric complex. Independently, the ATG4 protease cleaves soluble LC3-I at the C-terminus, exposing the glycine residue that is conjugated to phosphatidylethanolamine (PE) by ATG7 and ATG3 (E2-like), thus generating the membrane-bound LC3-II[22]. Both of these ubiquitin-like systems are required for elongation and closure of the phagophore. However, the molecular mechanisms controlling the late stages of autophagy, including the autophagosome-lysosome fusion and the regulation of lysosomal function, remain poorly understood. It is s thought that additional ATGs, specific SNARE, and Rab proteins as well as cytoskeletal elements may be involved in these fusion events[17]. Recently, it has been demonstrated that in the course of autophagy, lysosomal function is upregulated via a dual mechanism involving mTORC1 suppression and autophagosome-lysosome fusion[23].

One of the best-characterized endogenous inhibitors of autophagy is the upstream serine/threonine kinase mTOR (mammalian target of rapamycin)[24,25]. Under normal nutrient conditions, mTOR represses autophagy through direct phosphorylation of Unc-51 like autophagy activating kinase 1 (ULK1), a kinase essential for autophagosome formation, and sequestration of the ULK1-Atg13-FIP200 complex in an inactive state at the mTOR complex 1 (mTORC1)[24,26]. Upon amino acid depletion, AMPK inhibits mTORC1 (by phosphorylating TSC2 and raptor) and inactivation of mTOR is followed by cytosolic release of ULK1 and its relocation to sites of autophagosome biogenesis. Once amino acids become available again, mTOR reactivation suppresses autophagy[26]. Another crucial regulator of starvation-induced autophagy is c-Jun N-terminal kinase (JNK-1) that mediates the phosphorylation of Bcl-2 to release Beclin-1 in response to nutrient deprivation[19].

1.2.3 Autophagy regulates lipid metabolism in liver

Despite the fact that the most studied autophagic activity is protein breakdown, mobilization and degradation of other cellular energy stores have gained considerable attention in recent years. In particular, lysosomal breakdown of hepatic lipids has been demonstrated to occur through a selective form of autophagy known as lipophagy[27]. In addition to the well-characterized arrival of extracellular lipids to lysosomes through endocytosis, intracellular triglycerides (TG) and cholesterol stored in the form of lipid droplets (LD) can also be delivered to the lysosomes. The sequestration of lipid droplets by autophagosomes and the subsequent fusion of these autophagosomes with lysosomes result in LD hydrolysis by lipases in the lysosomal lumen, producing free fatty acids (FFAs) which may be used as cellular fuel in case of prolonged nutrient scarcity. This link between autophagy and lipid metabolism has been confirmed by both *in vitro* and *in vivo* experiments[27]. In cultured hepatocytes, pharmacological or genetic ablation of autophagy leads to increased LD size, number and TG accumulation under basal

conditions, as well as in response to starvation or lipogenic stimulus[27]. Interestingly, increased hepatocellular TG stores have been shown to result from decreased lipolysis of lipid stores due to decreased delivery of lipid cargo into the lysosomes, as witnessed in genetically modified mice with liver specific ablation of ATG7, and not from increased hepatocellular TG synthesis or a reduction in secretion in the form of VLDL[28]. Clearly, autophagy is directly involved in the maintenance of lipid homeostasis by regulating lipid metabolism (primarily in hepatocytes), either directly via lipophagy or indirectly through other autophagy-mediated regulatory functions[27,28].

1.2.4 Autophagy and lipoprotein metabolism

Since the discovery that autophagy serves to mobilize and degrade intracellular lipid stores[27], its potential contribution to regulation of lipoprotein metabolism has been an emerging concept with important implications in regards to diseases associated with dyslipidemias, such as diabetes and atherosclerosis. In recent studies, it has been demonstrated that autophagy stimulation in primary hepatocytes leads to increased VLDL production[29], while hepatic-specific ablation of the autophagy gene Atg7 in mice decreases triglycerides secreted as VLDL with a concomitant increase in liver lipid content[27]. Furthermore, during hepatic VLDL assembly, autophagy is able to degrade apoB aggregates that accumulate on LDs[30], although it is not clear whether activated lipophagy mediates lysosomal degradation of apoB and how apoB accumulation on LDs could trigger autophagy[31]. Given that apoB availability is a key determinant of VLDL plasma levels, autophagy could modulate the number of liver secreted VLDL particles by controlling the pool of hepatic apoB in response to metabolic stimuli (e.g. oxidative stress)[32,33]. Assumingly, autophagic manipulation might hold potential therapeutic value in modulating lipoprotein metabolism, but still, full lipoprotein profiles of liver-specific autophagy-deficient mice crossed into different disease mouse models require further investigation in order to elucidate the exact role of autophagy in lipoprotein metabolism.

1.3 Hepatic autophagy and metabolic diseases

1.3.1 Hepatic autophagy in fatty liver disease and metabolic syndrome

The dynamic cross-talk between liver autophagic function and lipid metabolism has been reported to play a rather important role in non-alcoholic fatty liver disease (NAFLD)[12,34,35], the hepatic manifestation of metabolic syndrome[36]. The first step in the pathogenesis of NAFLD is the development of a fatty liver or hepatic steatosis, where the characteristic accumulation of lipids may be due to increased lipid influx in this organ, enhanced *de novo* synthesis of lipids and decreased mobilization and utilization of hepatic lipid stores[35]. Even though hepatic deposition of triglycerides is a critical step, additional parameters such oxidative stress and inflammation lead to steatohepatitis (NASH) and liver injury[37]. Autophagic impairment in the liver might be a cause or a consequence of the disease (Figure

1.3a) and as NAFLD progresses, it is still a matter of debate whether compromised autophagy comes from primary or secondary defects.

The implication of autophagic function in NAFLD has been partially attributed to its protective role against lipotoxicity[27,38]. In response to a short-term increase in lipid availability, hepatocytes upregulate autophagy leading to a greater breakdown of stored lipids to supply fatty acids for β-oxidation or other uses[27]. In contrast, sustained lipogenic challenge or even acutely elevated abnormal lipid levels result in compromised macrolipophagic function, eventually leading to hepatotoxicity and development of fatty liver[27,38,39]. Thus, failure of autophagy in NAFLD could be explained by these observations, where hepatic lipid accumulation through chronic exposure to a high-fat diet or acute exposure to a cholesterol-enriched diet actually inhibits autophagy[27,38]. Similar phenotypes have been observed in a genetic (ob/ob mice) mouse model of chronic obesity and insulin resistance, as sustained increase in lipid availability results in markedly decreased hepatic autophagy[40–42]. In these mice, defective autophagy and lipotoxicity may further deteriorate obesity-induced ER stress (Figure 1.3a) in the face of continuous energy and nutrient stress and contribute to insulin resistance, a known consequence of ER stress[40,43]. Interestingly, restoration of Atg7 expression results in significant ER stress reduction in the liver of ob/ob mice and decreases liver triglyceride content[40]. These findings, along with the high prevalence of NAFLD in individuals with type 2 diabetes or with morbid obesity, suggest common pathogenic mechanisms for all these conditions and that autophagic failure might underlie the metabolic syndrome[35].

Several mechanisms might contribute to the impairment of hepatic autophagy in steatotic liver and obesity (Figure 1.3). A recent study demonstrates that autophagic degradation of both lipid and protein cargos is compromised following lipid-enriched diets, primarily due to changes in the membrane lipid composition of autophagosomes and lysosomes. These alterations, which disturb membrane fluidity, lead to decreased autophagosomal and lysosomal fusion and, as a consequence, inhibit the completion of the autophagic process[38]. In addition, diet-induced changes may negatively affect CMA by reducing the stability of its receptor in lysosomes, a condition similar to that observed in physiological ageing[44]. Induction or suppression of autophagy upon a lipid challenge depends not only on the intensity and duration of the challenge, but also on the types of lipids (e.g. unsaturated and saturated fatty acids induce and supress autophagy[39], respectively). In obese mice with hepatic steatosis and insulin resistance, induction of hepatic autophagy might be compromised due to altered cellular signalling (Figure 1.3b). To begin with, obesity-induced increase in the calcium-dependent protease calpain 2 leads to Atg7 downregulation, while the sustained activation of mTOR, presumably due to increased amino acid concentrations following overnutrition may also negatively regulate hepatic autophagy[42,45]. Autophagy in the liver is normally suppressed by insulin-mTOR signaling[46], suggesting that conditions with insulin resistance, such as NAFLD and obesity, should lead to augmented hepatic autophagy. However,

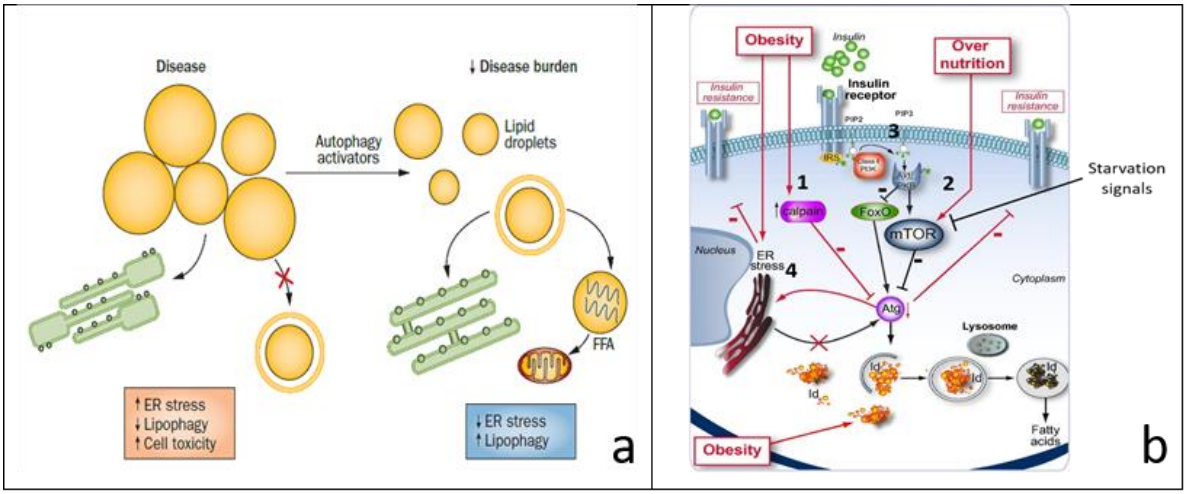

Figure 1.3 Impaired hepatic autophagy in fatty liver disease and metabolic syndrome.

(a) Left: failure of autophagy in NAFLD could be an underlying cause for the accumulation of lipids in this organ or a consequence, as abnormal increase in lipid content has been shown to exert an inhibitory effect on autophagy. Defective autophagy and lipotoxicity may contribute to ER stress. Right: in NAFLD, steatosis is reduced upon autophagic activation which promotes mobilization of lipid droplets and reduces ER stress. Adopted from published review[12].

(b) Autophagy is normally (**black arrows**) inhibited by the insulin-, amino acid–mTOR signaling pathway via both short-term and long-term mechanisms. Short-term inhibition can be produced by the mTOR complex. Long-term regulation occurs via the transcription factors FoxO and TFEB, which control the transcription of autophagic genes and are inhibited by insulin-induced activation of Akt/PKB and mTOR, respectively. In obesity (**red arrows**), autophagy is impaired in hepatocytes, which can be attributed to several mechanisms: **(1)** Obesity-induced increase in the calcium-dependent protease calpain 2, which leads to down-regulation of Atg7 and consequently to defective autophagy. Acute inhibition of calpain is able to restore Atg7 expression. **(2)** In obese mice, the autophagy inhibitor mTOR is over-activated in the liver, presumably as a result of an increased amino acid concentration following over-nutrition. The over-activation of mTOR by itself can result in liver and muscle insulin resistance because of phosphorylation, and inhibition of IRS1 by S6K, a downstream target of mTOR. **(3)** Although controversial, hyperinsulinemia may also contribute to down-regulation of autophagy in obese mice. Indeed, elevated activation of Akt/PKB, a key molecule in the insulin pathway, decreases autophagy in the liver of obese mice. **(4)** In obesity, a defect in hepatic autophagy and its associated decrease in lysosomal degradation contribute to further increasing the ER stress induced by nutrient overload, in an inflammatory milieu. Together, a decline in autophagy and increased ER stress lead to insulin resistance. Adopted from published review[34].
Atg, autophagy genes; ld, lipid droplet; ER, endoplasmic reticulum; FFA, free fatty acid; mTOR, mammalian target of rapamycin; PKB, protein kinase B.

experimental evidence has shown that liver autophagy is suppressed in mouse models of obesity with insulin resistance and hyperinsulinaemia through a decrease in FoxO1-mediated expression of essential Atgs[40,41]. Interestingly, restoration of normal hepatic autophagic function in the context of obesity is sufficient to rescue the defects in insulin receptor signaling and restore peripheral glucose tolerance[40]. This unexpected outcome, autophagy modulating sensitivity to its own inhibitor, insulin, seems to originate from the ability of this pathway to attenuate ER stress, as previously reported[40].

Despite the well-recognized malfunction of liver autophagy in NAFLD and metabolic syndrome, it is still unclear whether compromised autophagy strikes in the onset of metabolic syndrome, followed by

hepatic steatosis, lipotoxictiy and metabolic dysregulation or whether autophagic impairment is a consequence of obesity and changes in insulin sensing. Although examples of both scenarios have been documented[27,39,41,47,48], distinguishing between cause and consequence might not always be possible. Even so, the identification of this bidirectional interplay between autophagy and lipids in liver complications points towards breaking this vicious cycle, either by enhancing autophagy function or reducing the inhibitory effect of the metabolic imbalance (lipids and insulin) on this pathway, and justifies the current interest in exploring the use of chemical modulators of autophagy in treatment of metabolic syndrome[35,38].

1.4 Hypothesis

Apolipoprotein A-I (ApoA-I) plays important role in lipid metabolism through its known function in reverse cholesterol transport (RCT)[8]. Despite the strong link between cellular ApoA-I, circulating HDL levels, and coronary heart disease risk, ApoA-I cell dynamics (e.g. transport, degradation, recycling) have remained largely unexplored. ApoA-I deficiency has been shown to sensitize mice to diet-induced hepatic triglyceride accumulation and lead to increased levels of steatosis[10]. As autophagy regulates lipid metabolism in hepatocytes[27], with liver-specific ablation of Atg7 leading to the development of fatty liver, a phenotype similar to that observed in ApoA1-/- mice upon a high-fat challenge, we hypothesized the possible regulation of intracellular ApoA-I by the autophagic pathway that could impact on hepatic lipid homeostasis.

1.4.1 Aims

The present Master's project aims to determine whether intracellular ApoA-I levels are affected upon autophagy modulation. It further attempts to provide mechanistic insights into the autophagic regulation of ApoA-I by molecular dissection of the steps that mediate autophagy and may affect intracellular protein levels in hepatocytes.

1.4.2 Methodology used

In the current study, we mainly used the human hepatocellular carcinoma cell line HepG2 as cell model, in which liver cells express *APOA1* per se. To address whether hepatic ApoA-I levels change during autophagy modulation, HepG2 cells were cultured in full media in the presence of pharmacological inhibitors or inducers of autophagic process. As illustrated in Figure 1.4, 3-methyladenine (3-MA) results in inhibition of class III PI3K, which is required for the induction of autophagy, while later steps of the pathway can be impaired by interrupting the autophagosome-lysosome fusion (e.g. by raising the lysosomal pH with the lysosomal proton pump inhibitor bafilomycin_A1) or by inhibiting lysosome-mediated proteolysis (e.g. with chloroquine). In contrast, treatment of cells with Torin2 leads to induction of autophagy by suppressing mTORC1, which normally phosphorylates and inactivates

ULK1, a kinase required for autophagy initiation. Some of these experiments were also performed in HEK293 cells transfected with a plasmid of WT *APOA1*, in order to substantiate the regulation of ApoA-I expression through autophagy in a different experimental system.

To monitor autophagic flux (readout of autophagy modulation) in HepG2 cells, we systemically analyzed by western blotting MAP1LC3B (the lipidated form of LC3 that is associated with autophagosomes), and evaluated degradation of another autophagy substrate (p62), whenever possible. In addition to immunoblotting and band densitometry analysis, expression levels of ApoA-I, as well as patterns of subcellular protein localization, were further investigated by immunofluorescence experiments. In these experiments, we set out to examine the association of cellular ApoA-I with autophagosomal and lysosomal structures by assessing the co-localization of hepatic ApoA-I with LC3 (marker for autophagosome) and LAMP-1 (marker for late endosome/lysosome), respectively, upon autophagy blockage. What is more, in an attempt to provide biochemical evidence of ApoA-I putative association with LC3, we performed immunoprecipitation experiments in HepG2 and *APOA1*-transfected HEK293 cells using either anti-ApoA-I or anti-LC3 antibodies.

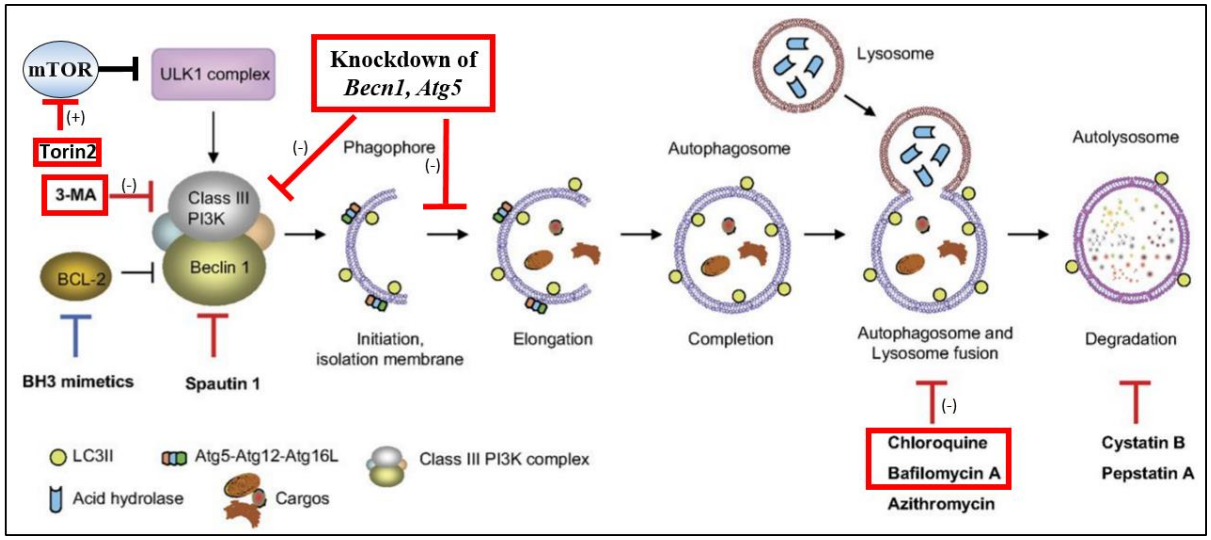

Figure 1.4 Genetic and pharmacological approaches employed for inhibition or induction of autophagy.
Based on the understanding of ATG protein function through autophagic process, many pharmacological inhibitors have been used in order to modulate autophagy (**in red boxes**: those used in the current study). Inhibition of class III PI3K, which positively regulates autophagy via bridging Beclin1, UVRAG and ATG14, by **3-methyladenine (3-MA)** can inhibit autophagy under certain context. In addition, there are drugs to inhibit the late phase of autophagy. Lysosomotropic agents that enhance lysosomal pH such as **chloroquine** inhibit lysosomal enzymes and also prevent the fusion of autophagosome and lysosome, resulting in inhibition of autophagy. **Bafilomycin A1** inhibits the fusion of autophagosome and lysosome by inhibiting vacuolar ATPase (V-ATPase) located in the lysosomal membrane. In contrast, **Torin2** leads to induction of autophagy by inhibiting mTOR, a component of the mTORC1 complex that phosphorylates and inactivates ULK1 which is essential for autophagy initiation. Apart from pharmacological inhibitors**, genetic**

knockdown of *Beclin1* or *Atg5* inhibits autophagy at the nucleation and elongations steps, respectively. The figure was adjusted from published review[49].

Finally, in an effort to study the underlying mechanism(s) by which autophagy modulates intracellular ApoA-I expression in hepatocytes, we impaired autophagic process by knocking down *Beclin1* and *Atg5*, key players in autophagy initiation and elongation, respectively. In particular, we transiently transfected HepG2 cells with small interfering RNA (siRNA) targeting *Beclin1* or *Atg5* genes and examined *APOA1* expression at both mRNA (by RT-qPCR) and protein level (by immunoblotting). We confirmed the effect of these genetic knockdowns on autophagy by evaluating LC3 and p62 protein levels. Interestingly, we demonstrated that ApoA-I is regulated by autophagy in a *Becn1*-dependent and *Atg5*-independent manner.

1.4.3 Importance and implication in dyslipidemias

In spite of the critical role that ApoA-I plays in plasma lipoprotein metabolism, to date very little is known on its post-translational regulation of expression as well as its intracellular effects on lipid metabolism, especially in the liver. In this study, we tested whether hepatic intracellular ApoA-I levels are affected upon autophagy modulation. Our results demonstrated for the first time that pharmacological and genetic inhibition of autophagy (*Becn1*-deficient HepG2 cells) cause accumulation of intracellular ApoA-I levels. We also show that blockage of autophagy at late steps of this process enhances the association of ApoA-I with autophagosomal and lysosomal structures. Following the discovery of lipophagy and its underlying role in lipid metabolism, the potential role of autophagy in regulating lipoprotein metabolism is an emerging concept that has important implications in regards to diseases associated with dyslipidemias, such as diabetes and atherosclerosis. Given that ApoA-I availability is a key determinant of HDL metabolism, hepatic autophagy could possibly modulate HDL plasma levels in part by controlling the intracellular pool of hepatic ApoA-I in response to metabolic stimuli.

2 Materials and Methods

2.1 Antibodies and reagents

The following primary antibodies were used: rabbit anti-LC3B/MAP1LC3B antibody (Novus Biological Inc.; used for WB, IP), rabbit anti-Beclin-1, -4E-BP1, -phospho-4E-BP1 (Thr37/46), -LC3B (used for IF) antibodies (Cell Signaling), mouse anti-actin and goat anti-ApoA-I antibody (used for WB, IF)(Millipore/ Chemicon), mouse anti-ApoA-I antibody (G-11, Santa Cruz; used for IP), mouse anti-GAPDH antibody (Sigma), rabbit anti-APG5L/ATG5 antibody (Abcam), mouse anti-p62 lck ligand antibody (BD Biosciences) and mouse anti-CD107a (LAMP-1) antibody (BD Pharmingen™; a gift from Chamilos lab, Department of Medicine, University of Crete). The following secondary antibodies were used for western blot detection with enhanced chemiluminescence (ECL, Western Lightning™ Chemiluminescence Reagent *Plus* was purchased from PerkinElmer): HRP-linked anti-mouse IgG and anti-rabbit IgG (Cell Signaling), as well as HRP-linked anti-goat antibody (Invitrogen). For immunofluorescence experiments, donkey anti–goat and donkey anti–mouse Alexa Fluor 488–conjugated antibodies, donkey anti–goat Alexa Fluor 568–conjugated antibody and goat anti–rabbit Alexa Fluor 594–conjugated antibody were all purchased from Invitrogen, while ProLong® Gold Antifade Reagent with DAPI was ordered from Cell Signaling. For distinct treatment conditions, 5mM 3-methyladenine (3-MA; Sigma), 50nM bafilomycin A1 (Sigma), 50μM chloroquine diphosphate (Sigma), and 250nM Torin2 (Cayman Chemical) were used. The SigmaFAST Protease Inhibitor Tablets were purchased from Sigma and used for protein extraction purposes. For immunoprecipitation (IP) experiments, Protein G Sepharose® 4 Fast Flow beads (Sigma, GE17-0618-01) were used.

2.2 Cell culture and transfection assays

Cell culture growth media, antibiotics (penicillin/streptomycin, or P/S) and fetal bovine serum (FBS) were purchased from Invitrogen. HepG2 cells were grown in DMEM low glucose (1 g/L) GlutaMAX™ medium, HEK293 cells were grown in DMEM high glucose (4.5 g/L) GlutaMAX™ medium and both were supplemented with 100 U/ml penicillin,100 mg/ml streptomycin and 10% FBS. Cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

The pAdTrack-CMV-*APOA1* WT plasmid was a generous gift from Kardassis lab (Department of Medicine, University of Crete). For plasmid transfection, HEK293 cells were seeded in 24-well plate ($0.5x10^5$ cells per well) and transfected with a total of 0.1μg or 0.5μg of the indicated vectors using the Lipofectamine 2000 method according to manufacturer's protocol (Life Technologies). Following 36-40 hours of incubation (37°C, 5% CO2) with the transfection cocktail, media were aspirated, cells were

washed once with PBS, subjected to trypsinisation and plated appropriately in order to proceed with further treatment (e.g. bafilomycin)

For siRNA transfections, HepG2 cells were seeded in 24-well plate ($0.6x10^5$ cells per well) and cells were reverse-transfected with 10pmol of siRNA (final per well) in the presence of Lipofectamine RNAimax reagent (Life Technologies), according to the procedure provided by the manufacturer. The siRNA, obtained from Life Technologies (Invitrogen), were for *BECN1* (NM_003766.3) Silencer® Select siRNA (ID: s16537 and s16538) named HS716464 and for *ATG5* (NM_004849.2) *Silencer® Select* siRNA (ID: s18160) named HS486063. si*luc* was used as negative control for sequence independent effect.

2.3 Immunoblotting and densitometry analysis of bands

Cells were placed on ice, washed once with cold PBS and then lysed with RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM Nacl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 1X protease inhibitor solution). A volume of 50 µL lysis buffer was used in each of 24-well plate. Gentle agitation at 4° C was applied for 15 minutes to lyse the cells and then lysates were transfered to a pre-cooled microcentrifuge tube. Lysates were then centrifuged for 20 min at 13,000 rpm in a 4°C pre-cooled centrifuge. The supernatant was transferred to a new tube and protein contents in the supernatant were quantified using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific), according to the manufacturer's instructions. For electrophoresis, samples were prepared by mixing 3X SDS loading buffer (150 mM Tris HCl, pH 6.8, 6% SDS, 30% glycerol, 9% β-mercaptoethanol, 0.3% bromophenol blue) with 15–30 µg of cell lysates. Equal protein amounts were loaded onto 12% acrylamide resolving gels (12% acrylamide mix (11.6% acrylamide, 0.4% bis). Separated by SDS-PAGE proteins (120V,~ 2h30) were transferred (280mA, 1h40) to nitrocellulose membranes (Amersham Protran 0.2µm, GE Healthcare Life Sciences). Nitrocellulose membranes were blocked with 5% non-fat milk containing 1% BSA (Sigma) for 30 min and then incubated with primary antibody with gentle agitation overnight at 4°C. After 3 washes (5 min each) with TBS-T (20 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20), membrane was incubated and gently agitated for 1 hour in secondary antibody in 1% non-fat milk with 0.2% BSA. Nitrocellulose membranes were then incubated for 1 minute with chemiluminescent substrate (PerkinElmer) and developed with Molecular Imager ® Gel Doc™ XR System (Bio-Rad). Protein signal intensities (densitometric values of protein bands) were normalized against a GAPDH or β-actin loading control for each sample. Densitometric values were determined and quantified on Western blots at nonsaturating exposures using the Image Lab™ 5.0 Software (Bio-Rad). All the graphs were generated using PRISM (GraphPad Software Inc.)

2.4 Immunoprecipitation

HepG2 or *APOA1* transfected HEK 293 cells were placed on ice and rinsed twice with ice-cold PBS prior to lysis in NP-40 lysis buffer (50mM Tris-HCl, pH 7.4, 250mM NaCl, 5mM EDTA, 1% Nonidet P-40 (v/v), supplemented with SigmaFAST Protease Inhibitor mixture tablets (Sigma) and 1mM PMSF (Sigma). A volume of 300μl lysis buffer was used in each of 6-well plate, while adherent cells were scrapped off the wells. Lysates were cleared by centrifugation (16,000 x g in a precooled centrifuge for 15 minutes) followed by a 2h incubation with protein G-sepharose beads (Sigma) on an orbital shaker. Following bead removal by centrifugation (14,000 x g, 10 min), the precleared protein lysates were quantified using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Pre-cleared diluted (with PBS to reduce the concentration of the detergents) lysates (1μg/μl) were then incubated with the indicated primary antibodies overnight at 4°C and then with protein G-sepharose beads for additional 6 h. Precipitated immunocomplexes were washed three times with lysis buffer and eluted by boiling for 5 min in 2X loading buffer (w/o β-mercaptoethanol). The beads are collected by centrifugation (200 x g, 1 min), and SDS-PAGE is performed with the supernatant, as described in previous section (Immunoblotting).

2.5 Immunofluorescence staining

To analyze colocalization of ApoA-I with autophagy and lysosome markers (MAP1LC3B, LAMP1), HepG2 cells (0.6x105 cells per well) were seeded on a sterile coverslip in a 24-well plate. Cultivation and treatment conditions ended up in culture media removal by washing with PBS and cell fixation with 4% paraformaldehyde for 15 min at RT (followed by PBS washes and further fixation with 100% methanol for 10 min at -20°C, in the case of LC3 staining).Coverslips were washed twice with PBS before cell permeabilization and blocking with 0.3% Triton X-100 (Sigma), 5% horse serum in PBS for 60min (ApoA-I, LC3 double staining) or 0.1% saponin, 2% BSA (Sigma) in PBS for 15min (ApoA-I, LAMP-1 double staining). Primary antibody incubation (1:200 LC3B, 1:100 ApoA-I) in 1% BSA, 0.3% Triton X-100 (in PBS) was performed overnight at 4°C or sequentially in 2% BSA, 0.1% saponin for ApoA-I (1:100, 1h at 37oC) and LAMP-1 (1:100, 1h at RT) antibodies. These incubations were followed by 3 PBS (or 2% BSA, 0.1% saponin, respectively) washing steps and secondary antibody incubation (listed above) in 1% BSA, 0.3% Triton X-100 (in PBS) or 2% BSA, 0.1% saponin (in PBS) for 1h-1h30 at RT. Coverslips were washed three times (10min each) and mounted on microscope slides using Prolong® Gold AntiFade Reagent with DAPI from Cell Singaling. For image acquisition, the AxioObserver Z1inverted fluorescence microscope (Carl Zeiss) equipped with the ApoTome.2 (Carl Zeiss) and an EC Plan-Neofluar objective (100x/1.30 oil lens, Carl Zeiss) as well as a charge-coupled device (CCD) camera (AxioCam MR Rev3; Carl Zeiss) and the Zen lite software were used (Carl Zeiss).

Finally, maximal projections of z stack images (individual channels and merged) were generated using Image J software. The same settings of light source intensity and exposure time were used between samples in order to be able to compare the intensity of fluorescent signals.

2.6 RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated from HepG2 cells using NucleoSpin RNA Kit (Macherey-Nagel) according to the manufacturer's protocol. RNA concentrations was measured spectrophotometrically and total RNA (300ng) was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Quantitative Real-Time PCR reactions were performed by using TaqMan Gene Expression Assays (Applied Biosystems) for *APOA1* (Hs00985000_g1) and for *ACTB* (Hs99999903_m1), in the Applied Biosystems ViiA™ 7 Real-Time PCR System. The relative gene expression was calculated using the comparative CT method (ΔΔCT Method). APOA1 mRNA expression was normalized to *ACTB* and represented as arbitrary units.

2.7 Statistical analysis

Statistical analysis was performed using PRISM (GraphPad Software Inc.). Results are expressed as mean± SD. Statistical significance was calculated using Student's t-test, or for comparisons involving more than two groups, one-way analysis of variance (ANOVA) with a post hoc Tukey multiple comparison test being used to assess the differences between the groups. Statistical significance was defined as the conventional p value of < 0.05.

3 Results

3.1 Pharmacological inhibition of autophagy leads to ApoA-I accumulation

Despite the key role that ApoA-I plays in lipoprotein metabolism in plasma, to date very little is known on its regulation of expression as well as its intracellular effects on lipid metabolism, especially in the liver. Given the recent discovery that autophagy regulates lipid content in hepatocytes[27], we speculated the possible regulation of ApoA-I through autophagy. To determine whether ApoA-I levels are affected during autophagy modulation, we used HepG2 hepatocellular carcinoma as cell model. Cells were cultured in normal conditions in the presence of bafilomycin A1 (BAF), which is an inhibitor of the vacuolar ATPase and blocks the fusion of autophagosomes with lysosomes, leading to an accumulation of autophagosomal structures[50]. As shown in Figure 3.1a, bafilomycin treatment at 6h caused a pronounced increase of endogenous ApoA-I levels, indicating its possible regulation via autophagy. We confirmed, by immunoblot and protein quantification (Figure 3.1b), that bafilomycin resulted in the accumulation of the LC3 lipidated form and a slight increase of p62. LC3 is involved during the late steps of autophagy after the isolation membrane has formed[51]. The unmodified LC3-I form is cytosolic, whereas LC3-II is presumably covalently attached to phosphatidylethanolamine (PE) at its COOH terminus and is tightly bound to autophagosomal membranes, serving as an important marker for

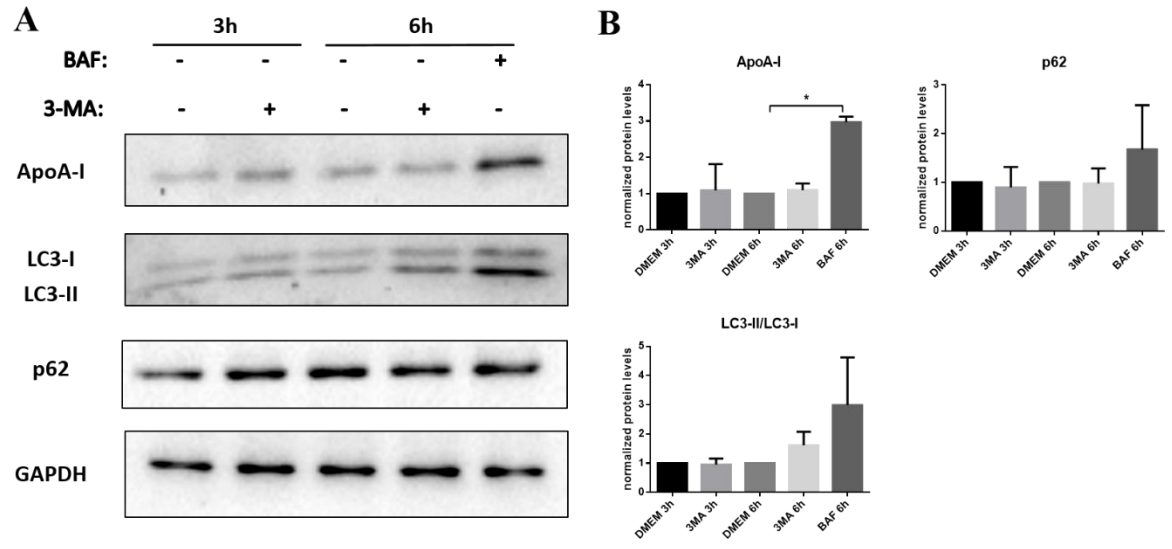

Figure 3.1 Pharmacological inhibition of autophagy at late stages increases intracellular ApoA-I levels in HepG2 cells.

(A) The levels of ApoA-I, LC3-II and p62 change with autophagic activity. HepG2 cells were either left untreated or 3-methyladenine (3-MA; 5mM) or bafilomycin A1 (BAF; 50nM) was added for the indicated time points. Immunoblots were sequentially probed using ApoA-I, LC3, p62, and GAPDH antibodies. Blots are representative of 2 independent experiments. (B) Relative quantification of ApoA-I, LC3-II/ LC3-I and p62 protein levels; histograms represent band densitometry analysis of indicated proteins, normalized to GAPDH and expressed as means of fold-change (of expression levels compared to the control) ± SD of 2 independent experiments. *P < 0.05.

autophagy[52]. Levels of p62/Sqstm1 are often also used as an indirect measure of functional autophagy because p62/Sqstm1 accumulates when autophagy is defective[53], although autophagy-independent regulation of p62/Sqstm1 makes this a less robust readout of autophagic flux[54]. HepG2 cells were also cultured in the presence of 3-methyladenine (3-MA), which inhibits the sequestration step during autophagy, but it did not have any effect on ApoA-I levels; more surprisingly, this treatment seemed to have an adverse effect on autophagic flux, as documented at 6h by the increase in LC3-II/LC3-I ratio compared to untreated cells (Figure 3.1), suggesting that it may induce autophagy. In fact, 3-MA has been shown to have a dual regulatory effect on autophagy: while 3-MA is capable of inhibiting autophagy under certain context, prolonged treatment of 3-MA under nutrient rich conditions may promote autophagy via suppression of the Class I PI3K-AKT-mTORC1 pathway[55]. Thus, given the lack of specificity and off-target effects of 3-MA treatment, we were unable to interpret these data.

To further investigate the relationship between autophagy and ApoA-I degradation, *APOA1*-tranfected HEK293 cells were cultured in normal conditions in the presence of autophagy inhibitors, either bafilomycin A1 (BAF) or chloroquine (CHLQ). Chloroquine is believed to reduce lysosomal acidification after its accumulation inside the acidic compartments, inhibiting lysosomal enzymes and also preventing the fusion of autophagosome with lysosome[56]. In agreement with the endogenous protein experiments in HepG2, both treatments resulted in an accumulation of ApoA-I in transfected cells (Figure 3.2). A Western blot with an antibody against LC3 revealed a strong induction of LC3-II upon treatment of cells with bafilomycin or chloroquine, while p62 levels could not serve as a robust

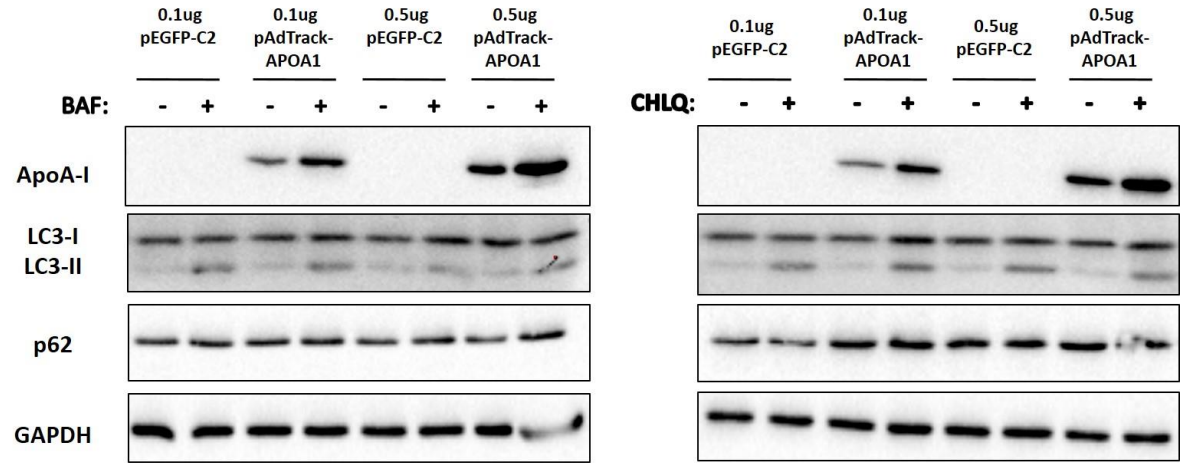

Figure 3.2 Accumulation of ApoA-I upon inhibition of autophagy in *APOA1*-transfected HEK293 cells.

HEK293 cells were transiently transfected with *EGFP-C2* (empty vector) or *APOA1* alone using different concentrations of plasmid DNA (0.1μg and 0.5μg of total plasmid DNA). Cells were either left in normal medium or in the presence of autophagy inhibitors (at late stages), bafilomycin A1 (BAF) or chloroquine (CHLQ) for 6h. Total cellular lysates were extracted and ApoA-I, LC3 and p62 protein levels were detected by immunoblotting. GAPDH levels served as internal loading control.

readout of autophagic flux in this case. As previously stated, p62 may be modulated independent of autophagy[54], and thus a negative result with changes in p62 levels is not always meaningful.

3.2 ApoA-I partially co-localizes with LC3, an autophagosomal marker

LC3 is associated with the isolation membrane during its formation and remains on the membrane after a spherical autophagosome has formed. LC3 staining has, therefore, been shown to be a very good marker for autophagy, as its localization changes from diffuse to a punctuate or dotted pattern when

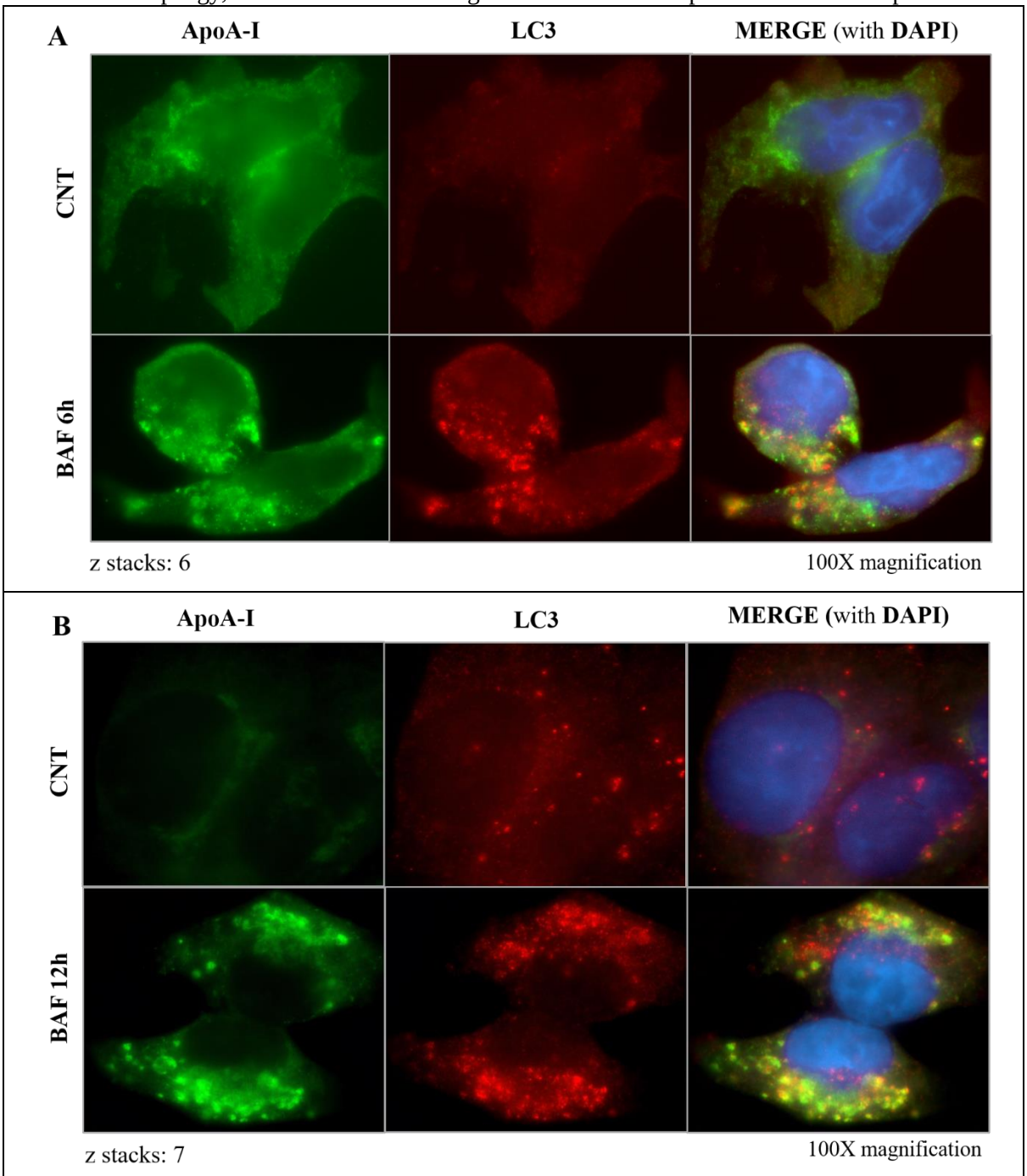

Figure 3.3 ApoA-I partially co-localizes with LC3 upon autophagy blockage.

HepG2 cells were cultured in full serum DMEM media (CNT) or treated with 50nM bafilomycin A1 (BAF) for (A) 6h and (B) 12h. Cells were fixed, permeabilized and then ApoA-I and LC3 were

detected by immunofluorescence using primary antibodies against human ApoA-I and LC3, and Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488 donkey anti-goat Ab (green for ApoA-I) and Alexa Fluor 594 goat anti-rabbit Ab (red for LC3)) and by fluorescence microscopy (Zeiss ApoTome). Micrograph 100X images of representative cells from 2 independent experiments are shown (same settings of light source intensity and exposure time); taking advantage of ApoTome structured illumination, optical sections (z stacks) of the specimen were used to generate maximal projection of the stacks (Image J software).

autophagy is induced[52].LC3 dots represent isolation membranes and autophagosomes[52]. Our finding of a link between ApoA-I degradation and autophagy modulation prompted the question whether this protein would localize to autophagosomal structures upon autophagy blockage. To test this, HepG2 cells were either left untreated or cultured in the presence of bafilomycin A1, which blocks autophagosome-lysosome fusion and leads to an accumulation of autophagosomal structures[50], and the cells were fixed and stained for endogenous ApoA-I and endogenous LC3. Fluorescence microscopy (Zeiss ApoTome) confirmed the Western blot data and further showed that ApoA-I and LC3 are partially colocalized, probably within autophagosomes (Figure 3.3). Control conditions (Figure 3.3a&b, upper panels) demonstrated the staining pattern for both LC3 (red, diffuse) and ApoA-I (green, mostly perinuclear), and did not illustrate any levels of co-localization of the two proteins in the merged images (no yellow-orange spots detected). In contrast, when LC3 and ApoA-I immunofluorescent images for bafilomycin treated cells were merged (Figure 3.3A&B, lower panels), the images showed yellow-orange structures in BAF-treated versus control cells, which indicates that autophagy blockage leads to the co-localization of ApoA-I with LC3. Similar to that shown in Figure 3.1, bafilomycin A1 increased cellular LC3 and ApoA-I levels relative to control at 6h of treatment, with even more pronounced differences at 12h. Furthermore, fluorescent images showed LC3 to be localized to punctate structures within the cell indicating its impaired degradation, and ApoA-I to be found in aggregate-like structures. Together, the aforementioned results suggest a relatively close association between LC3 and ApoA-I aggregates and that a fraction of these 'ApoA-I bodies' are degraded by autophagy.

3.3 ApoA-I is not found to directly interact with LC3

The results of partial fluorescence co-localization between ApoA-I and LC3 could imply the presence of direct or indirect interactions between these proteins. By using the iLIR bioinformatics tool[57] for prediction of Atg8-family interacting proteins, we identified 3 putative LC3-interacting region (LIR) motifs shared between human and mouse ApoA-I (data not shown). We therefore wanted to examine whether ApoA-I could directly interact with LC3. To this end, we transiently expressed ApoA-I in HEK293 cells or used HepG2 cells (endogenous expression of ApoA-I) and treated cells with autophagy inhibitors, chloroquine or bafilomycin A1 respectively. Then, cell extracts were subjected to immunoprecipitation using an anti-LC3 antibody. In both cases, co-immunoprecipitation (co-IP) of ApoA-I was assessed by immunoblotting with a monoclonal anti-ApoA-I antibody. As shown in Figure 3.4a&b, ApoA-I and LC3-II/LC3-I levels increase upon autophagy blockage in whole cell lysates (input; left and right upper blots), but there is no evident co-IP of transfected or endogenous ApoA-I

with endogenous LC3. It should be noted that p62, a known interaction partner of LC3, was found to co-IP with endogenous LC3 and to accumulate upon autophagy inhibition in both IP experiments. Next, we performed immunoprecipitation in HepG2 cell extracts, formerly treated with bafilomycin, using an anti-ApoA-I antibody; again, no co-immunoprecipitation of LC3 with ApoA-I was detected by immunoblotting with a monoclonal anti-LC3 antibody (Figure 3.4c). Despite the close association of ApoA-I with LC3, these results reveal that a direct interaction between these proteins cannot be detected, possibly due to either weak or indirect interactions.

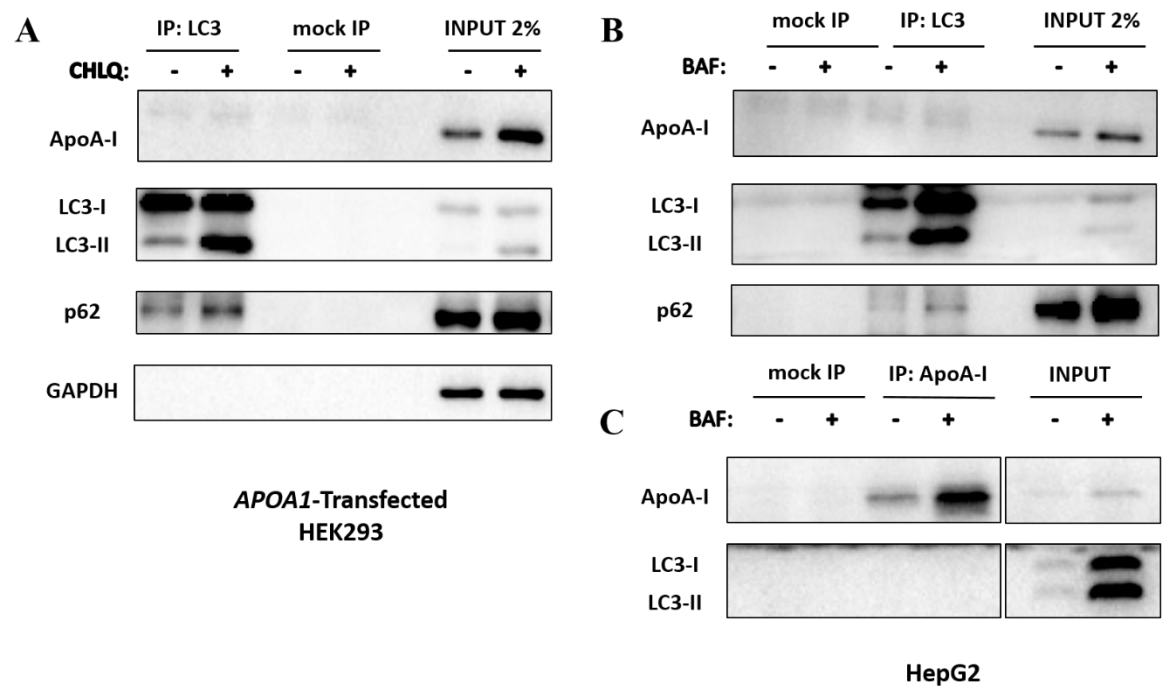

Figure 3.4 Neither LC3 nor ApoA-I co-immunoprecipitates (co-IPs) with each other.

(A) Endogenous p62, but not ApoA-I, coimmunoprecipitates (co-IPs) with LC3 from *APOA1*-transfected HEK293 cell extracts. HEK293 cells transiently transfected with pAdTrackCMV-APOA1 were either cultured in normal medium or in the presence of chloroquine (CHLQ) for 16h as indicated. After transfection and drug treatment, endogenous LC3 was immunoprecipitated (*IP*) from total cellular extracts (*input*, right blots). ApoA-I, LC3 and p62 were visualized by immunoblotting. Also, total cellular extracts incubated only with protein G beads (*mock IP*), and not anti-LC3, were used as negative control. (B) Endogenous p62, but not endogenous ApoA-I, coimmunoprecipitates (co-IPs) with LC3 from HepG2 cell extracts. HepG2 cells were either left untreated or cultured in the presence of bafilomycin A1 (BAF) for 12h as indicated. Then, endogenous LC3 was immunoprecipitated (*IP*) from total cellular extracts (*input*, right blots). Endogenous ApoA-I, LC3 and p62 were detected by immunoblotting. Also, total cellular extracts incubated only with protein G beads (*mock IP*) were used as negative control. (C) Endogenous LC3 does not coimmunoprecipitate (co-IP) with ApoA-I from HepG2 cell extracts. HepG2 cells were either cultured in full DMEM media or in the presence of bafilomycin A1 (BAF) for 12h as indicated. After drug treatment, endogenous ApoA-I was immunoprecipitated (*IP*) from total cellular extracts (*input*, right blots). Endogenous ApoA-I and LC3 were detected by immunoblotting. Also, total cellular extracts incubated only with protein G beads (*mock IP*) were used as negative control.

3.4 Autophagy blockage leads to ApoA-I accumulation within LE/ lysosomes

Autophagy is a major catabolic pathway for delivery of proteins and organelles to lysosomes or the vacuole, where they are degraded and recycled. To further analyze whether ApoA-I partly localizes to

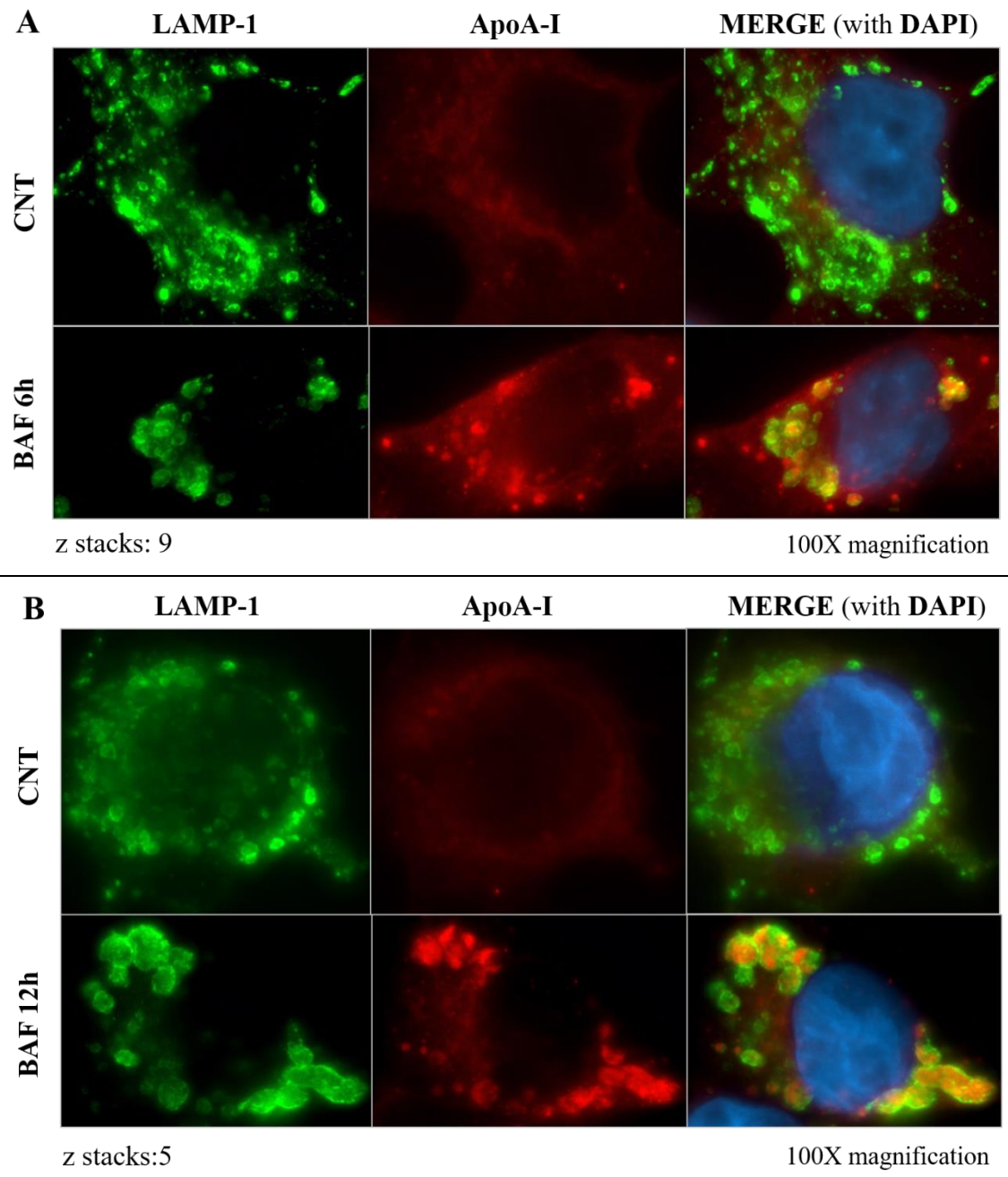

Figure 3.5 Pharmacological inhibition of autophagy leads to accumulation of ApoA-I within late endosomes/lysosomes.

HepG2 cells were maintained in the absence (CNT) or presence of bafilomycin A1 (BAF), and stained with antibodies against LAMP-1 and ApoA-I. Representative micrograph 100X images after (A) 6h and (B) 12h of pharmacological inhibition of autophagy. Individual channels and merged images are shown (same settings of light source intensity and exposure time); taking advantage of ApoTome structured illumination, optical sections (z stacks) of the specimen were used to generate maximal projection of the stacks (Image J software).

the lysosomal compartment, we performed immunofluorescence for ApoA-I and lysosomal-associated membrane protein (LAMP)-1 as marker for late endosomes (LE)/lysosomes. As shown in Figure 3.5(lower panels), rings of LAMP-1 staining often encircled the ApoA-I aggregates only in the presence of bafilomycin A1, which is a specific V-ATPase inhibitor and is thus able to abolish lysosomal acidification. In contrast, under basal conditions (CNT) with functional autophagic flux, no engulfment of ApoA-I by LAMP-1-decorated rings was apparent (Figure 3.5, upper panels). These observations confirmed that bafilomycin A1 caused accumulation of ApoA-I within late endosomes/lysosomes, probably due to the inefficient recycling and catabolism of autophagosomes. The latter suggestion requires further investigation, but it might be supported by the marked increase of lysosomes in diameter upon inhibition of autophagy (Figure 3.5), as in mammalian cells lysosome is considered to be smaller in size than autophagosome[58].

3.5 Torin2, a potent mTOR inhibitor, decreases intracellular ApoA-I levels

To further investigate the connection between ApoA-I and autophagy, we monitored ApoA-I levels upon pharmacological induction of autophagy. In particular, we used Torin2, a catalytic inhibitor of mTOR (mechanistic target of rapamycin), a serine/threonine kinase) that, among others, negatively regulates autophagy[59,25]. mTORC1 activity, which mediates the autophagy-inhibitory effect of amino acids, was monitored by following the phosphorylation (p4E-BP1) of one of its substrates, EIF4EBP1 (4E-BP1/PHAS-I) at Thr37 and Thr46 sites. This protein directly interacts with eukaryotic translation initiation factor 4E (eIF4E), which is a limiting component of the multi-subunit complex that recruits 40S ribosomal subunits to the 5' end of mRNAs, and this interaction inhibits complex assembly and represses cap-dependent translation[60]. As shown in Figure 3.6a, the mTORC1-dependent phosphorylation of 4EBP1 could not be detected by western blotting in Torin2-treated samples compared to untreated cells, where a molecular mass shift can be seen, indicating that Torin2 abolishes mTORC1 activity. As expected, following Torin2 treatment, ApoA-I protein levels were severely diminished over time (Figure 3.6). To measure the autophagic flux in HepG2 cells, we performed our treatments with Torin2 and full media controls and then additionally inhibited the formation of the autolysosome with Baf A1. Performing densitometric analysis, we observed an accumulation of LC3-II (increased LC3-II/I ratios) in BAF A1-treated autophagy-activated cells, whereas without lysosomal inhibitor LC3-II partially degraded (Figure 3.6b, middle graph). Unfortunately, p62 levels could not serve as a robust readout of autophagic flux, except 6h of treatment (right graph). To our surprise, a combined treatment of autophagy induction and inhibition with Torin2 and BAF A1 did not fully prevent Torin2-mediated ApoA-I downregulation. These data suggest that, although Torin2 treatment led to induction of autophagy, reduced ApoA-I levels were not entirely due to autophagy modulation, indicating the presence of other degradation mechanisms or decreased protein synthesis or elevated secretion.

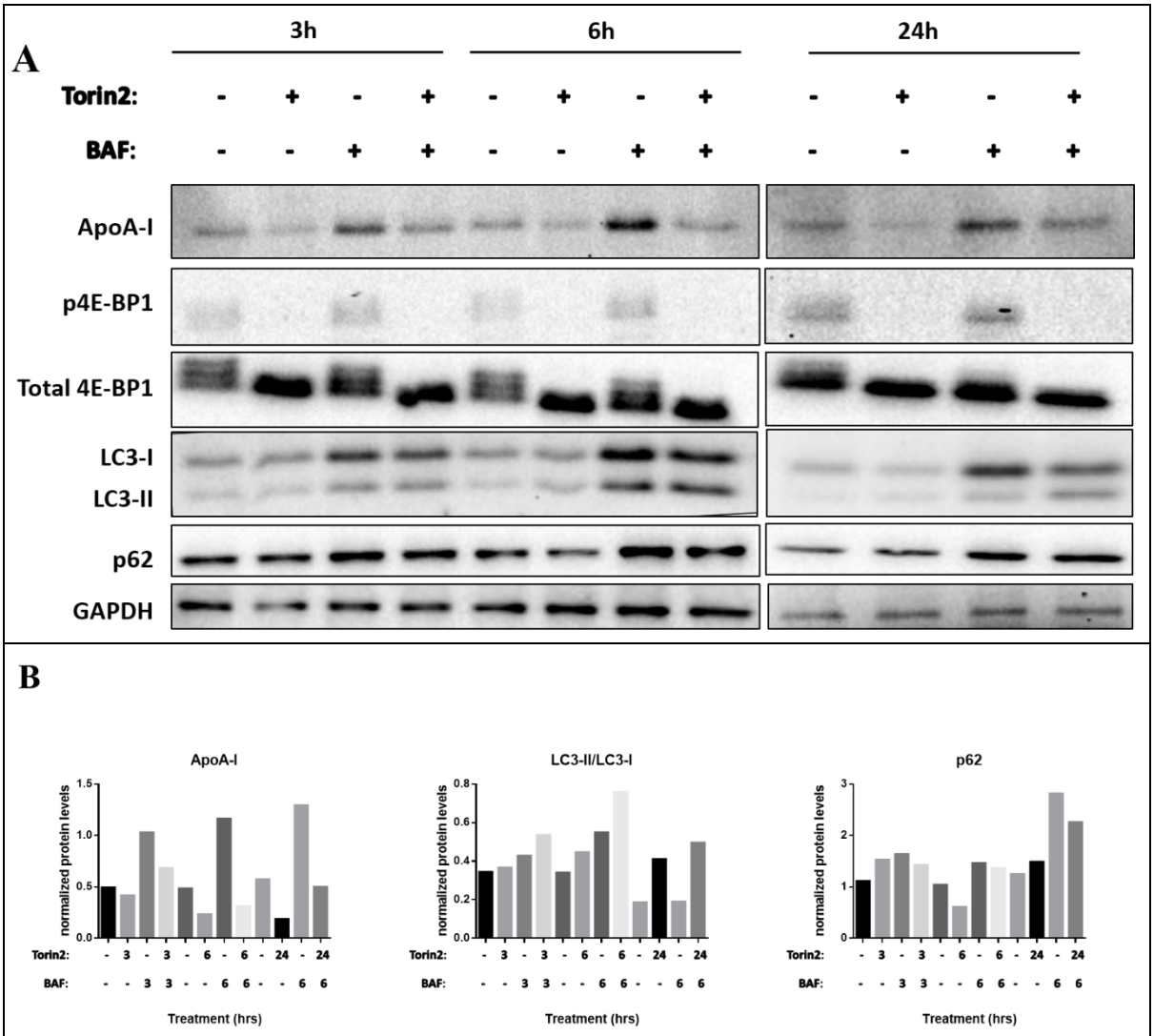

Figure 3.6 Torin2, a potent mTOR inhibitor, decreases ApoA-I levels in HepG2 cells.

(A) HepG2 cells were either left untreated or treated with 50nM Bafilomycin A1 (BAF) or 250nM Torin2 or both for the indicated time. Total cellular lysates were extracted and analysed for the indicated proteins by western blot. GAPDH served as internal loading control. (B) Relative quantification of ApoA-I, LC3-II/ LC3-I and p62 protein levels from the same experiment; histograms represent band densitometry analysis of indicated proteins, normalized to GAPDH and expressed in arbitrary units.

3.5.1 mTOR inhibition does not prevent from ApoA-I accumulation upon autophagy blockage

Since pharmacological inhibition of autophagy leads to ApoA-I accumulation, we sought to examine the extent to which Torin2-dependent ApoA-I degradation is due to autophagy. To this end, HepG2 cells were treated with BAF for 2h and Torin2 was added to the BAF-treated cells for 6h prior harvesting (8h BAF in total). Consistent with previous experiments, mTORC1 inhibition, as witnessed by the absence of phosphorylation of its downstream target (4E-BP1), attenuated ApoA-I accumulation (Figure 3.7b) in BAF-treated cells, suggesting that other pathways (related to protein synthesis or degradation) may partially contribute to ApoA-I downregulation. In any case, the relative increase or decrease in ApoA-I expression was not due to transcription, as APOA1 mRNA levels remained

unaffected by bafilomycin or Torin2 treatments (Figure 3.7a). Intriguingly, BAF lysosomal inhibitor (Figure 3.7b and c) inhibited ApoA-I and p62 degradation in the control hepatocytes (lanes 1 vs. 3), and such inhibition was similar in the BAF-, Torin2-treated HepG2 cells (lanes 2 vs. 4). We also observed a partial degradation of LC3-II in BAF-treated autophagy-activated cells, whereas without mTOR inhibitor LC3-II strongly accumulated (Figure 3.7c, LC3-II graphs), implying that prior treatment of HepG2 with bafilomycin did not fully block autolysosome fusion. These data indicate that partial amounts of ApoA-I are constitutively degraded in the lysosome via autophagic pathway, despite the possible contribution of other processes that may regulate its expression.

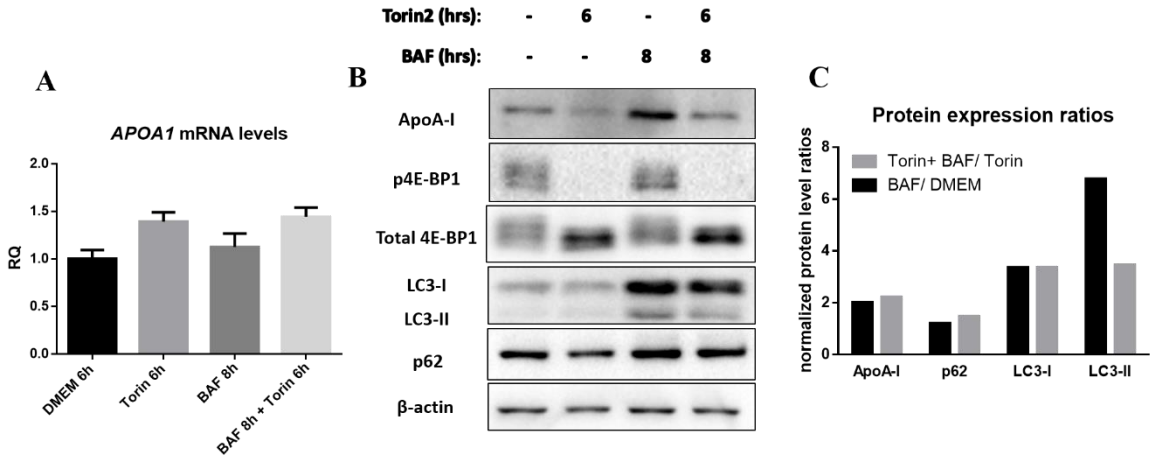

Figure 3.7 mTORC1 inhibition does not prevent from ApoA-I accumulation upon autophagy blockage.

HepG2 cells were cultured in full media or treated with Bafilomycin A1 (50nM) or Torin2 (250nM) or a combination of both for the indicated time. (A)RNA of cells was isolated and mRNA expression of *APOA1* was analyzed using qPCR; mRNA levels were normalized to β actin mRNA levels. DMEM sample is set as 1. Data represent the mean ± SD of duplicates of 1 individual experiment. (B) Total cellular lysates were extracted and analyzed by western blot (WB) using antibodies specific for the indicated proteins. Actin b served as internal loading control. (C) Densitometric analysis was performed for ApoA-I, p62, LC3-I and LC3-II bands detected by WB. The graphs represent the ratios of normalized (to β actin) abundances for each protein in BAF-treated cells vs. untreated cells (black columns) and in BAF+Torin2-treated cells vs. Torin2-treated cells (grey columns).

3.6 ApoA-I is regulated in a *Becn1*-dependent and *Atg5*-independent manner

The molecular mechanism of autophagy involves several conserved autophagy-related genes (Atgs), among which Beclin 1 (*Becn1*) and *Atg5* are considered to be essential for mammalian macro-autophagy; constitutive and starvation-induced autophagy is impaired in genetically modified mice lacking *Becn1*[61,62] or *Atg5*[63]. To investigate the mechanism underlying regulation of ApoA-I expression through autophagy, we generated a transient siRNA-mediated knockdown of *Becn1* or *Atg5* in HepG2 cells. Functional knockdown of both autophagy-related genes was determined on protein level by western blot analysis (Figure 3.8b). In *Becn1*-silenced conditions, ApoA-I protein levels were highly elevated (almost 2-fold) compared to control (si*luc*), indicating that Beclin 1 may mediate autophagic

regulation of ApoA-I expression, although it might possibly affect *APOA1* transcription to a smaller extent (slightly elevated mRNA levels). The mammalian Beclin 1, an ortholog of the Atg6 vacuolar protein sorting (Vps)-30 protein in yeast, is important for localization of autophagic proteins to a pre-autophagosomal structure (PAS), depending on interaction with the class III type phosphoinositide 3-kinase (PI3KC3)/Vps34[64]. Although a significant amount of basal LC3-II was still present in *Becn1* KD HepG2 cells, which is consistent with the fact that yeast atg6 is not required for Atg8 (LC3 mammalian homolog)-PE formation[65], the level of endogenous p62, another autophagy substrate, was higher in si*Becn1* cells than that of control (Figure 3.8b and c), indicating that depletion of *Becn1* caused impaired autophagic degradation.

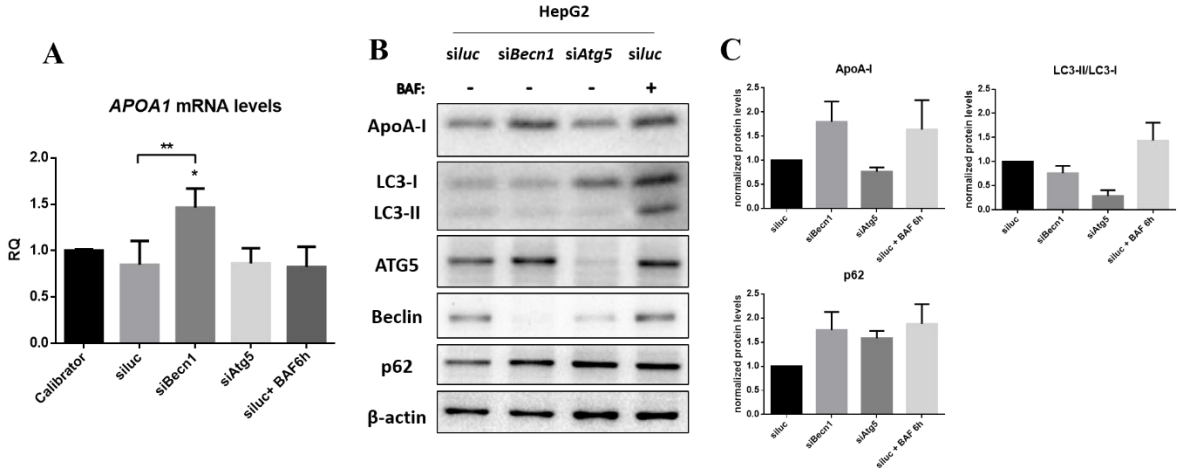

Figure 3.8 *Becn1*-dependent and *Atg5*-independent regulation of ApoA-I levels.

HepG2 cells transiently transfected with si*Becn1*, si*ATG5* or si*luc* (control) were cultured in full media and si*luc* cells were also treated with bafilomycin (50nM) for 6h. (A) RNA was isolated and mRNA expression of *APOA1* was analyzed using qPCR. mRNA levels were normalized to β actin mRNA levels. Data represent the mean ± SD of duplicates of at least 2 individual experiments. Calibrator was set as 1 and served as an external control between qPCR experiments (*P < 0.05; **P < 0.005). (B) Total cellular lysates were extracted and analyzed for the indicated proteins by western blot. β-actin served as internal loading control. (C) Relative quantification of ApoA-I, LC3-II/ LC3-I and p62 protein levels; histograms represent band densitometry analysis of indicated proteins, normalized to β-actin and expressed as means of fold-change (compared to the control) ± SD of 2 independent experiments.

In contrast, *Atg5* knockdown did not display any effect on ApoA-I levels, despite the apparent decrease in LC3-II levels and concomitant LC3-I accumulation. ATG5 protein in a conjugated form with ATG12, and ATG8 (LC3) are involved in the early stages of autophagosome formation[58]. The ATG12–ATG5 conjugate is required for the proper function of the LC3 ubiquitin-like conjugation system, and therefore, processing of LC3-I to LC3-II[58]. The levels of p62 are also increased in *Atg5*-depleted HepG2 cells, further demonstrating defective autophagic activity due to the absence of ATG5. As expected, pharmacological inhibition of autophagy in si*luc* HepG2 cells led to ApoA-I and p62 accumulation and increase in LC3-II/LC3-I ratio, without affecting *APOA1* mRNA levels (Figure 3.8a, b and c). Together, all the aforementioned results suggest that autophagy regulates turnover of intracellular ApoA-I through

a *Becn1*-dependent and *Atg5*-independent pathway. Interestingly, besides *Atg5/Atg7*-dependent conventional pathway, mammalian macroautophagy has been also shown to occur through an *Atg5/Atg7*-independent alternative pathway, which is still regulated by ULK1 and Beclin 1[66].

4 Discussion

To date, the vast majority of published studies have focused almost exclusively on the effects of ApoA-I expression on plasma lipid levels and atherosclerosis, whereas intracellular roles for ApoA-I and its cellular dynamics (e.g. transport, degradation) have remained elusive. A recent study has implicated ApoA-I in modulating processes associated with diet-induced hepatic lipid deposition and non-alcoholic fatty liver disease (NAFLD) development in mice[10]. Also, it has become known that autophagy mediates the elimination of stored lipid droplets in the liver (a selective type of autophagy termed lipophagy)[27] and that inhibition of autophagy leads to the development of hepatosteatosis[27,28,47]. In view of these findings, it was tempting to speculate the possible regulation of hepatic ApoA-I through autophagy. Thus, the main objective for the present study was to evaluate if intracellular ApoA-I levels are affected upon autophagy modulation in model hepatic cells. We show that autophagy mediates degradation of ApoA-I, which is regulated through a Becn1-dependent, Atg5-independent pathway.

4.1 Autophagic degradation of ApoA-I

Our current study, as well as previous work performed in our lab, provide several lines of evidence suggesting that ApoA-I is an autophagy substrate. First of all, western blotting, followed by densitometry analysis of bands, revealed a dramatic increase in ApoA-I levels, when autophagosome-lysosome fusion (treatment with bafilomycin A1) or lysosomal acidification (treatment with chloroquine) was blocked in HepG2 cells. These findings were also confirmed in a different experimental system, in which HEK293 cells, which do not produce ApoA-I *per se*, were transiently transfected with a construct of WT *APOA1*. Secondly, fluorescence microscopy showed that BAF-treated hepatocytes had a much higher fluorescence intensity in endogenous ApoA-I, which was found in aggregate-like structures, compared to untreated cells (subcellular ApoA-I localization was mostly perinuclear). All these data suggest that blockage of autophagosome-lysosome fusion leads to impaired ApoA-I degradation and concomitant accumulation within hepatocytes. What is more, amino acid depletion has been previously shown to regulate ApoA-I via autophagy, while its protein levels are not affected in the presence of proteasomal inhibitor (Konstantina Georgila; personal communication).

In neurodegenerative diseases, both autophagy and the lysosomal pathway have been implicated in the clearance of protein aggregates including those of α-synuclein, tau, and mutant huntingtin proteins[67,68]. In our study here, we attempted to monitor whether autophagic-lysosomal pathway is involved in the degradation of hepatic ApoA-I aggregates upon autophagy blockade. Therefore we performed immunofluorescence experiments, which indicated that blockage of autophagosome-lysosome fusion

results in partial co-localization of ApoA-I with LC3, an autophagosomal marker, while it leads to ApoA-I accumulation within lysosomal structures as determined by LAMP-1 (marker for late endosomes/lysosomes) staining. Extended treatments (over 12h) with bafilomycin A1 have been shown to result in an obvious separation between LC3-labelled vesicles (autophagosomes) and LAMP-1-stained (late endosomes/lysosomes) vesicles[69], which suggests that conditions with blocked autophagosome-lysosome fusion should lead to accumulation of autophagy substrates within autophagosomes or amphisomes (fused endosome-autophagosome). Although our findings may seem contradictory at first glance, it is likely that our treatments with bafilomycin A1(<12h) cause accumulation of autophagosomal material (like ApoA-I aggregates) within autolysosomes, and without the use of additional markers (e.g. mcherry-GFP-LC3)[70] it is not possible to determine whether accumulated ApoA-I is found within autolysosomes or amphisomes. In line with our data, but in a discrete pathological context, a recent study reported that degradation of internalized ApoA-I_{Iowa} fibrils occurs via the autophagy-lysosomal pathway[71]. Taken together, our results indicate that the autophagy-lysosomal pathway contributes to the clearance of hepatic ApoA-I under basal conditions, a function that could have further implications in pathological conditions. Elucidating the detailed mechanisms of intracellular transport and degradation of apolipoprotein A-I is an important challenge for the future.

In the mammalian system, cargo receptors involved in selective autophagy, such as p62 and NBR1, are specifically recruited to the inner surface of the phagophore via LIR (LC3-interacting region) interactions[70,72]. Interestingly, both human and mouse ApoA-I have been predicted to carry putative LIR motifs ($X_{-2}X_{-1}$[WY] X_1X_2[LIV]) (prediction was achieved by using the iLIR bioinformatics tool), indicating a possible association with LC3. In view of this observation, further supported by the partial immunofluorescence co-localization of ApoA-I with LC3 upon autophagy blockage, we analyzed whether LC3 interacts with ApoA-I using co-immunoprecipitation experiments. Unfortunately, immunoprecipitation of either endogenous LC3 or ApoA-I showed lack of association between these proteins, which can be attributed to the nature of protein-protein interactions (low-affinity, transient or indirect). However, it would be interesting to determine in the future whether ApoA-I degradation in the course of autophagy is mediated through cargo receptors such as p62.

MTORC1 has been well established as the key negative regulator of autophagy, via suppression of the ULK1 complex at the initiation stage of autophagy[24,26]. Thus, we checked the impact of Torin2, a potent mTOR inhibitor[59], on ApoA-I expression levels in hepatocytes. We found that Torin2 does not affect *APOA1* transcription, as cellular mRNA levels remained unchanged over the 24-hour incubation, while it significantly decreased ApoA-I protein levels. Surprisingly, blockade of autophagosome-lysosome fusion did not fully rescue Torin2-mediated ApoA-I degradation. These findings suggest that, although Torin2 led to induction of autophagy, reduction in ApoA-I levels was not entirely due to autophagic pathway, indicating the presence of other degradation mechanisms or decreased protein synthesis or elevated secretion. As ApoA-I protein is reduced by autophagy activation (either through starvation or

pharmacological modulation) and accumulated upon autophagy inhibition, further experiments are required in order to monitor ApoA-I stability and delineate the degradation pathways involved in its regulation. In a recent study, inhibition of mTOR with Torin1 was demonstrated to rapidly increase the degradation of long-lived cell proteins, but not short-lived ones, by stimulating proteolysis by proteasomes, in addition to autophagy[73]. However, our observation that blockade of lysosomal proteolysis attenuated, to the same extent, the degradation of ApoA-I and p62, in spite of mTOR inhibition, indicates that an intracellular pool of ApoA-I is constitutively degraded in the lysosome via autophagic pathway.

4.2 Regulation of ApoA-I via *Atg5*-independent macroautophagy

Aside from pharmacological modulation of autophagy, we performed transient knockdowns of Atgs (*Becn1*, *Atg5*) essential for mammalian macroautophagy in HepG2 cells, in order to provide direct insights into the mechanism underlying autophagic regulation of hepatic ApoA-I. In mice lacking *beclin 1*[61,62] and *Atg5*[63], both constitutive and starvation-induced autophagy are impaired. Intriguingly, we showed here that HepG2 cells deficient in *Becn1* display accumulation of intracellular ApoA-I levels, while we could not observe any effect on ApoA-I levels in *Atg5* depleted cells. In both cases, we confirmed a defective autophagic clearance of p62, a well-established autophagy substrate, suggesting that autophagy-mediated degradation of p62 occurs via the conventional pathway. This finding has been also demonstrated *in vivo*, with liver specific ablation of *Atg5* having no effect on hepatic ApoA-I levels (Konstantina Georrgila, personal communication). In agreement with these observations, it has been recently reported that mammalian macroautophagy can occur through an Atg5/Atg7-independent alternative pathway, in addition to the Atg5/Atg7-dependent conventional pathway[66]. Indeed, this alternative process of autophagy has been shown to be regulated by Unc-51-like kinase 1 (Ulk1) and beclin 1, with overexpression of human beclin 1 being able to restore macroautophagy in *Becn1*-silenced *ATG5*-/- mouse embryonic fibroblasts[66]. Our findings indicate that autophagic regulation of hepatic ApoA-I is more complex than initially anticipated. Taking into account that alternative macroautophagy can be triggered by cellular stress in vitro, it could possibly function in the autophagic clearance of hepatic ApoA-I in conditions of disturbed lipid homeostasis. To better understand the relationship between autophagy and regulation of ApoA-I, it is crucial to further map autophagy-related molecules according to their effect on degradation as well as secretion of hepatic ApoA-I.

4.3 Possible roles of ApoA-I autophagic regulation in atherosclerosis and non-alcoholic fatty liver disease

To date, the majority of studies uncovering a protective role for autophagy in atherosclerosis progression, have focused almost exclusively on macrophage autophagy function in atherogenic mouse models[74–76]. However, the physiological role of liver degradation pathways, such as autophagy, in the modulation of circulating ApoA-I levels has remained largely unexplored. Low circulating ApoA-I levels are considered to be an independent risk factor for cardiovascular disease and are closely

associated with low HDL cholesterol levels[77]. Recently, a study demonstrated that ApoA-I secretion is regulated through cellular autophagic pathways in response to the expression of a hepatocyte ADP-receptor, $P2Y_{13}$[78]. In an independent study, this receptor has been demonstrated to regulate HDL metabolism, reverse cholesterol transport and to confer protection against atherosclerosis[79]. Thus, it would be tempting to speculate that hepatic autophagy could partially modulate HDL plasma levels by controlling ApoA-I availability, a key determinant of HDL metabolism, in response to metabolic stimuli. These observations indicate an interrelationship between secretion and autophagic degradation of ApoA-I, which could have an impact on the atherosclerotic pathology and requires further investigation in the future.

In addition to its well-characterized properties in atheroprotection, ApoA-I has been shown to modulate diet-induced hepatic lipid deposition and non-alcoholic fatty liver disease (NAFLD) development in mice[10]. In fact, reduced HDL cholesterol levels and NAFLD are present in patients with metabolic syndrome[36,80–82], while no mechanistic link between these conditions has been described in the literature so far. Given that autophagy regulates hepatic lipid stores as well as intracellular ApoA-I levels, this dynamic cross-talk could have a direct bearing to NAFLD. Importantly, our findings raise the interesting possibility that compromised liver autophagy in conditions where hepatocytes face a sustained lipid challenge[27,38,39](NAFLD, chronic obesity and insulin resistance) may lead to accumulation of intracellular ApoA-I pool, possibly affecting its secretion in the plasma. Therefore, ApoA-I might not be able to exert its functions as a modulator of lipoprotein metabolism in plasma and its accumulation within hepatocytes may further deteriorate diet-induced lipotoxicity in the face of increased lipid influx in this organ and decreased mobilization of hepatic lipid stores due to impaired autophagy[27]. It is essential that future studies delineate ApoA-I's contribution to disease pathogenesis in regards to its autophagic regulation.

4.4 Concluding remarks and future prospects

In summary, our study shows that hepatic ApoA-I is regulated by autophagy, as its protein levels are decreased upon induction of autophagy (either through starvation or pharmacological modulation) and increased following blockade of autophagic process. In fact, a pool of intracellular ApoA-I is constitutively degraded in the lysosome via autophagic pathway, despite the possible contribution of other degradation mechanisms. Autophagy-mediated degradation of ApoA-I seems to occur through a Beclin-dependent, Atg5-independent alternative pathway.

Despite these exciting findings, a number of questions need to be addressed. It is still unclear how autophagosomes sequester intracellular ApoA-I on its way for secretion. An interesting possibility is that ApoA-I aggregates, which accumulate in cytoplasm upon autophagy blockade, could be sequestered by cargo receptors, such as p62, which are specifically recruited to the inner surface of the phagophore. However, it remains to be determined how intracellular ApoA-I aggregation (within

cytoplasm) is induced (e.g. lipid challenge, cellular stress, UPR pathway), as it might affect its secretion and serve as a signal for autophagosome sequestration. Another integral question is to explore the possibilities of crosstalk between lipophagy, which degrades sequestered lipid droplets, and ApoA-I, which has been reported to modulate diet-induced hepatic lipid deposition. In humans, it is conceivable that prolonged consumption of diets rich in fat, such as the western diet, can impair autophagy through the effects of lipids on autophagosome–lysosome fusion[38] perpetuating a vicious circle of further hepatic fat accumulation. Thus, it would be very interesting in future studies to investigate whether proper autophagic regulation of hepatic ApoA-I confers protection against lipotoxicity, in the setting of fatty liver, and if it influences HDL circulating levels, in the setting of atherosclerosis.

5 References

1. Oda, M. N. & Ryan, R. O. in *High-Density Lipoproteins* 1–23 (Wiley-VCH Verlag GmbH & Co. KGaA, 2007). doi:10.1002/9783527625178.ch1

2. Brunham, L. R. *et al.* Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J. Clin. Invest.* **116,** 1052–1062 (2006).

3. Timmins, J. M. *et al.* Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J. Clin. Invest.* **115,** 1333–1342 (2005).

4. von Eckardstein, A. & Kardassis, D. *High Density Lipoproteins*. **224,** (2015).

5. Zannis, V. I., Chroni, A. & Krieger, M. Role of apoA-I, ABCA1, LCAT, and SR-BI in the biogenesis of HDL. *J. Mol. Med. (Berl).* **84,** 276–294 (2006).

6. Boden, W. E. High-density lipoprotein cholesterol as an independent risk factor in cardiovascular disease: assessing the data from framingham to the veterans affairs high-density lipoprotein intervention trial. *Am. J. Cardiol.* **86,** 19–22 (2016).

7. Rosenson, R. S. *et al.* Dysfunctional HDL and atherosclerotic cardiovascular disease. *Nat. Rev. Cardiol.* 1–13 (2015). doi:10.1038/nrcardio.2015.124

8. Cuchel, M. & Rader, D. J. Macrophage Reverse Cholesterol Transport. *Circulation* **113,** 2548–2555 (2006).

9. Duffy, D. & Rader, D. J. Update on strategies to increase HDL quantity and function. *Nat. Rev. Cardiol.* **6,** 455–463 (2009).

10. Karavia, E. & Papachristou, D. Apolipoprotein A-I modulates processes associated with diet-induced nonalcoholic fatty liver disease in mice. *Mol. Med.* **18,** 901–912 (2012).

11. Wiggins, D. & Gibbons, G. F. The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of very-low-density lipoprotein and its response to hormones and sulphonylureas. *Biochem. J.* **284,** 457–462 (1992).

12. Schneider, J. L. & Cuervo, A. M. Liver autophagy: much more than just taking out the trash. *Nat. Rev. Gastroenterol. Hepatol.* **11,** 187–200 (2014).

13. Stolz, A., Ernst, A. & Dikic, I. Cargo recognition and trafficking in selective autophagy. *Nat. Cell Biol.* **16,** 495–501 (2014).

14. Singh, R. & Cuervo, A. M. Autophagy in the cellular energetic balance. *Cell Metab.* **13,** 495–504 (2011).

15. Kraft, C., Peter, M. & Hofmann, K. Selective autophagy: ubiquitin-mediated recognition and beyond. *Nat. Cell Biol.* **12,** 836–841 (2010).

16. Yang, Z. & Klionsky, D. J. Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* **12,** 814–22 (2010).

17. He, C. & Klionsky, D. J. Regulation Mechanisms and Signalling Pathways of Autophagy. *Annu. Rev. Genet.* **43,** 67 (2009).

18. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The role of Atg proteins in autophagosome formation. *Annu. Rev. Cell Dev. Biol.* **27,** 107–32 (2011).

19. Wei, Y., Pattingre, S., Sinha, S., Bassik, M. & Levine, B. JNK1-Mediated Phosphorylation of Bcl-2 Regulates Starvation-Induced Autophagy. *Mol. Cell* **30,** 678–688 (2008).

20. Petiot, A. *et al.* Distinct Classes of Phosphatidylinositol 3'-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in Distinct Classes of Phosphatidylinositol 3 ˜-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in HT-29 Cells. *J. Biol. Chem. 2000, 275992-998* **275,** 1–8 (2000).

21. Tassa, A., Roux, M. P., Attaix, D. & Bechet, D. M. Class III phosphoinositide 3-kinase--Beclin1 complex mediates the amino acid-dependent regulation of autophagy in C2C12 myotubes. *Biochem. J.* **376,** 577–586 (2003).

22. Kabeya, Y. *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19,** 5720 LP-5728 (2000).

23. Zhou, J. *et al.* Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. *Cell Res.* **23,** 508–23 (2013).

24. Jung, C. H. *et al.* ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Mol. Biol. Cell* **20,** 1992–2003 (2009).

25. Jung, C. H., Ro, S. H., Cao, J., Otto, N. M. & Kim Do-Hyung, D. H. MTOR regulation of autophagy. *FEBS Lett.* **584,** 1287–1295 (2010).

26. Egan, D., Kim, J., Shaw, R. J. & Guan, K.-L. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. *Autophagy* **7,** 643–644 (2011).

27. Singh, R. *et al.* Autophagy regulates lipid metabolism. *Nature* **458,** 1131–5 (2009).

28. Singh, R. in (eds. Meyerhof, W., Beisiegel, U. & Joost, H.-G.) 35–46 (Springer Berlin Heidelberg, 2010). doi:10.1007/978-3-642-14426-4_4

29. Skop, V. *et al.* Autophagy-lysosomal pathway is involved in lipid degradation in rat liver. *Physiol. Res.* **61,** 287–297 (2012).

30. Fujimoto, T. & Ohsaki, Y. Proteasomal and autophagic pathways converge on lipid droplets. *Autophagy* **2,** 299–301 (2006).

31. Singh, R. & Cuervo, A. M. Lipophagy: Connecting autophagy and lipid metabolism. *Int. J. Cell Biol.* **2012,** (2012).

32. Fisher, E. A. The degradation of apolipoprotein B100: multiple opportunities to regulate VLDL triglyceride production by different proteolytic pathways. *Biochim. Biophys. Acta* **1821,** 778–781 (2012).

33. Pan, M. *et al.* Presecretory oxidation, aggregation, and autophagic destruction of apoprotein-B: a pathway for late-stage quality control. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 5862–5867 (2008).

34. Rautou, P.-E. *et al.* Autophagy in liver diseases. *J. Hepatol.* **53,** 1123–1134 (2010).

35. Koliaki, C. & Roden, M. Hepatic energy metabolism in human diabetes mellitus, obesity and non-alcoholic fatty liver disease. *Mol. Cell. Endocrinol.* **379,** 35–42 (2013).

36. Marchesini, G. *et al.* Nonalcoholic Fatty Liver Disease. *Diabetes* **50,** 1844 LP-1850 (2001).

37. Day, C. P. & James, O. F. Steatohepatitis: a tale of two 'hits'? *Gastroenterology* **114,** 842–845 (1998).

38. Koga, H., Kaushik, S. & Cuervo, A. M. Altered lipid content inhibits autophagic vesicular fusion. *FASEB J.* **24,** 3052–3065 (2010).

39. Mei, S. *et al.* Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. *J. Pharmacol. Exp. Ther.* **339,** 487–498 (2011).

40. Yang, L., Li, P., Fu, S., Calay, E. S. & Hotamisligil, G. S. Defective hepatic autophagy in obesity

promotes ER stress and causes insulin resistance. *Cell Metab.* **11,** 467–478 (2010).

41. Liu, H.-Y. *et al.* Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. *J. Biol. Chem.* **284,** 31484–31492 (2009).

42. Codogno, P. & Meijer, A. J. Autophagy: A potential link between obesity and insulin resistance. *Cell Metab.* **11,** 449–451 (2010).

43. Ozcan, U. *et al.* Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306,** 457–461 (2004).

44. Cuervo, A. M. & Dice, J. F. Age-related decline in chaperone-mediated autophagy. *J. Biol. Chem.* **275,** 31505–31513 (2000).

45. Newgard, C. B. *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab.* **9,** 311–326 (2009).

46. Mortimore, G. E. & Mondon, C. E. Inhibition by insulin of valine turnover in liver. Evidence for a general control of proteolysis. *J. Biol. Chem.* **245,** 2375–2383 (1970).

47. Ding, W.-X. *et al.* Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. *Gastroenterology* **139,** 1740–1752 (2010).

48. Rodriguez-Navarro, J. A. *et al.* Inhibitory effect of dietary lipids on chaperone-mediated autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **109,** E705–E714 (2012).

49. Nakahira, K. & Choi, A. M. K. Autophagy: a potential therapeutic target in lung diseases. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **305,** L93–L107 (2013).

50. Yamamoto, A. *et al.* Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct.* **23,** 33–42 (1998).

51. Klionsky, D. J. The molecular machinery of autophagy: unanswered questions. *J. Cell Sci.* **118,** 7–18 (2005).

52. Mizushima, N. Methods for monitoring autophagy. *Int. J. Biochem. Cell Biol.* **36,** 2491–2502 (2004).

53. Bjorkoy, G. *et al.* Chapter 12 Monitoring Autophagic Degradation of p62/SQSTM1. *Methods Enzymol.* **451,** 181–197 (2009).

54. Moscat, J. & Diaz-Meco, M. T. p62 at the Crossroads of Autophagy, Apoptosis, and Cancer. *Cell* **137,** 1001–1004 (2009).

55. Wu, Y.-T. *et al.* Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J. Biol. Chem.* **285,** 10850–10861 (2010).

56. Homewood, C. a, Warhurst, D. C., Peters, W. & Baggaley, V. C. Lysosomes, pH and the anti-malarial action of chloroquine. *Nature* **235,** 50–2 (1972).

57. Kalvari, I. *et al.* iLIR: A web resource for prediction of Atg8-family interacting proteins. *Autophagy* **10,** 913–925 (2014).

58. Mizushima, N., Ohsumi, Y. & Yoshimori, T. Autophagosome Formation in Mammalian Cells. *Cell Struct. Funct.* **27,** 421–429 (2002).

59. Liu, Q. *et al.* Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR. *Cancer Res.* **73,** 2574–2586 (2013).

60. Pause, a *et al.* Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371,** 762–767 (1994).

61. Yue, Z., Jin, S., Yang, C., Levine, A. J. & Heintz, N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15077–82 (2003).

62. Qu, X. *et al.* Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J. Clin. Invest.* **112,** 1809–1820 (2003).

63. Kuma, A. *et al.* The role of autophagy during the early neonatal starvation period. *Nature* **432,** 1032–1036 (2004).

64. Kang, R., Zeh, H. J., Lotze, M. T. & Tang, D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ.* **18,** 571–80 (2011).

65. Suzuki, K. *et al.* The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* **20,** 5971–5981 (2001).

66. Nishida, Y. *et al.* Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* **461,** 654–8 (2009).

67. Bahr, B. A. & Bendiske, J. The neuropathogenic contributions of lysosomal dysfunction. *J. Neurochem.* **83,** 481–489 (2002).

68. Ravikumar, B., Duden, R. & Rubinsztein, D. C. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* **11,** 1107–1117 (2002).

69. Klionsky, D. J., Elazar, Z., Seglen, P. O. & Rubinsztein, D. C. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy* **4,** 849–850 (2008).

70. Pankiv, S. *et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. *J. Biol. Chem.* **282,** 24131–24145 (2007).

71. Kameyama, H., Nakajima, H., Nishitsuji, K. & Mikawa, S. Iowa Mutant Apolipoprotein A-I (ApoA-I Iowa) Fibrils Target Lysosomes. *Nat. Publ. Gr.* 1–11 (2016). doi:10.1038/srep30391

72. Kirkin, V. *et al.* A Role for NBR1 in Autophagosomal Degradation of Ubiquitinated Substrates. *Mol. Cell* **33,** 505–516 (2009).

73. Zhao, J., Zhai, B., Gygi, S. P. & Goldberg, A. L. mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 15790–15797 (2015).

74. Le Guezennec, X. *et al.* Wip1-dependent regulation of autophagy, obesity, and atherosclerosis. *Cell Metab.* **16,** 68–80 (2012).

75. Liao, X. *et al.* Macrophage autophagy plays a protective role in advanced atherosclerosis. *Cell Metab.* **15,** 545–553 (2012).

76. Razani, B. *et al.* Autophagy links inflammasomes to atherosclerotic progression. *Cell Metab.* **15,** 534–544 (2012).

77. Lewis, G. F. & Rader, D. J. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ. Res.* **96,** 1221–1232 (2005).

78. Chatterjee, C. & Sparks, D. L. Extracellular nucleotides inhibit insulin receptor signaling, stimulate autophagy and control lipoprotein secretion. *PLoS One* **7,** (2012).

79. Goffinet, M. *et al.* P2Y13 Receptor Regulates HDL Metabolism and Atherosclerosis In Vivo. *PLoS One* **9,** e95807 (2014).

80. Preiss, D. & Sattar, N. Non-alcoholic fatty liver disease: an overview of prevalence, diagnosis, pathogenesis and treatment considerations. *Clin. Sci.* **115,** 141 LP-150 (2008).

81. Hamaguchi, M. *et al.* The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann. Intern. Med.* **143,** 722–728 (2005).

82. Browning, J. D. *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **40,** 1387–1395 (2004).