Inhibition of ATG3 ameliorates liver steatosis by increasing mitochondrial function

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Conflict of interest

The authors declare that they have no conflicts of interest related to the study.

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Authors' contributions

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Abstract

Background & Aims: Autophagy-related gene 3 (ATG3) is an enzyme mainly known for its actions in the LC3 lipidation process, which is essential for autophagy. Whether it plays a role in lipid metabolism or contributes to nonalcoholic fatty liver disease (NAFLD) remains unknown.

Methods: By performing a liver proteomic analysis from mice with genetic manipulation of hepatic p63, a regulator of fatty acid metabolism, we identified ATG3 as a new target downstream of p63. ATG3 was evaluated in liver samples of people with NAFLD. Further, genetic manipulation of ATG3 in human hepatocyte cell lines, primary hepatocytes and in the liver of mice was performed.

Results: ATG3 expression is induced in the liver of animal models and patients with NAFLD as compared with those without liver disease. Moreover, genetic knockdown of ATG3 in mice and human hepatocytes ameliorates p63- and diet-induced steatosis, while its overexpression increases the lipid load in hepatocytes. The inhibition of hepatic ATG3 improves fatty acid metabolism by reducing c-Jun N-Terminal Protein Kinase 1 (JNK1), which increases sirtuin 1 (SIRT1), carnitine palmitoiltransferase I (CPT1a), and mitochondria function. Hepatic knockdown of SIRT1 and CPT1a blunts the effects of ATG3 on mitochondrial activity. Unexpectedly, these effects are independent of an autophagic action.

Conclusions: Collectively, these findings indicate that ATG3 is a novel protein implicated in the development of steatosis.

Lay summary

We show that autophagy-related gene 3 (ATG3) contributes to the progression of NAFLD in humans and mice. The hepatic knockdown of ATG3 ameliorates the development of NALFD, by stimulating SIRT1, CPT1a and mitochondrial function. Thus, ATG3 is an important gene implicated in steatosis.

Highlights

- ATG3 levels are elevated in the liver of mice and patients with NALFD.
- Inhibition of ATG3 in human hepatocytes and in liver ameliorates steatosis by stimulating SIRT1, CPT1a, and mitochondrial function.
- The hepatic knockdown of SIRT1 or CPT1a blunts the ability of the inhibition of ATG3 to increase mitochondrial activity and to alleviate steatosis.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a major health threat in both developed and developing countries and is a precursor of the more advanced liver diseases, including nonalcoholic steatohepatitis (NASH), liver cirrhosis, and liver cancer. Current, understanding the multiple and complex molecular pathways implicated in NAFLD onset and progression is a major goal.

The transcription factor p63, which belongs to a family comprising p53, p63, and p73 [1], is one of many factors that contributes to the development of liver steatosis from NAFLD. The role of p63 is as a tumor suppressor and in cell maintenance and renewal is well studied [2]; however, we have recently reported that it is also relevant in the control of lipid metabolism [3]. p63 encodes multiple isoforms that can be simplified as being isoforms with an acidic transactivation domain (TA) or without this domain (domain negative, DN). The TAp63α isoform is elevated in the liver of animal models of NAFLD as well as in liver biopsies from obese NAFLD patients. Furthermore, downregulation of p63α in the liver attenuates liver steatosis in diet-induced obese (DIO) mice, while the activation of TAp63α increases hepatic fat content, mediated by the activation of IKKβ and ER stress [3].

A specialized form of autophagy that degrades lipid droplets, termed “lipophagy”, is a major pathway of lipid mobilization in hepatocytes. The levels of lipophagy are elevated in hepatoma cells upon exposure to free fatty acids [4], and lipophagy reduces the fatty acid load in mouse hepatocytes [5]. Its impairment has been associated with the development of fatty liver and insulin resistance [4, 6]; during the activation of hepatic stellate cells, the autophagic flux is increased [7].

Identifying target genes and proteins controlled by p63 under lipid overload is an important step in dissecting the p63 gene network and unmasking new targets involved

in NAFLD. In the present study, we performed proteomics in the liver of mice with genetic knockdown or overexpression of TAp63α to gain insight into novel proteins modulating lipid metabolism in a global and unbiased manner. Strikingly, we found that autophagy-related gene 3 (ATG3) was modified by TAp63α activation and downregulated after p63α inhibition. Further *in vitro* and *in vivo* experiments demonstrated that ATG3 is elevated in several animal models of NAFLD and in the liver of patients with NAFLD, who also show a positive correlation between ATG3 and steatosis grade and NAS score. Genetic overexpression of ATG3 increased the lipid load in hepatocytes, while its repression alleviated TAp63α- and diet-induced steatosis but not steatohepatitis. Unexpectedly, although ATG3 exerted its role in lipid metabolism by regulating SIRT1 and mitochondrial function, it acted independent of any autophagic actions. Collectively, these findings indicate that ATG3 is a novel gene protein implicated in the development of steatosis whose effects mirror those of TAp63α in hepatic lipid accumulation and prevention of steatosis *in vitro* and *in vivo*.

Material and Methods

Animals and diets

Mice experiments were conducted to the standards protocols approved by the Committee at the University of Santiago de Compostela, and mice received the highest levels of humane care. C57BL/6J (8-week-old male) mice were housed in rooms under constant temperature (22 °C), a 12:12 h light/dark cycle and with *ad libitum* access to standard diet (SD) (U8200G10R, SAFE), a choline-deficient high-fat diet (CDHFD) (D05010402; 45 kcal% fat, Research Diets), a methionine- and choline-deficient (MCD) diet (A02082002BR, Research Diets), or a high-fat diet (HFD) (D12492; 60 kcal% fat,

Research Diets) used during specified times and experiments. Food intake and body weight were measured weekly during all experimental phases.

Cohort of human patients with NAFLD

The study population included 33 biopsy-proven NAFLD patients (25 with nonalcoholic fatty liver [NAFL], and 8 with nonalcoholic steatohepatitis [NASH]), and 32 patients with histologically normal liver (NL) (Table S1). Patients consumed <20 g alcohol/day, did not take potentially hepatotoxic drugs, had no analytical evidence of iron overload, and were seronegative for autoantibodies and for hepatitis B/C viruses and human immunodeficiency virus. All participants underwent a liver biopsy during programmed cholecystectomy (Santa Cristina University Hospital, Madrid). Minimal criteria for NASH included the combined presence of grade 1 steatosis, hepatocellular ballooning, and lobular inflammation with or without fibrosis. This study was performed in agreement with the Declaration of Helsinki and with local and national laws. The Human Ethics Committees of the hospital approved the study procedures, and all participants voluntarily signed an informed written consent before inclusion in the study.

Con formato: Resaltar

Results

Hepatic ATG3 is regulated by p63 and increased in diet-induced animal models of steatosis

We previously found that hepatic overexpression of TAp63α isoform induces steatosis, while inhibition of p63α ameliorates diet-induced steatosis [3]. We now analyzed these two animal models, in which hepatosteatosis was modulated by p63 genetic manipulation, by proteomics (Fig. 1A-1D). Volcano plots shows that multiple changes in hepatic protein levels were triggered by the manipulation of p63 (Fig. 1A-B). We

specifically searched for proteins oppositely expressed in mice following the overexpression or knockdown of p63; we found that p63 positively-regulated 43 proteins, and negatively-regulated 2 proteins. Analyzing this protein set (of changes induced by p63 in liver steatosis) by a Reactome Enrichment revealed a significant overrepresentation (44%) of metabolism-related proteins, with mitochondria identified as the most affected cell component (Fig. 1C).

Next, we studied the expression of this set of proteins through a heatmap of proteins up- or downregulated as compared to the control group. Interestingly, several proteins involved in metabolism, and particularly lipid metabolism, showed a strong positive regulation by p63 (Fig. 1D). Further, we found marked changes in ATG3 (Fig. 1D). Given the relevant role of lipophagy in regulating fatty acid metabolism, and the fact that ATG3 has not been previous related to liver function, we selected this candidate for further investigation. We corroborated that ATG3 mRNA expression and protein levels were upregulated when TAp63α was induced in the liver (Fig. 1E). In line with this, both ATG3 mRNA and protein levels were reduced when hepatic p63α was inhibited in diet-induced obese (DIO) mice (Fig. 1E). Thus, ATG3 was consistently regulated by p63 in gain- and loss-of-function models.

Next, we evaluated the expression of ATG3 in livers from mice fed a HFD or a choline-deficient, high-fat diet (CDHFD) for 6 or 52 weeks. In all conditions, ATG3 mRNA was elevated compared as compared to livers from mice fed a standard diet (SD) (Fig. 1F). These results indicate that ATG3 expression is consistently increased in conditions of steatosis and steatohepatitis.

ATG3 is increased in the liver of patients with nonalcoholic fatty liver (NAFL)

Since ATG3 levels were increased in NAFLD and NASH preclinical models, we next evaluated its expression in liver biopsies from people with NAFLD (NASH or NAFL) or not diseased (Table S1). ATG3 mRNA expression was significantly higher in liver from patients with NAFL than from those with NASH or not diseased (Fig. 2A). Further, ATG3 positively correlated with steatosis grade (Fig. 2B), NAS score (Fig. 2C), and fatty liver index (Fig. 2D). Further, we found that ATG3 levels were similarly e in in non-obese (BMI<30) and obese (BMI>30) patients (Fig. 2E), with no correlation to BMI (Fig. 2F). Altogether, these results indicate that higher ATG3 levels are present in patients with NAFL that are not influenced by adiposity.

Comentado [NPR1]: add new patients

ATG3 increases lipid content and reduces β-oxidation in human hepatocytes

THLE2 cells treated with oleic acid (OA) presented increased ATG3 mRNA expression (Fig. 3A) and lipid content (Fig. 3B). However, inhibition of ATG3 reduced OA-induced lipid storage (Fig. 3B and Fig. S1A). As previously reported [3], TAp63α overexpression increased the lipid droplets in hepatocytes, but this effect was blunted when ATG3 was silenced (Fig. 3C). The transfection efficiency of the plasmid for p63, and siRNA for ATG3, were corroborated (Fig. S1B). In a gain-of-function experiment using a plasmid overexpressing ATG3 (Fig. S1C), we found that ATG3 increased the lipid content in THLE2 cells (Fig. 3D). Importantly, performing the loss- and gain-of-function experiments in HepG2 cells gave the same results: the OA-induced lipid content was reduced in cells in which ATG3 was silenced (Fig. 3E), while ATG3 overexpression augmented the lipid load (Fig. S1D and Fig. 3F). We next measured the fatty acid β-oxidation in hepatocytes by using ^{14}C-palmitate and found that the overexpression of ATG3 decreased the β-oxidation rate (Fig. 3G), confirming that ATG3 modulates fatty acid storage.

ATG3 regulates mitochondrial activity in hepatocytes

The overexpression of ATG3 in HepG2 cells led to a decrease in the oxygen consumption rate (Fig. 4A). More specifically, we observed a decrease in the basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare capacity, and non-mitochondrial oxygen consumption at 24-h after ATG3 overexpression, as compared to control cells (Fig. 4A). These results were consistent with reduced protein levels of the mitochondrial complexes II (II-SDHB), III, and V (Fig. 4B). No changes were observed in protein levels of fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), or carnitine palmitoyl transferase 1a (CPT1a) (Fig. 4C). However, the induction of ATG3 led to decreased levels of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and sirtuin 1 (SIRT1) (Fig. 4D), two proteins that promote mitochondrial function and fatty acid oxidation [8]. These results were corroborated in isolated hepatocytes, where the overexpression of ATG3 (Fig. S1E) also reduced protein levels of SIRT1 and PGC1α (Fig. 4E).

ATG3 inhibition in HepG2 cells increased the oxygen consumption rate, with significant stimulation of basal respiration, ATP-linked respiration, proton leak, maximal respiration, and non-mitochondrial oxygen consumption (Fig. 4F). This was in agreement with increased levels of mitochondrial complexes I, II (II-SDHB), and IV (IV-COX II) (Fig. 4G), and the upregulation of CPT1a, PGC1α and SIRT1 levels after silencing ATG3 (Fig. 4H, 4I). The inhibition of ATG3 (Fig. S1F) also triggered protein levels of SIRT1 and PGC1α in primary hepatocytes (Fig. 4J).

All results obtained in HepG2 cells were also corroborated in THLE2 cells: ATG3 overexpression led to a decrease in the oxygen consumption rate and in the levels of mitochondrial complexes I and II, as well as of PGC1α and SIRT1 (Fig. S2A-S2D).

Conversely, ATG3 silencing in THLE2 cells increased the oxygen consumption rate and the protein levels of mitochondrial complexes I, II, and IV, CPT1a, PGC1α, and SIRT1 (Fig. S2E-S2H).

Inhibition of hepatic ATG3 ameliorates CDHFD-induced steatosis

After demonstrating *in vitro* that ATG3 regulates lipid content and mitochondrial activity, we investigated the *in vivo* relevance of these findings. For this, mice were fed a choline-deficient, high-fat diet (CDHFD; 45% kcal from fat) for 16 weeks; at week 8, mice were injected in the tail vein with a lentivirus encoding for a scrambled shRNA or shRNA against ATG3. No significant differences in body weight or food intake were observed over the 16-week period (Fig. 5A-5B). ATG3 protein levels in liver were inhibited after the lentiviral injection (Fig. 5C). Although the liver mass was not affected by the knockdown of hepatic ATG3 (Fig. 5D), serum levels of AST and ALT were significantly lower as compared to the control group (Fig. 5E). Circulating TG and NEFAs were not affected (Table S2). Consistent with this, hepatic triglyceride content and Oil Red O (ORO) staining in liver sections of mice after ATG3 knockdown showed a lower amount of lipids, with no differences in fibrotic content (Sirius Red staining) (Fig. 5F), indicating hepatic function improvement. Furthermore, despite unchanged levels of pACC, ACC, FAS, and LPL, the inhibition of hepatic ATG3 increased protein levels of CPT1a, PGC1α, and SIRT1 (Fig. 5G-5H), as well as the mitochondrial complexes I (I-NDUFB8) and II (II-SDHB) (Fig. 5I) and the activity of mitochondrial complex II (Fig. 5J), as compared to the control group. Functional assays revealed an increased β-oxidation rate in liver of mice with ATG3 knockdown (Fig. 5K). These effects were independent of cellular proliferation since the expression of cyclin D1 (CCD1) and cyclin D2 (CCD2) and the immunostaining of Ki67, all of them surrogate

markers of proliferation, were unchanged between the two groups (Fig. S3A-S3B). The knock down of ATG3 did not affect mRNA expression of fibrotic markers such as collagen alpha 1, collagen alpha 2, collagen III or αSMA (Fig. S4A). Overall, these results indicate that the liver-specific ATG3 inhibition exerts a beneficial effect in alleviating diet-induced steatosis.

Inhibition of hepatic ATG3 ameliorates TAp63α-induced steatosis

We next evaluated the effects of ATG3 inhibition in TAp63α-induced steatosis in mice. For this, mice fed a SD were injected via the tail vein with AAV8-TAp63α; at week 8, they were injected with either AAV8-shATG3 (to knockdown ATG3) or AAV8-GFP (as a control) and maintained on the same diet for another 8 weeks. Body weight (Fig. 6A) and food intake (Fig. 6B) remained unchanged during the experiment. Administration of AAV8-TAp63α or lentivirus-shATG3 caused an increase of p63 or a decrease of ATG3, respectively (Fig. 6C). Even though liver mass was unchanged (Fig. 6D), significantly higher circulating levels of AST and ALT levels were observed after TAp63α overexpression as compared to the control group; ATG3 inhibition reduced the AST and ALT levels (although that of ALT was not statistically significant; $p = 0.0513$) (Fig. 6E). Serum TG and NEFAs were similar among all groups (Table S3).

As previously reported [3], the overexpression of TAp63α caused liver steatosis, an effect that was blunted by ATG3 knockdown (Fig. 6F). At a biochemical level, AAV8-TAp63α-treated mice had evaluated levels of the FAS protein, unaltered levels of pACC, ACC, and LPL, and reduced levels of CPT1a, PGC1α, and SIRT1 (Fig. 6G). Genetic inhibition of ATG3 restored the levels of CPT1a, PGC1α, and SIRT1 to those of the control group (Fig. 6G-6H). Finally, hepatic overexpression of TAp63α decreased all mitochondrial complexes levels, while ATG3 inhibition normalized the liver levels

of complexes I and II (Fig. 6I). In these groups, mRNA expression of fibrotic markers such as collagen alpha 2, collagen III or αSMA remained unaltered, although collagen alpha 1 was elevated by TAp63α and reduced when ATG3 was inhibited (Fig. S4B). Taken together, these results show that ATG3 modulates the *in vivo* effects of TAp63α on lipid metabolism in the liver.

Knockdown of hepatic ATG3 does not affect fibrosis

We next evaluated whether ATG3 knockdown in liver alleviates fibrosis by injecting mice into the tail vein with a lentivirus encoding a shRNA-ATG3 or shRNA-scrambled (as control); mice were fed a SD for 4 week and a methionine- and choline-deficient (MCD) diet for another 4 weeks, and were sacrificed at week 8 [9]. Body weight was similar between ATG3-knockdown and control mice (Fig. S5A). As expected, ATG3-knockdown significantly reduced ATG3 mRNA levels as compared to control mice (Fig. S5B). No differences were observed between ATG3-knockdown and control mice for liver mass (Fig. S5C), serum levels of AST or ALT (Fig. S5D), inflammatory or profibrotic markers (e.g., collagen and ACTA2) (Fig. S5E), or ORO or Sirius Red staining (Fig. S5F). To note, ATG3 was highly expressed in isolated hepatic stellate cells (HSCs) compared to hepatocytes or Kupffer cells (Fig. S6A) and in LX2 cells (a human hepatic cell line) compared to THLE2 and HepG2 cells (Fig. S6B). However, the treatment of LX2 cells with TGFβ did not change ATG3 expression, and the TGFβ-induced expression of fibrotic markers was not affected after silencing ATG3 (Fig. S6C-S6D). These *in vivo* and *in vitro* data indicate that ATG3 knockdown did not exert any beneficial effects on diet-induced fibrosis.

ATG3 requires SIRT1 for modulating lipid metabolism and mitochondrial activity

As our previous *in vitro* and *in vivo* results indicated that SIRT1 was negatively regulated by ATG3, we next assessed the functional relevance of SIRT1 as a modulator of the actions of ATG3. Similar to the results shown in Fig. 3B, silencing ATG3 in HepG2 cells ameliorated OA-induced lipid load (Fig. S7A). However, when SIRT1 was also silenced, siATG3 failed to decrease OA-induced lipid content (Fig. S7A). These results were also corroborated in THLE2 cells co-transfected with siATG3 and siSIRT1 (Fig. S7B). Moreover, the higher oxygen consumption rate induced by siATG3 in THLE2 cells was also reversed after silencing SIRT1 (Fig. S7C). In addition, ATG3-induced lipid content was blunted by resveratrol (1 ug), a pharmacological activator of SIRT1 in both THLE2 cells (Fig. S8A) and primary hepatocytes (Fig. S8B). This is in line with the effects at mitochondrial level in isolated hepatocytes, because ATG3 reduced OCR while resveratrol blunted this effect (Fig. S8C). In line with these results, silencing ATG3 increased SIRT1 activity and reduced acetylated levels of PGC1α, while its overexpression reduced SIRT1 activity in isolated hepatocytes (Fig. S9A-S9C).

Next, we evaluated the *in vivo* relevance of SIRT1 as a mediator of the effects of ATG3. For this, mice fed a CDHFD were injected with a lentivirus with shRNA-scrambled (as a control) or shRNA-ATG3; at week 4, a second lentiviral vector with shRNA-scrambled or shRNA-SIRT1 was injected; mice were sacrificed at week 8. Body weight and food intake remained unchanged between the four groups (Fig. 7A-7B). ATG3 and SIRT1 levels were reduced in liver after the injections of shRNA-ATG3 and shRNA-SIRT1 respectively (Fig. 7C). Even though liver weight was not affected, AST levels were reduced after ATG3 knockdown; this reduction was abolished when both ATG3 and SIRT1 were silenced (Fig. 7D-7E). Moreover, the reduced lipid content and hepatic

triglycerides in mice with ATG3 knockdown was blunted when SIRT1 was subsequently reduced (Fig. 7F). Similar to the findings described above, the knockdown of hepatic ATG3 caused an increase in CPT1a protein levels as well as a higher activity of complex II, but these effects were blocked with co-silencing ATG3 and SIRT1 (Fig. 7G-7I). On the other hand, the expression of fibrotic markers remained unchanged between the groups (Fig. S4C). Overall, our *in vitro* and *in vivo* data suggest that SIRT1 mediates the effects of ATG3 on hepatic fatty acid metabolism.

ATG3 regulates protein levels of SIRT1 via JNK1

A key mechanism for regulating SIRT1 protein levels is c-Jun N-terminal kinase 1 (JNK1), whose activation induces SIRT1 protein degradation [10]; and JNK2 that induces SIRT1 protein stability [11]. Therefore, we measured JNK1 and JNK2 protein levels in the liver of mice fed a CDHFD where ATG3 was knocked down in the liver and detected that the inhibition of ATG3 reduced hepatic levels of JNK1 but not JNK2 (Fig. S10A). Consistent with *in vivo* data, silencing ATG3 in THLE2 cells reduced JNK1 protein levels (Fig. S10B), while the induction of ATG3 increased JNK1 (Fig. S10C). We next silenced ATG3 in primary hepatocytes and found reduced JNK1 (Fig. S10D), while its overexpression increases JNK1 protein levels (Fig.S10E). Finally, the overexpression of ATG3 in isolated hepatocytes increased lipid content, reduced OCR and SIRT1 activity; while the co-treatment with a JNK1 inhibitor named SP600125 blunted ATG3-induced actions (Fig. S10F-10H). Although a previous report found that modulation of ATG3 in the adipose tissue activates the Nrf2/Keap1 signaling pathway in the liver [12], the expression of Nrf2 and Keap1 in the WAT and liver of mice fed a CD-HFD where ATG3 was knocked down remained unaltered (Fig. S11A-11C). Similar results were found in the WA of mice where Tap63α was manipulated (Fig.

S11D) and in isolated hepatocytes after silencing ATG3 (Fig. S11E). Altogether, these results indicate that ATG3 regulates SIRT1 activity and ultimately lipid content in hepatocytes through JNK1.

ATG3 requires CPT1a for modulating lipid metabolism and mitochondrial activity

As our results indicated that the genetic disruption of hepatic ATG3 increased the levels of CPT1a (Fig. 6G-7G), we assessed the *in vivo* importance of CPT1a to regulate the actions of ATG3. CPT1a floxed mice (fed a CDHFD) were injected with AAV8-GFP (control) or AAV8-Cre; at and week 4, groups were injected with either scrambled shRNA-scrambled (control) or shRNA-ATG3; mice were sacrificed at week 8. Body weight remained unchanged among the three groups (Fig. S12A). CPT1a and ATG3 levels in liver were reduced after AAV8-Cre or shRNA-ATG3 treatment, respectively (Fig. S12B). Liver mass was not affected, but AST and ALT levels were lower after ATG3 knockdown; these levels returned to baseline when both ATG3 and CPT1a were silenced (Fig. S12C-12D). Moreover, the reduced lipid and hepatic triclyceride content in mice with ATG3 downregulation was blunted when CPT1a was also reduced (Fig. S12E). As before, the knockdown of hepatic ATG3 increased the levels of complex II and IV, but these effects were blocked by co-silencing ATG3 and CPT1a (Fig. S12F). The expression of fibrotic markers was not altered between these groups (Fog. S12G). Overall, these results suggest that CPT1a mediates the effects of ATG3 on hepatic fatty acid metabolism.

ATG3 regulates hepatic lipid content in an autophagy-independent manner

To further explore whether ATG3 exerts its hepatic actions via an autophagy-dependent process, we used both THLE2 and HepG2 hepatocyte cell lines to manipulate ATG3.

Upon induction of autophagy, microtubule-associated protein 1 light chain 3 (LC3) is lipidated, and this LC3-phospholipid conjugate (LC3-II) is recruited to autophagosomal membranes and fuses with lysosomes to form autolysosomes, which degrade intra-autophagosomal components and LC3-II [13]. In other words, LC3-II formation depends on the autophagy conjugation systems [14, 15]. As expected, silencing ATG3 in HepG2 cells decreased LC3-II levels (Fig. 8A). We then monitored the autophagic flux by analyzing LC3-II turnover by Western blot, in the presence or absence of the inhibitor of lysosome-mediated proteolysis, chloroquine (CQ). CQ administration induced the expected LC3-II accumulation (Fig. 8A). Hepatocytes treated with OA and transfected with siATG3 stored fewer lipids, and CQ treatment increased the lipid content (Fig. 8B). Strikingly, however, if cells overexpressed ATG3, inhibition of autophagy by CQ did not affect ATG3-induced lipid droplets in THLE2 or HepG2 cells (Fig. 8C-8D). Thus, these results suggested that the lipid accumulation induced by ATG3 is independent of autophagy. This was confirmed *in vivo*, as ATG3 knockdown in our two animal models of steatosis (e.g., induced by TAp63α or CDHFD) reduced LC3-II accumulation but did not affect protein and mRNA levels of well-established markers of autophagy. such as ATG5, ATG7, and p62 (Fig. 8E-8F). Overall, these data indicate that the effects of ATG3 on lipid content are independent of its autophagic action. This is also supported by the fact that autophagy contributes to the turnover of lipid droplets, and thereby to an increased fatty acid oxidation, a process named lipophagy [16, 17]. Thus, we see that the overexpression of ATG3 reduced beta oxidation, while the inhibition of ATG3 stimulated beta oxidation, reinforcing the hypothesis that ATG3-mediated effects are autophagy-independent.

Discussion

In this work, we identified for first the time the role of ATG3 on fatty acid metabolism and its implications in the development of NAFLD. ATG3 is elevated in the liver of animal models as well as patients with NAFLD. Moreover, *in vitro* and *in vivo* genetic functional studies indicated that the overexpression of ATG3 favors lipid deposition, while its silencing alleviates steatosis induced by OA, p63, or diet. These novel findings further our knowledge about the molecular mechanisms involved in liver steatosis.

Autophagy has a well-established role in hepatic lipid metabolism, insulin sensitivity, and cellular injury, suggesting different potential mechanistic roles for autophagy in NAFLD [4, 18-21]. Our initial hypothesis was that ATG3, an enzyme that catalyzes the LC3 lipidation process essential for autophagocytosis [22], exerts its effects through an autophagic action, in particular given that p63 (as well as its family co-members p53 and p73) can induce autophagy [23-25]. Contrary to our expectations, we found that ATG3 modulates lipid metabolism via non-autophagic mechanisms but in a JNK1- and SIRT1-dependent manner. ATG3 increases JNK1 protein levels, which are known to induce SIRT1 protein degradation [10]. The lower protein levels of SIRT1, concomitant to its inhibited activity in human hepatocytes and murine liver reduced fatty acid oxidation and mitochondrial function, leading to a higher lipid load. In agreement with this, the inhibition of ATG3 decreased JNK1 and induced protein levels of SIRT1 and mitochondrial function, ultimately decreasing lipid content. Indeed, many reports have demonstrated a key role of SIRT1 in stimulating mitochondrial function [26] and fatty acid oxidation [27, 28], which contribute to attenuating hepatic steatosis [29]. Our findings show that the protection against steatosis induced by the inhibition of ATG3 was lost when SIRT1 and CPT1a were also suppressed in cells and mice, indicating a functional role for SIRT1 and CPT1a in the anti-steatotic action, which was not

associated with changes in autophagic flux. Our results in the liver are opposite to the actions of ATG3 in adipose tissue, where depletion of ATG3 triggered the Nrf2/Keap1 pathway in the liver [12]. However, it is important to note that mice lacking ATG3 in adipose tissue displayed increased adipose tissue inflammation, systemic insulin resistance and accumulation of lipid peroxides, which are known to cause a marked impact in liver metabolism. On the other hand, it has also been reported that there is a tissue-specific autophagy response [30-32].

It is important to highlight that most, if not all, components of the molecular machinery for autophagy mediate effects that do not depend on lysosomal degradation of autophagy substrates [33, 34]. As a consequence, numerous, distinct non-autophagic biological functions for different ATG proteins have been reported, including cell survival, modulation of cellular transport, secretory processes, signaling, transcriptional/translational responses, and membrane reorganization [33, 34]. More specifically, ATG3 has been shown to modulate in an autophagic-independent manner diverse cellular processes such as phagocytosis, secretion and exocytosis and cell proliferation [34]. Our study indicating that the effects of ATG3 are modulated by JNK1, SIRT1 and CPT1a points to another, previously-undescribed non-autophagic action. Importantly, our results also propose that different ATGs have opposite actions in the liver, at least in terms of fatty liver disease. While we demonstrated that inhibition of ATG3 ameliorates NAFLD, liver-specific deletion of ATG5, ATG7, or ATG14 exacerbate the sensitivity of mice to develop NAFLD in response to a HFD [35-38]. However, the loss of autophagic factors does not always involve negative actions. For example, mice lacking ATG5 specifically in the liver are protected against acetaminophen-induced liver injury [39]. These paradoxical actions of autophagy might occur after long periods in response to cellular compensatory mechanisms.

The ameliorated steatosis after ATG3 knockdown, and subsequent increased SIRT1 and CPT1a protein levels, was associated with stimulated mitochondrial function. The link between ATG3 and mitochondrial homeostasis has been studied before in the context of cell death, pluripotency acquirement, and maintenance of embryonic stem cells [40, 41]. Although those studies suggested that the effects of ATG3 on mitochondria are dependent on autophagy, disruption of the ATG3/ATG12 conjugation does not affect starvation-induced autophagy [41], supporting an autophagic-independent role of ATG3.

In addition to the unexpected mechanism used by ATG3, another important feature is that ATG3 levels are elevated in liver of different animal models and patients with steatosis, but it is not increased in patients with NASH and that its genetic inhibition (in mice) only alleviated steatosis but did not alter fibrosis. Although ATG3 does not seem to be relevant in late stages of NAFLD, it is important to take into account that a fraction of people with NAFL will develop NASH or advanced liver fibrosis [42-44], implying that specific signaling mechanisms are involved in liver disease progression. Thus, understanding mechanism dysregulated in early stages of NAFLD might help to identify potential biomarkers.

In summary, our findings show for the first time that: a) ATG3 expression increased in the liver of mouse models and of people with NAFLD, who also show a positive correlation between ATG3 with steatosis grade and NAS score; b) ATG3 overexpression induced the lipid load, and its inhibition in the liver ameliorated TAp63α- and diet-induced liver steatosis; c) this anti-steatotic action is mediated by reduction of JNK1 and increased levels of SIRT1, CPT1a, fatty acid oxidation, and mitochondrial function, in an autophagic-independent manner. Overall, our results point towards ATG3 as a novel molecule implicated in the development of steatosis.

REFERENCES

[1] Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305-316.

[2] Costanzo A, Pediconi N, Narcisi A, Guerrieri F, Belloni L, Fausti F, et al. TP63 and TP73 in cancer, an unresolved "family" puzzle of complexity, redundancy and hierarchy. FEBS Lett 2014;588:2590-2599.

[3] Porteiro B, Fondevila MF, Delgado TC, Iglesias C, Imbernon M, Iruzubieta P, et al. Hepatic p63 regulates steatosis via IKKbeta/ER stress. Nat Commun 2017;8:15111.

[4] Amir M, Czaja MJ. Autophagy in nonalcoholic steatohepatitis. Expert Rev Gastroenterol Hepatol 2011;5:159-166.

[5] Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. Nature 2009;458:1131-1135.

[6] Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab 2010;11:467-478.

[7] Thoen LF, Guimaraes EL, Dolle L, Mannaerts I, Najimi M, Sokal E, et al. A role for autophagy during hepatic stellate cell activation. J Hepatol 2011;55:1353-1360.

[8] Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. EMBO J 2007;26:1913-1923.

[9] Fondevila MF, Fernandez U, Gonzalez-Rellan MJ, Da Silva Lima N, Buque X, Gonzalez-Rodriguez A, et al. The L-alpha-lysophosphatidylinositol/GPR55 system induces the development of non-alcoholic steatosis and steatohepatitis. Hepatology 2020.

[10] Gao Z, Zhang J, Kheterpal I, Kennedy N, Davis RJ, Ye J. Sirtuin 1 (SIRT1) protein degradation in response to persistent c-Jun N-terminal kinase 1 (JNK1) activation contributes to hepatic steatosis in obesity. J Biol Chem 2011;286:22227-22234.

[11] Ford J, Ahmed S, Allison S, Jiang M, Milner J. JNK2-dependent regulation of SIRT1 protein stability. Cell Cycle 2008;7:3091-3097.

[12] Cai J, Pires KM, Ferhat M, Chaurasia B, Buffolo MA, Smalling R, et al. Autophagy Ablation in Adipocytes Induces Insulin Resistance and Reveals Roles for Lipid Peroxide and Nrf2 Signaling in Adipose-Liver Crosstalk. Cell Rep 2018;25:1708-1717 e1705.

[13] Nakatogawa H. Mechanisms governing autophagosome biogenesis. Nat Rev Mol Cell Biol 2020;21:439-458.

[14] Tsuboyama K, Koyama-Honda I, Sakamaki Y, Koike M, Morishita H, Mizushima N. The ATG conjugation systems are important for degradation of the inner autophagosomal membrane. Science 2016;354:1036-1041.

[15] Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M. Atg8 family LC3/GABARAP proteins are crucial for autophagosome-lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. J Cell Biol 2016;215:857-874.

[16] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451:1069-1075.

[17] Martinez-Lopez N, Singh R. Autophagy and Lipid Droplets in the Liver. Annu Rev Nutr 2015;35:215-237.

[18] Hazari Y, Bravo-San Pedro JM, Hetz C, Galluzzi L, Kroemer G. Autophagy in hepatic adaptation to stress. J Hepatol 2020;72:183-196.

[19] Ueno T, Komatsu M. Autophagy in the liver: functions in health and disease. Nat Rev Gastroenterol Hepatol 2017;14:170-184.

[20] Madrigal-Matute J, Cuervo AM. Regulation of Liver Metabolism by Autophagy. Gastroenterology 2016;150:328-339.

[21] González-Rodríguez A, Mayoral R, Agra N, Valdecantos MP, Pardo V, Miquilena-Colina ME, et al. Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD. Cell Death Dis 2014;5:e1179.
[22] Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell 2008;19:2092-2100.
[23] Kenzelmann Broz D, Spano Mello S, Bieging KT, Jiang D, Dusek RL, Brady CA, et al. Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. Genes Dev 2013;27:1016-1031.
[24] Kouwenhoven EN, van Heeringen SJ, Tena JJ, Oti M, Dutilh BE, Alonso ME, et al. Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. PLoS Genet 2010;6:e1001065.
[25] Napoli M, Flores ER. The family that eats together stays together: new p53 family transcriptional targets in autophagy. Genes Dev 2013;27:971-974.
[26] Tang BL. Sirt1 and the Mitochondria. Mol Cells 2016;39:87-95.
[27] Gillum MP, Erion DM, Shulman GI. Sirtuin-1 regulation of mammalian metabolism. Trends Mol Med 2011;17:8-13.
[28] Nogueiras R, Habegger KM, Chaudhary N, Finan B, Banks AS, Dietrich MO, et al. Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. Physiol Rev 2012;92:1479-1514.
[29] Li Y, Wong K, Giles A, Jiang J, Lee JW, Adams AC, et al. Hepatic SIRT1 attenuates hepatic steatosis and controls energy balance in mice by inducing fibroblast growth factor 21. Gastroenterology 2014;146:539-549 e537.
[30] Chapin HC, Okada M, Merz AJ, Miller DL. Tissue-specific autophagy responses to aging and stress in C. elegans. Aging (Albany NY) 2015;7:419-434.
[31] Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem 2007;282:18573-18583.
[32] Singh R. Autophagy and regulation of lipid metabolism. Results Probl Cell Differ 2010;52:35-46.
[33] Subramani S, Malhotra V. Non-autophagic roles of autophagy-related proteins. EMBO Rep 2013;14:143-151.
[34] Galluzzi L, Green DR. Autophagy-Independent Functions of the Autophagy Machinery. Cell 2019;177:1682-1699.
[35] Hammoutene A, Biquard L, Lasselin J, Kheloufi M, Tanguy M, Vion AC, et al. A defect in endothelial autophagy occurs in patients with non-alcoholic steatohepatitis and promotes inflammation and fibrosis. J Hepatol 2020;72:528-538.
[36] Xiong X, Tao R, DePinho RA, Dong XC. The autophagy-related gene 14 (Atg14) is regulated by forkhead box O transcription factors and circadian rhythms and plays a critical role in hepatic autophagy and lipid metabolism. J Biol Chem 2012;287:39107-39114.
[37] Liu K, Zhao E, Ilyas G, Lalazar G, Lin Y, Haseeb M, et al. Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization. Autophagy 2015;11:271-284.
[38] Ni HM, Woolbright BL, Williams J, Copple B, Cui W, Luyendyk JP, et al. Nrf2 promotes the development of fibrosis and tumorigenesis in mice with defective hepatic autophagy. J Hepatol 2014;61:617-625.
[39] Ni HM, Boggess N, McGill MR, Lebofsky M, Borude P, Apte U, et al. Liver-specific loss of Atg5 causes persistent activation of Nrf2 and protects against acetaminophen-induced liver injury. Toxicol Sci 2012;127:438-450.
[40] Liu K, Zhao Q, Liu P, Cao J, Gong J, Wang C, et al. ATG3-dependent autophagy mediates mitochondrial homeostasis in pluripotency acquirement and maintenance. Autophagy 2016;12:2000-2008.

[41] Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, et al. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell 2010;142:590-600.
[42] McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. J Hepatol 2015;62:1148-1155.
[43] Adams LA, Ratziu V. Non-alcoholic fatty liver - perhaps not so benign. J Hepatol 2015;62:1002-1004.
[44] Pais R, Charlotte F, Fedchuk L, Bedossa P, Lebray P, Poynard T, et al. A systematic review of follow-up biopsies reveals disease progression in patients with non-alcoholic fatty liver. J Hepatol 2013;59:550-556.

Fig. legends

Fig. 1. Hepatic ATG3 is upregulated in different mouse models of NAFLD. (A, B) Volcano plots of hepatic protein expression determined by LC-MS/MS proteomics of (A) mice fed a standard diet (SD), with TAp63α overexpressed specifically in liver, as compared to the control group (n = 3 per group). Upregulated proteins are indicated in blue, and downregulated proteins, in brown; or **(B)** mice fed a choline deficient high-fat diet (CDHFD) for 12 weeks, with p63α downregulated specifically in liver, as compared to the control group (n = 3 per group). Upregulated proteins are indicated in orange, and downregulated, in violet. Values obtained for ATG3 are represented in the bar graph. **(C)** Reactome pathway classification of deregulated proteins in liver of mice from (A) and (B), showing the number of proteins included in each category and the associated false discovery rate (FDR). The same proteins were also classified according to the cellular component using FunRich tool. **(D)** Heatmap representation of the levels of the individual proteins differentially expressed in the groups from (A) and (B). **(E)** mRNA levels of p63 and ATG3, as well asATG3 protein levels, in mice in the conditions as in (A) and (B). (n = 4 per group). **(F)** mRNA levels of ATG3 in liver of mice fed a (SD), a high-fat diet (HFD), or CDHFD (n = 9–14 per group). HPRT and GAPDH were used to normalize mRNA and protein levels, respectively. Lines indicate splicing in the same gel. Data are presented as mean ± standard error of the mean (SEM); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, using a Student's *t*-test.

Fig. 2. ATG3 is increased in the liver of patients with NAFL. (A) ATG3 mRNA expression in the liver of patients without NAFLD (normal, NL) or with NAFL or NASH. Data are presented as mean ± standard deviation (SD). **(B)** Correlation between ATG3 and steatosis grade. **(C)** Correlation between ATG3 and NAS score. **(D)**

Correlation between ATG3 and fatty liver index. **(E)** ATG3 mRNA expression in the liver of non-obese *versus* obese patients with NAFLD. **(F)** Correlation between ATG3 and body mass index. Data are presented as mean ± SD.

Comentado [MFF2]: Please, add:
1) Number of samples.
2)statistical analysis (t student, anova and pearson tests?)

Fig. 3. ATG3 regulates lipid accumulation in two human hepatic cell lines. (A) ATG3 mRNA levels in THLE2 cells treated with oleic acid (OA; 1 mM) for 24 h (n = 6 per group). **(B–F)** Representative oil Red O (ORO) staining of (B) THLE2 cells with siATG3 treated with OA or vehicle (n = 6 per group); (**C**) TAp63α-upregulated THLE2 cells after ATG3 silencing (n = 15 per group); and (**D**) THLE2 cells with overexpression of ATG3 (n = 12 per group). **(E, F)** Representative ORO staining of HepG2 cells with ATG3 downregulated (E) or ATG3 overexpressed (F), treated with oleic acid or vehicle for 24 h (n = 4–7 per group). **(G)** Palmitate oxidation rate (partial and complete oxidation) in HepG2 cells overexpressing ATG3. The n represents the number of independent experiments. Data are presented as mean ± standard error mean (SEM); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, using a Student's *t*-test (A) (D) (F) (G) or one-way ANOVA followed by a Newman-Keuls Multiple Comparison Test (B) (C) (E).

Fig. 4. ATG3 modulates mitochondrial function in hepatocytes. HepG2 cells overexpressing ATG3 were analyzed for **(A)** oxygen consumption rate (OCR), **(B)** protein levels of OXPHOS complex subunits I–V, **(C)** lipid metabolism markers, and **(D)** protein levels of PGC1α and SIRT1 (n = 6 per group). **(E)** PGC1α and SIRT1 protein levels of primary cultures of hepatocytes overexpressing ATG3 (n=5 per group). HepG2 cells downregulating ATG3 were analyzed for **(F)** OCR, **(G)** protein levels of OXPHOS complex subunits I–V, **(H)** lipid metabolism markers, and **(I)** protein levels of PGC1α and SIRT1 (n = 5–6 per group). **(J)** PGC1α and SIRT1 protein levels of

primary cultures of hepatocytes downregulating ATG3 (n=6 per group). The n represents the number of independent experiments. GAPDH was used to normalize protein levels. Dividing lines indicate splicing in the same gel. Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, using a Student's *t*-test.

Fig. 5. Liver-specific downregulation of ATG3 ameliorates NAFLD induced by CDHFD. **(A)** Cumulative body weight change, **(B)** food intake, **(C)** hepatic ATG3 protein levels, and **(D)** liver mass of mice fed CDHFD for 16 weeks, with tail vein injection (TVI) of lentivirus of shRNA ATG3 or shRNA–scrambled at week 8, as indicated. **(E)** Serum levels of AST and ALT. **(F)** Representative microphotographs of Hematoxylin and Eosin (upper panel), Oil Red O staining (middle panel) and Sirius Red staining (lower panel) of liver sections. Lipids in Oil Red O stained sections (red area) and collagen deposition in Sirius Red stained sections (pink area) were quantified using ImageJ. Hepatic triglycerides content was also directly measured. **(G)** Hepatic protein levels of lipid metabolism markers. **(H)** Liver proteins levels of PGC1α and SIRT1. **(I)** Liver protein levels of OXPHOS complex subunits I–V. **(J)** Complex II activity in liver. **(K)** Hepatic palmitate oxidation rate (partial and complete oxidation). GAPDH and HPRT were used to normalize protein and mRNA levels, respectively. Dividing lines indicate splicing in the same gel. Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, using a Student's *t*-test (n = 7–15 per group).

Fig. 6. Liver-specific downregulation of ATG3 ameliorated NAFLD induced by TAp63α. **(A)** Cumulative body weight change, **(B)** food intake, **(C)** hepatic levels of TAp63α mRNA and ATG3 protein, and **(D)** liver mass of mice fed a SD for 16 weeks, with TVI of AAV8–TAp63α or AAV8–GFP at week 1, and a second TVI of lentivirus

encoding the shRNA-ATG3 or shRNA-scrambled control at week 8, as indicated. **(E)** Serum levels of AST and ALT. **(F)** Representative microphotographs of Hematoxylin and Eosin (upper panel) and Oil Red O staining (lower panel) of liver sections. Oil Red O staining sections (red area) were quantified using ImageJ. Hepatic triglycerides content was also directly measured. **(G)** Hepatic protein levels of lipid metabolism markers. **(H)** Liver protein levels of PGC1α and SIRT1. **(I)** Liver protein levels of OXPHOS complex subunits I–V. Protein levels were normalized to GAPDH and HPS70, and mRNA levels, to HPRT. Dividing lines indicate splicing in the same gel. Data are presented as mean ± SEM; $*p < 0.05$, $**p < 0.01$, using one-way ANOVA followed by a Newman-Keuls Multiple comparison test (n = 7–10 per group).

Fig. 7. Inhibition of SIRT1 blunts the effects of liver-specific downregulation of ATG3 in NAFLD. (A) Cumulative body weight change, **(B)** cumulative food intake, **(C)** hepatic levels of ATG3 protein and SIRT1 mRNA, and **(D)** liver mass in mice fed CDHFD for 8 weeks, with TVI of lentivirus with shRNA-ATG3 or shRNA-scrambled at week 1, and a second TVI of lentivirus with shRNA-SIRT1 or shRNA-scrambled at week 4, as indicated. **(E)** Serum levels of AST and ALT. **(F)** Representative microphotographs of Oil Red O staining (upper panel) and Hematoxylin and Eosin staining (lower panel) of liver sections. Oil Red O staining (red area) was quantified using ImageJ. Hepatic triglycerides content was also directly measured. **(G)** Hepatic protein levels of lipid metabolism markers. **(H)** Liver protein levels of OXPHOS complex subunits I–V. **(I)** Hepatic Complex II activity. GAPDH was used to normalize Protein levels were normalized with GAPDH, and mRNA levels, with HPRT. Dividing lines indicate splicing in the same gel. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, using one-

way ANOVA followed by a Newman-Keuls Multiple Comparison Test (n = 9–10 per group).

Fig. 8. ATG3 regulates hepatic lipid accumulation in an autophagy-independent manner. HepG2 cells silencing ATG3 were treated for 24 hours with 1 mM oleic acid (OA) and 60 µM chloroquine (CQ) (added 18 h after OA) (n=3-4 per group). **(A)** LC3-I and LC3-II protein levels. **(B)** Representative microphotographs of Oil Red O staining. Oil Red O staining (red area) was quantified using ImageJ and normalized to the total number of nuclei per field. **(C)** HepG2 cells and **(D)** THLE2 cells overexpressing ATG3 were treated for 24 hours with 1 mM OA and 60 µM CQ (added 18 h after OA) (n=4-7 per group). Representative microphotographs of Oil Red O staining. **(E)** Protein levels of autophagy markers and mRNA levels of ATG5 and ATG7 in the liver of mice fed a SD wtih a TVI of AAV8-TAp63α or AAV8-GFP at week 1, and a second TVI of lentivirus with shRNA-ATG3 or shRNA-scrambled at week 8 (n=7-10 per group). **(F)** Protein levels of autophagy markers and mRNA levels of ATG5 and ATG7 in liver of mice with liver-specific downregulation of ATG3, fed a CDHFD for 16 weeks (n = 7-15 per group). The n represents the number of independent experiments. Protein levels were normalized with GAPDH, and mRNA levels, with HPRT. Dividing lines indicate splicing in the same gel. Data are presented as mean ± SEM; $*p < 0.05$, $**p < 0.01$, using a Student's *t*-test (A) (B) (C) (D) (F) or one-way ANOVA followed by a Newman-Keuls Multiple Comparison Test (E).