Absence of Atg7 in the liver disturbed hepatic regeneration after liver injury

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Abstract

Background and aims: Autophagy is a critical process in cell survival and the maintenance of homeostasis. However, the implementation of therapeutic approaches based on autophagy mechanisms after liver damage is still challenging.

Methods: We used a hepatospecific Atg7-deficient murine model to address this question.

Results: We showed that the proliferation and regeneration capacity of Atg7-deficient hepatocytes was impaired. On the one hand, Atg7-deficient hepatocytes showed steady-state hyperproliferation. On the other hand, external triggers such as partial hepatectomy (PHx) or cell transplantation did not induce hepatocellular proliferation or liver repopulation. After PHx, hepatocyte proliferation was strongly decreased, accompanied by high mortality. This increase in mortality could be overcome by pharmacological mTOR inhibition. In accordance with hepatocyte hypoproliferation after damage, Atg7-deficient hepatocytes failed to repopulate the liver in a hepatic injury model. Atg7-deficient mice showed hepatic hypertrophy, transient cellular hypertrophy, and high transaminase levels followed by strong perisinusoidal/pericellular fibrosis with age. Their elevated modified hepatic activity index (mHAI) was almost exclusively due to apoptosis without any inflammation. These parameters were associated with variations in the triglyceride content and compromised lipid droplet formation after PHx. Mechanistically, we also observed a modulation of HGF, PAK4, NOTCH3 and YES1, which are proteins involved in cell cycle regulation.

Conclusion: We demonstrated the important role of autophagy in the regeneration capacity of hepatocytes. We showed the causative relationship between autophagy and triglycerides that is essential for promoting liver recovery. Finally, pharmacological
1 | INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is a cellular survival mechanism that targets cytosolic components to lysosomes. Autophagy catabolizes and reutilizes energy generation and sustains cellular homeostasis. This delivery is performed by the autophagosome, a double-membraned spherical vesicle. The formation of the autophagosome is orchestrated by several evolutionarily well-conserved ‘Autophagy’ genes (ATGs). Autophagy is permanently active at low levels and is induced as a response to intra- or extracellular stimuli. Plenty of evidence indicates that autophagy participates in the pathogenesis of different human diseases, including neurodegenerative, cardiovascular and infectious diseases, metabolic disorders, and cancer. Nevertheless, the role of autophagy in several diseases is considered a ‘double-edged sword.’

Autophagy is considered of crucial importance in liver metabolism. Autophagy degrades and recycles protein and cell organelles supplying amino acids to cellular processes, delivers glycogen to vacuoles during starvation, and breaks down lipid droplets, providing free fatty acids and intracellular triglycerides. Several studies have shown that hepatic autophagy is dysregulated in metabolic liver diseases such as nonalcoholic fatty liver disease and obesity.

Atg7 is involved in autophagosome formation and is thus an essential gene for autophagy. Therefore, there is no autophagy in Atg7-deficient cells. In this study, we used a hepatospecific Atg7-deficient murine model. We observed steady-state hepatocyte hyperproliferation. In contrast, after partial hepatectomy (PHx), hepatocyte proliferation was strongly decreased. Even more pronounced was the 50% mortality after this intervention that could be reversed by pharmacological mTOR inhibition. In accordance with hepatocyte hypoproliferation and impaired regeneration capacity after injury, Atg7-deficient hepatocytes failed to repopulate the liver in a Fah (fumarylacetoacetate hydrolase) hepatic injury model. Mechanistically, we showed that the triglyceride content varied with hepatic hypoproliferation and poor liver regeneration. Additionally, cell cycle-related proteins were specifically regulated after liver damage in the serum of Atg7-deficient mice.

2 | METHODS

2.1 | Ethics statement

All animal care and experiments were performed in accordance with institutional and national guidelines. All animal experiments were performed according to protocols approved by the animal welfare commission of the Hannover Medical School and the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany).

2.2 | Mice

All animals were kept at the central animal facility of the Hannover Medical School (Hannover, Germany) under the previously described conditions. Atg7fl/fl mice were obtained from Masaaki Komatsu. Atg7fl/fl mice were bred with C57BL/6- Tg(Alb-Cre)21Mgn mice to generate Atg7fl/fl Alb-cre+ and Atg7fl/fl Alb-cre− (controls) animals. Fah−/− Rag2−/− Il2rg−/− (FRG) mice were generated as previously described. Drinking water was supplemented with NTBC (2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione) at a concentration of 7.5 mg/mL until transplantation.

2.3 | Histology and immunostaining

Freshly dissected liver tissues were fixed, processed and paraffin embedded. Five-micron sections were stained with haematoxylin and eosin or processed further for immunohistochemistry. Histological scoring for the modified hepatic activity index (mHAI) was performed by a pathologist in a blinded fashion as shown before. Ki-67, BrdU, Sirius Red, Oil Red O, Fah and β-catenin staining were performed using standard immunohistochemistry protocols. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to identify apoptotic cells was performed as described previously.

Lay Summary

- Autophagy is a cleaning and recycling cell process which plays an important role in liver regeneration
- Absence of autophagy in hepatocytes impairs lipid droplet formation disturbing the hepatic regeneration capacity
- Pharmacological treatment after liver injury overcomes the high mortality caused by autophagy deficiency

mTOR inhibition overcame the impact of autophagy deficiency after liver damage and prevented mortality.
2.4 | Statistical analysis

The unpaired Student’s two-tailed t-test, Kaplan-Meier survival analysis and two-way analysis of variance (ANOVA) with Tukey’s post test were performed using Prism 8 software (GraphPad). Data are presented as the mean ± standard deviation or as the median in violin plots. Alternatively, for multiple variables, the unpaired Student’s two-tailed t-test with an implemented Benjamini-Hochberg multiplicity correction was performed using Qlucore Omics Explorer software 3.5 (Qlucore, Lund, Sweden). Heat maps represent the multiple protein expression profiles ($P < .05$; $q < 0.05$). Significant differences with $P \leq .05$ are indicated by *, very significant differences ($P \leq .01$) are indicated by **, extremely significant differences ($P \leq .001$) by *** or by **** ($P \leq .0001$). $P > .05$ was considered to be not significant (ns).

3 | RESULTS

3.1 | Hepatocyte-specific autophagy loss leads to hepatic hypertrophy in Atg$7^{+/+}$ Alb-cre$^+$ mice

To investigate the impact of autophagy on the liver as a whole and hepatocytes in detail, we crossed Atg$7^{+/+}$ mice with mice harbouring the liver-specific albumin-cre (Alb-cre) transgene, thereby generating Atg$7^{+/+}$ Alb-cre$^+$ animals expressing no Atg7 in hepatocytes (data not shown). The controls used were littermates that

![FIGURE 1](image.png)
express no Alb-cre (Atg7fl/fl Alb-cre+) males and their counterpart littermates were sacrificed. A, Representative pictures of β-catenin immunohistochemistry showing stained cellular membranes. Atg7fl/fl Alb-cre+ livers displayed transient cellular hypertrophy. Violin plots display the huge variation in hepatocyte size in those mice at younger age. B, Representative pictures of Sirius Red immunohistochemistry exhibiting fibrotic tissue in Atg7fl/fl Alb-cre+ livers. C, Hydroxyproline measurement in the liver confirmed the higher collagen content. D, Ki-67 and BrdU immunohistochemistry was assessed to analyse hepatocyte proliferation. Quantification revealed great proliferation in Atg7fl/fl Alb-cre+ livers. The magnification bar represents 100 μm. n = 5-6

FIGURE 2  Atg7 deficiency enormously increased the steady-state proliferation of hepatocytes. Eight- and sixteen-week-old Atg7fl/fl Alb-cre+ males and their counterpart littermates were sacrificed. A, Representative pictures of β-catenin immunohistochemistry showing stained cellular membranes. Atg7fl/fl Alb-cre+ livers displayed transient cellular hypertrophy. Violin plots display the huge variation in hepatocyte size in those mice at younger age. B, Representative pictures of Sirius Red immunohistochemistry exhibiting fibrotic tissue in Atg7fl/fl Alb-cre+ livers. C, Hydroxyproline measurement in the liver confirmed the higher collagen content. D, Ki-67 and BrdU immunohistochemistry was assessed to analyse hepatocyte proliferation. Quantification revealed great proliferation in Atg7fl/fl Alb-cre+ livers. The magnification bar represents 100 μm. n = 5-6

express no Alb-cre (Atg7fl/fl Alb-cre+) and therefore have normal Atg7 levels (data not shown). Autophagy deficiency was validated by an impaired LC3-II formation (Figure S1). This newly generated model is slightly different from published Atg7fl/fl Mx-cre or Atg5fl/fl Mx-cre models with a less specific promoter and/or different targeted gene. Nonetheless, and in line with these models, examination of the Atg7fl/fl Alb-cre+ mice revealed that they were significantly lighter than the controls at 8 weeks of age (Figure 1A). At 16 weeks of age, this difference persisted; the Atg7fl/fl Alb-cre+ mice displayed a 40% body weight gain in 8 weeks, while the control mice displayed a 32% gain. Upon closer examination of the liver, we observed that the liver weight of Atg7fl/fl Alb-cre+ mice was significantly increased compared to that of controls at 8 weeks of age, and the difference was even greater at the sixteenth week of life (Figure 1A). The Atg7fl/fl Alb-cre+ mice gained a mean liver weight of 92% in 8 weeks, whereas the control mice gained only 19%. Overall, the liver weight gain in Atg7fl/fl Alb-cre+ mice was ten times more rapid than that in control mice. Due to the reciprocal relationship between body weight and liver weight, the proportion of liver to total body weight increased from 4.7% in controls to 14.2% in Atg7fl/fl Alb-cre+ mice and increased even more with advancing age (Figure 1A). This extraordinary liver enlargement was also macroscopically visible (Figure 1B), but the bilirubin values were rather unremarkable (Figure 1C). Interestingly, a significant increase in hepatocyte size was evident at 8 weeks of age with Atg7 loss, but this difference was not observed 8 weeks later (Figure 1D). These observations led to the question of whether the hepatic hypertrophy in Atg7fl/fl Alb-cre+ livers was a result of hepatocyte hypertrophy or augmented hepatocyte proliferation.
3.2 | Atg7 loss leads to increased hepatocyte proliferation and transient cellular hypertrophy followed by perisinusoidal/pericellular fibrosis

Corroborating our previous observations (Figure 1), we were able to attest that there was a strong hypertrophy of the majority of hepatocytes in Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> mice at the eighth week of life, which was associated with a disarray of liver cell plates (Figure 2A). However, while this disarray remained, this transient difference in hepatocyte size was no longer evident at 16 weeks of age. Although, there was focal condensation of β-catenin-positive cell membranes in Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> mice at 16 weeks of age, which was associated with areas of small hepatocytes and strong perisinusoidal/pericellular fibrosis, as demonstrated by collagen I and III fibre staining (Figure 2B). This effect was also reflected in the largely increased content of the major component of protein collagen hydroxyproline at 16 weeks of age but not at 8 weeks (Figure 2C). Because transient hepatocyte hypertrophy does not explain the extreme increase in liver weight and hepatocyte hypertrophy observed later, we immunohistochemically examined hepatocyte proliferation with the independent markers Ki-67 and BrdU. Both labelling indices showed consistent hyperproliferation of hepatocytes at both time points in the histology of Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> livers (Figure 2D). However, this proliferation was lower at 16 weeks of age than at 8 weeks but was still exceptionally elevated. These data suggest that Atg7 deficiency promotes hepatocyte hyperproliferation and cellular hypertrophy but only transient.

3.3 | The regenerative capacity of the hepatocytes of Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> mice is severely impaired despite greater steady-state proliferation of hepatocytes

The strong proliferation of hepatocytes and the extreme liver weight in Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> mice suggest a very high regenerative capacity. Therefore, we were interested in determining whether hepatocyte proliferation could be further increased by additional regenerative stimuli, such as PHx. To address this question, we partially hepatectomized Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> and control mice at the time of peak baseline proliferation. In contrast to the controls, approximately 50% of Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> mice died within 48 hours after surgery (Figure 3A). Interestingly, an analysis of the liver weight of the survivors showed that the liver mass was always higher in these mice than in the controls after PHx (Figure 3B). As the initial size and mass of the Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> livers were well above those of the controls, data normalization was performed. Therefore, the ‘basal’ time point in all animals corresponds to a value of 1 in Figure 3C. Immediately after PHx, this value dropped to 0.3 at time point 0 hours as a result of the two-thirds
PHx. We noticed that the Atg7fl/fl Alb-cre+ livers, only regained 47% of the starting weight one week after PHx, whereas the controls regained 70% and 3%-4% of the total body weight (Figure 3C; data not shown). This observation implies that liver regeneration was reduced to less than 50% of that in the controls. Importantly, at time points after 48 hours, the Atg7fl/fl Alb-cre+ mice included in the analysis represent a positive selection, since half of the animals have already died (Figure 3A). This also explains the inconspicuous bilirubin levels in Atg7fl/fl Alb-cre+ animals, as only surviving animals were tested, resulting in a form of positive selection (Figure 3D).

To exclude environmental influences and to examine only the effect on hepatocytes, we have established a hepatocyte transplantation model using immunosuppressed Fah− Rag2− Il2rg− (FRG) mice.6,11 These mice lack B, T and NK cells and have a genetic defect that leads to an accumulation of a toxic metabolite during tyrosine catabolism, which subsequently causes acute liver failure. The drug NTBC blocks this accumulation and therefore prevents liver damage in mice.12 One million hepatocytes from 8-week-old Atg7fl/fl Alb-cre+ or Atg7fl/fl Alb-cre− mice were intrasplenically transplanted into FRG mice, which allows nearly complete liver repopulation with wild-type

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**FIGURE 4** Atg7-deficient hepatocytes did not repopulate Fah immunosuppressive livers. One million hepatocytes isolated from 8-wk-old Atg7fl/fl Alb-cre+ males and their counterpart littermates were intrasplenically injected into FRG mice. A, Illustration of the hepatocyte transplantation approach. B, Survival of FRG mice after hepatocyte transplantation. C, Representative pictures of Fah immunohistochemistry and quantification of repopulated livers. Red-stained hepatocytes were the transplanted hepatocytes. n = 3-7
hepatocytes. Following transplantation, NTBC treatment was withdrawn, creating a strong proliferative environment for transplanted Fah-expressing cells (Figure 4A). After 8 weeks, 62% of the FRG mice transplanted with control hepatocytes survived; however, the survival after transplantation with Atg7fl/fl Alb-cre+ hepatocytes was significantly lower (37%; Figure 4B). Fah immunostaining revealed multiple proliferating hepatocytes in liver sections of both groups 4 weeks after transplantation. Interestingly, 8 weeks after transplantation, 50% of the livers were repopulated with control hepatocytes; in contrast, Atg7fl/fl Alb-cre+ hepatocytes were unable to repopulate the FRG livers (Figure 4C). Together, these data imply that Atg7 deficiency in hepatocytes significantly impairs their proliferation and regenerative capacity.

3.4 | Hepatocyte proliferation after liver damage in Atg7-deficient mice is severely compromised, and cellular hypertrophy is reversible

The discrepancy between hepatocellular hyperproliferation during homeostasis and less liver regeneration after PHx and transplantation is conspicuous and requires further investigation.

Therefore, we additionally examined the regeneration and proliferation capacity of hepatocytes after PHx. The Ki-67 and BrdU immunostaining results revealed that the proliferation of hepatocytes in the Atg7-deficient mice was still greatly increased at the time of PHx (8 weeks of age). Surprisingly, this picture was reversed after 48 hours, and furthermore, hepatocyte proliferation returned to a level comparable to that in control mice after one week (Figure 5A). Notably, at 48 hours and later, only up to half of the mice survived and served as a benchmark. Immunostaining quantification revealed a strong increase in hepatocyte proliferation in the control livers that was not observed in Atg7fl/fl Alb-cre+ mice. As expected, control mice presented a hepatocyte proliferation peak at 48 hours, reaching levels of proliferation after one week similar to those before PHx. In contrast, Atg7fl/fl Alb-cre+ livers exhibited reduced hepatocyte proliferation after intervention compared to the basal time point (Figure 5B and 5D). Normalization of the data to fold change revealed the timeline of hepatocyte proliferation in control and Atg7-deficient livers (Figure 5C and 5E). Here, the data show that PHx did not induce the proliferation of Atg7fl/fl Alb-cre+ hepatocytes.

In addition, the hepatocyte enlargement seen in Atg7fl/fl Alb-cre+ livers was highly reversible, since the hypertrophic hepatocytes halved in size 48 hours after PHx, shifting back to normal size (Figure 5F).

3.5 | Atg7 deficiency promotes apoptosis and thereby enhances the transaminases and modified hepatic activity index in Atg7fl/fl Alb-cre+ mice

Homeostasis in the liver requires balanced hepatocyte apoptosis and mitosis in healthy individuals. Therefore, we wondered how Atg7 deficiency modulates apoptosis sensitivity in the liver. The TUNEL assay and its quantification revealed that approximately 10% of hepatocytes in Atg7fl/fl Alb-cre+ mice at 8 weeks of age were apoptotic, level which remained high with increasing age (Figure 6A).

The increased apoptosis rate of hepatocytes was correlated with a strong increase in aspartate transaminase and alanine transaminase (ALT) in Atg7fl/fl Alb-cre+ mice (Figure 6B). Additionally, pathological evaluation of liver sections showed a high mHAI (Figure 6C), almost exclusively due to apoptosis without any inflammation (data not shown).

After PHx, the number of apoptotic hepatocytes remained high in Atg7fl/fl Alb-cre+ livers (Figure 6D). At 48 hours, the transaminase levels were higher than the levels before PHx. Unexpectedly, after one week, the transaminase levels were only moderately increased (Figure 6E) and significantly lower than the levels before PHx (Figure 6A and 6E). In summary, the high pathological mHAI is due to a significantly increased hepatocyte apoptosis that is in line with the elevated transaminases in Atg7-deficient mice.

3.6 | Hyperproliferation and the high apoptosis rate are regulated at the molecular level

To elucidate the mechanism by which high hepatocyte proliferation and apoptosis occurred in Atg7fl/fl Alb-cre+ mice, we analysed the expression of 92 selected proteins in the serum. They are related to different biological processes, including cellular proliferation, apoptosis and metabolism. The steady-state hepatocyte hyperproliferation, cellular hypertrophy and increased hepatocyte apoptosis rate of Atg7fl/fl Alb-cre+ mice were accompanied by 57 differentially regulated proteins in the serum (Table S1A). The most interesting severely upregulated proteins are shown in Figure 7A and 7C. While HGF, PAK4 and NOTCH3 regulate p53 and therefore cell proliferation as well as HCC and metastasis, YES1 is part of the proto-oncogene tyrosine-protein (Src) kinase family. Nevertheless, increased apoptosis was associated with a 5-fold increase in FAS expression. Interestingly, the short-lived hepatocyte-stimulating factor IL-6 was also boosted in Atg7fl/fl Alb-cre+ mice. The second comparison shows the changes occurred between 0 and 48 hours after PHx in Atg7fl/fl Alb-cre+ serum (Figure 7B and 7D). A pool of 37 proteins was significantly regulated (Table S1B). Mechanistically, most of these proteins shown in Figure 7C were reversely regulated 48 hours after PHx, where hepatocyte proliferation was diminished (Figure 5A-5E). Contrary to expectations, FAS was also expressed at lower levels after 48 hours, while the apoptosis rate was unchanged (Figure 6A and 6D).

3.7 | mTOR inhibition improves survival in mice with Atg7 deficiency

Mechanistic target of rapamycin (mTOR) is a protein kinase identified as a central regulator of cell growth, proliferation and
Regeneration capacity of Atg7-deficient hepatocytes was impaired after PHx. Eight-week-old Atg7<sup>fl/fl</sup> Alb-cre<sup>+</sup> males and their counterpart littermates underwent PHx. A, Ki-67 and BrdU immunohistochemistry at the determined time points. B-E, Quantification and normalization revealed decreased hepatocyte proliferation in Atg7-deficient hepatocytes. F, β-catenin immunohistochemistry displayed a reduction in hepatocyte area, and violin plots show its strong variation after PHx. The magnification bar represents 100 μm. h = hours, 1 w = 1 week. The 0-h data correspond to 8-wk-old samples. n = 5-9.
survival as well as a key autophagy modulator. mTOR inhibition by everolimus treatment effectively prevents the proliferation of damaged hepatocytes without compromising the proliferation of healthy hepatocytes in the Fah mouse model. Because Atg7fl/fl Alb-cre⁺ mice displayed signals of liver injury, we wondered whether mTOR inhibition modulates the proliferation of damaged and/or healthy hepatocytes in livers with Atg7 deficiency. Atg7fl/fl Alb-cre⁺ mice and controls were treated with everolimus two days pre- and post-PHx. Remarkably, Atg7fl/fl Alb-cre⁺ mice displayed no mortality, indicating that mTOR inhibition improved mouse survival (Figure 8A). In Atg7fl/fl Alb-cre⁺ mice, everolimus treatment reduced hepatocyte proliferation at the 0-hour time point, a difference that disappeared at 48 hours (Figure 8B). Liver function was not disrupted, and liver transaminases and the mHAI remained consistent when compared with those in untreated mice (Figure 6, 8C and ). Taken together, these observations imply a mechanism for autophagy modulation since mTOR is a key factor and clinical target due to its inhibition improving the survival of Atg7fl/fl Alb-cre⁺ mice.

3.8 | Fewer protective triglycerides in Atg7fl/fl Alb-cre⁺ mice

Triglycerides in the liver protect against apoptosis and other damage by preventing the formation of free radicals. Therefore, we were interested in how Atg7 deficiency affects triglyceride levels in the blood and liver. At 8 weeks of age, the triglyceride content was inconspicuous in the blood of Atg7-deficient mice. Remarkably, Atg7fl/fl Alb-cre⁺ mice were lower than the levels before PHx. The magnification bar represents 100 μm. h = hours, 1 w = 1 week. n = 5-11
hepatic triglyceride metabolism, and mitochondrial stress and damage contribute to the development of fibrosis and inflammation in the liver following cell death. Consequently, we evaluated the mitochondrial respiration status in Atg7fl/fl Mx-cre hepatocytes. No significant differences in the oxygen consumption rate were observed (Figure S2).

**4 | DISCUSSION**

In this study, we report the importance of autophagy for hepatocellular regeneration in a new model with the liver-specific albumin promotor and Atg7 deficiency. We demonstrated for the first time an increased proliferation of hepatocytes in Atg7-deficient mice (Figure 2). Furthermore, we show that the extent of some liver injury abnormalities intensified with age (e.g., hepatocyte size, proliferation and fibrosis; Figure 1 and 2), while other pathological and biochemical parameters did not change. However, the most striking effects were shown for liver regeneration and repopulation after PHx (Figures 3, 5 and 8) and cellular Tx (Figure 4). Impaired liver regeneration was most prominent in the poor survival rate of Atg7fl/fl Alb-cre mice accompanied by impaired lipid droplet formation that might be causative. Nonetheless, the survivors had better transaminases, decreased proliferation, and reversal of transient cellular hypertrophy with a low hepatic triglyceride content. Surprisingly, poor survival could be fully recovered by pharmacological mTOR inhibition (Figure 8). Mechanistically, we observed the modulation of cell cycle-related proteins before and after PHx in serum (Figure 7).

The disproportional hyperproliferation of hepatocytes was also described by other authors for autophagy-deficient models. Notably, others have used different animal models. The models utilized were not based on Alb-cre but on MX1-cre system. The Alb-cre system is hepatospecific, while MX1 is also highly expressed in the lung, spleen, lymph nodes, heart, nervous system and other organs. This effect might cause interference within the organism, resulting in a discrepancy in the results. According to Biogps.org, albumin mRNA levels in the liver are approximately 90,000-fold higher than in the reference, while MX1 is approximately 5-fold higher. This observation is in line with our own findings; even though Atg7fl/fl Mx-cre mice had hepatocyte hyperproliferation, this increase was less pronounced than that in Atg7fl/fl Alb-cre mice (Figure S3). Although the observed
hyperhepatoproliferation was slightly reduced in Atg7fl/fl Alb-cre+ mice over time, the level of hepatocyte proliferation remained high (>10%), as also seen by others.22 In contrast, other studies/models could not reproduce this finding 5,10,23 or have shown the opposite.3 However, cellular hypertrophy and fibrosis have already been shown in other autophagy-deficient models.5,22 Corroborating our previous observations (Figure 1) and other studies,10,19 we were able to attest that there was strong hepatocyte hypertrophy at the eighth week of life, which was associated with a disarray of liver cell plates (Figure 2A). However, we were able to demonstrate for the first time that while this disarray remained, the difference in hepatocyte size could no longer be noticed at 16 weeks of age and therefore appeared to be transient.

The recovery rate after liver damage by PHx is a crucial parameter to study. A benign effect of PHx in our model was likely because the transaminases were strikingly reduced. However, since approximately 50% of Atg7fl/fl Alb-cre+ mice died after this intervention (Figure 3A), it cannot be considered; although, pharmacological mTOR inhibition resulted in complete survival after PHx (Figure 8A). Here, we described a disturbed tissue mass restoration (Figure 3C). Another autophagy study reported opposing results, but these mice were also Atg5-deficient as in the MX1-cre line, making a comparison difficult.10 Nevertheless, in our Atg7fl/fl Mx-cre+ mice, hepatocyte proliferation after PHx was also disturbed (Figure S4), but this disturbance was less pronounced than that observed with hepatocyte-specific Atg7 deficiency. The reduced liver regeneration in mice with disordered
autophagy might be due to various reasons: decreased hepatocyte proliferation or increased hepatocyte apoptosis. In contrast to wild-type mice and other compromised models, we observed a fulminant drop in hepatocyte proliferation after PHx in our Atg7fl/fl Alb-cre+ mice (Figure 5). Coincidentally, the apoptosis rate was slightly increased after PHx (Figure 6A and 6D). Others have shown that impaired hepatocyte regeneration in Atg7-deficient MX1-cre mice is due to premature senescence but not due to apoptosis activation.10

Our data after PHx displayed a decreased hepatocyte proliferation rate. This suggests that the remaining liver tissue might be sufficient to fulfill hepatic function and allow survival. Liver mass may limit regeneration through a negative feedback loop. However, Toshima et al showed that a massive hepatectomy (90% PHx) in Atg5-deficient mice increases mortality to 100% within one day.10 We and others also observed cellular hypertrophy in autophagy-deficient mice.30,31 Here, we could show that this cellular hypertrophy is only a transient effect. Contrary to our results, in Atg5-deficient mice, hepatocyte hypertrophy was observed one to two days after PHx; however, no later time points were analyzed. Therefore, this is not the cause of the disturbed hepatocyte function and regeneration.

Whether the doubled liver mass in Atg7-deficient mice after PHx hampers regeneration is a difficult question to address. Therefore, we transplanted Atg7fl/fl Alb-cre+ hepatocytes into FRG mice to specifically analyze how the loss of Atg7 affects cell cycle progression.6,13 We hypothesized that the transplanted Atg7-deficient hepatocytes should repopulate the damaged livers more efficiently than control hepatocytes because of their steady-state hyperproliferation. Surprisingly, Atg7-deficient hepatocytes failed to repopulate the liver. Therefore, we indirectly showed that disturbed liver regeneration is very unlikely to be due to a pure mass effect but is more likely due to a metabolic disorder.6,13,24,25 Together, these findings explain the reduction in regenerative capacity. To support this, we have shown a differential regulation of proteins involved in cell cycle control (Figure 7). Although little has been studied in the liver, PK4 has been shown to play a role in cell proliferation and cytoskeletal architecture modulation. Similar to NOTCH3, upregulation of PK4 has been associated with uncontrolled cell proliferation and cancer in different organs.27 Interestingly, cell proliferation activation by PK4 and NOTCH3 is performed through p53.28,29 Furthermore, YES1, a member of the SRC kinase family, interacts with YAP, facilitating its translocation to the nucleus and therefore its activation. YAP overexpression in the liver has been correlated with hepatocyte proliferation and hepatomegaly.30 Notably, these proteins were inversely regulated after PHx, where hepatocyte proliferation was largely decreased. This effect explains the impaired liver regeneration after PHx. HGF is associated with sustained hepatocyte proliferation in cancer.31 However, a study revealed that HGF stimulates PK4, possibly contributing to HGF-induced changes in cytoskeletal architecture in epithelial cells.32 The modulation of PK4 and HGF before and after PHx could elucidate transient cellular hypertrophy.

Although most of these findings are not clinically useful, they have important implications for the underlying mechanism of liver regeneration and autophagy; therefore, further detailed molecular characterization is needed.

Therefore, we evaluated the therapeutic options involving the inhibition of mTOR. One effect of the inhibition of mTOR is the activation of autophagy.33,34 We used everolimus to overcome the effect of Atg7 deficiency in hepatocytes. Although this therapy did not improve the mHAI, it led to complete survival of Atg7-deficient mice after PHx. This is the opposite effect described in Atg5/mTOR double knockout mice.35 These observations suggest that the pharmacological inhibition of mTOR might activate an Atg5/Atg7-independent autophagy process, leading to better mouse survival. An alternative Atg5/Atg7-independent process of autophagy without microtubule-associated protein 1A/1B light chain 3 (LC3) lipidation has been detected in several embryonic tissues.36 This revealed an important pharmacological mechanism to overcome the high mortality after liver injury.

There is controversy over the causality between autophagy and lipid storage. While some studies have shown an increase in lipid storage and triglyceride content in lipid droplets due to a lack of autophagy, others have shown the opposite.22,39,40 Impaired lipid droplet formation is considered to be a causative mechanism responsible for poor hepatic regeneration and hepatocyte hypoproliferation after liver injury. We have shown an almost complete absence of these hepatic lipid droplets after PHx in Atg7-deficient hepatocytes/mice (Figure 8). This observation is very remarkable in connection with the disturbed regeneration after PHx. This transient hepatic steatosis by lipid droplet formation is a crucial mechanism for successful hepatic regeneration after PHx and starvation.42 LC3 and ATG7 contribute to the fusion and growth of lipid droplets.43 LC3 is a central protein in autophagosome biogenesis. It has been shown that LC3-II is localized not only on the membrane of autophagosomes but also on the surface of lipid droplets in hepatocytes.39 These observations suggest that a nonfunctional ATG7 protein during autophagy leads to a nonfunctional LC3 conjugation system and lipid droplet formation and therefore to an impaired regeneration capacity after PHx.

Furthermore, triglycerides are radical scavengers and fulfill a protective function against toxicity.15 Different studies suggest that triglyceride deposition may be a guiltless witness or an initial cellular defence during lipotoxicity.44,45 Losing this protection mechanism results in the observed apoptosis of hepatocytes. This increase in hepatocyte cell death was confirmed by an increase in the mHAI and a disproportional increase in transaminase levels. Interestingly, specific autophagy deficiency in stellate cells attenuates fibrogenesis in the liver during CCl4 treatment,46 indicating that autophagy appears to be involved in hepatofibrogenesis. In this context, one might speculate whether the decrease in triglyceride content with age in Atg7fl/fl Alb-cre+ mice (Figure 8) is also responsible for the decrease in hepatocyte proliferation and perisinusoidal/pericellular fibrosis (Figure 2).

In conclusion, we demonstrated the important role of autophagy in the regeneration capacity of hepatocytes. We showed the causative relationship between autophagy and triglyceride content, which is essential for preventing hepatocyte cell death and
promoting liver recovery. Mechanistically, we observed a modulation of HGF, PAK4, NOTCH3 and YES1, which are proteins involved in cell cycle regulation. Finally, pharmacological mTOR inhibition after liver damage prevented mortality, overcoming the impact of hepatospecific autophagy deficiency in mice.

CONFLICT OF INTEREST
All authors claim that there is no conflict of interest including a desire for financial gain, prominence, professional advancement or a successful outcome.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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