GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Diverse Consequences in Liver Injury in Mice with Different Autophagy Functional Status Treated with Alcohol

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Alcoholic fatty liver disease is often complicated by other pathologic insults, such as viral infection or high-fat diet. Autophagy plays a homeostatic role in the liver but can be compromised by alcohol, high-fat diet, or viral infection, which in turn affects the disease process caused by these etiologies. To understand the full impact of autophagy modulation on alcohol-induced liver injury, several genetic models of autophagy deficiency, which have different levels of functional alterations, were examined after acute binge or chronic-plus-binge treatment. Mice given alcohol with either mode and induced with deficiency in liver-specific *Atg7* shortly after the induction of *Atg7* deletion had elevated liver injury, indicating the protective role of autophagy. Constitutive hepatic *Atg7*-deficient mice, in which *Atg7* was deleted in embryos, were more susceptible with chronic-plus-binge but not with acute alcohol treatment. Constitutive hepatic *Atg5*-deficient mice, in which *Atg5* was deleted in embryos, were more susceptible with acute alcohol treatment, but liver injury was unexpectedly improved with the chronic-plus-binge regimen. A prolonged autophagy deficiency may complicate the hepatic response to alcohol treatment, likely in part due to endogenous liver injury. The complexity of the relationship between autophagy deficiency and alcohol-induced liver injury can thus be affected by the timing of autophagy dysfunction, the exact autophagy gene being affected, and the alcohol treatment regimen. (*Am J Pathol* 2019, 189: 1744–1762; <https://doi.org/10.1016/j.ajpath.2019.05.011>)

The liver plays a crucial role in metabolism in the body.¹ When metabolic homeostasis is disturbed in the liver, fatty liver disease (FLD), which is characterized by steatosis, inflammation, and fibrosis, can occur. FLD can be subclassified as being caused by excessive alcohol intake (alcoholic fatty liver disease; AFLD) or by any non-alcohol-related etiology (nonalcoholic fatty liver disease; NAFLD).²

Autophagy is an evolutionarily conserved cellular degradation process that involves the delivery of cytoplasmic cargo (macromolecules or organelles) to the lysosome.³ Three types of autophagy have been defined: macroautophagy, microautophagy, and chaperone-mediated autophagy.⁴ Macroautophagy is quantitatively the most active form of autophagy and is referred to hereafter as autophagy. In macroautophagy, cytosolic materials are sequestered inside the autophagosome, and transported to,

and degraded in, the lysosome.⁵ Autophagy occurs at the basal level in the liver and can be further enhanced under conditions of stress. It can play an important role in the maintenance of normal liver function and in the pathogenesis of various liver diseases.^{6,7} In particular, autophagy could be activated or depressed in such common liver diseases as AFLD,^{8–12} NAFLD,^{13–15} and hepatitis viral infection.^{16–19}

The pathologic processes of AFLD are considered to be related to oxidative stress caused by the accumulation of acetaldehyde, increased NADH/NAD⁺ ratio, or generation of reactive oxidative species.^{20–22} During the progress of

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AFLD, oxidative stress may induce functional and structural mitochondrial changes that may affect oxidative phosphorylation, increase mitochondrial DNA damage, and alter mitochondrial protein profiles.^{23–28} Increased reactive oxygen species, together with alcoholic steatosis, lead to lipid peroxidation that can further enhance oxidative damage in AFLD.²⁹ As shown in previous studies, the effects of autophagy can vary at different pathologic stages of AFLD.^{8,9,12,15,30} Autophagy is activated in the liver *in vivo* and in cultured primary hepatocytes after acute alcohol treatment.^{8,9,30} This activation requires alcohol metabolism, and is mediated by reactive oxygen species and several signaling pathways. Acetaldehyde, a major alcohol metabolite and a pro-oxidant, has been implicated in the induction of autophagy.³¹ Meanwhile, alcohol-induced autophagy can be suppressed by antioxidants, such as *N*-acetyl cysteine.^{8,32} In addition, alcohol treatment alters the mammalian target of rapamycin, 5' AMP-activated protein kinase, and/or forkhead box O3a signaling pathway,^{33–35} which can contribute to changes in the autophagy process. Other autophagy stimulators, including alcohol-induced endoplasmic reticulum stress, proteasome inhibition, and minerals such as zinc, are also implicated in the dynamics of autophagy after alcohol treatment.^{36–38}

Interestingly, autophagy may be suppressed in chronic alcohol treatment or in other liver diseases. When mice were fed with a Lieber-DeCarli diet for 4 weeks, hepatic autophagy was stimulated at a lower dose of alcohol (accounting for 29% of the caloric need) but was inhibited at a higher alcohol dose (accounting for 36% of the caloric need).¹⁵ The suppression of autophagy by long-term alcohol use suggests that the promotion of autophagy can improve the condition. The cause of the autophagy suppression may include decreases in both the amount and the functioning of lysosomes, which were found in long-term alcohol-treated rat livers.^{10,11,39} Indeed, the findings from a recent study suggest that the level of transcription factor EB, which plays a crucial role in lysosomal biogenesis and autophagy, was decreased in the livers of mice given alcohol diet and in

patients with alcohol-induced hepatitis.¹² Disruption of hepatic transcription factor EB enhanced alcohol-induced liver injury in mice, but the increase in transcription factor EB level led to reduced alcohol-induced liver injury through elevated lysosomal biogenesis and mitochondrial bioenergetics.¹²

Nonetheless, the assessment of the contribution of autophagy to alcohol-induced liver injury had been mainly conducted through acute pharmacologic manipulation or acute knockout of certain autophagy-related genes. The impact of chronic autophagy deficiency on alcoholic liver injury has not been assessed. This effect may be important as ALDs are often complicated by other disease-causing factors, such as viral infection or high-fat diet, which can compromise autophagy. The present study thus used several genetic models of autophagy deficiency to assess the effects of alcohol in different autophagy functional statuses. The findings suggest that the complicated interaction between alcohol and autophagy in liver injury can be affected by the timing of autophagy dysfunction, the exact autophagy gene being affected, and the alcohol treatment regimen.

Materials and Methods

Mice

Atg5^{F/F} mice (*B6.129S-Atg5tm1Myok*)⁴⁰ and *Atg7^{F/F}* mice⁴¹ have been reported in previous studies. *Atg5^{Δhep}* and *Atg7^{Δhep}* mice were generated by crossing *Atg5^{F/F}* or *Atg7^{F/F}* with Alb:Cre transgenic mice, respectively (The Jackson Laboratory, Bar Harbor, ME). The inducible liver-specific *Atg7*-deficient mice (*Atg7^{Δhep-ERT2}*) were generated by crossing *Atg7^{F/F}* mice with Alb:Cre-ER^{T2} mice.⁴² The deletion of *Atg7* was induced by the administration of tamoxifen (6 mg/day s.c., for 2 days). Mice were maintained on a 12-hour dark/light cycle with free access to food and water. Both male and female mice were used in the studies. Age- and sex-matched mice were randomly assigned to the treatment or control group.

Table 1 Antibody List

Antibody name	Company	Catalog number	Host
ACTIN (8H10D10)	Cell Signaling Technology (Beverly, MA)	3700	Mouse
ADH	Santa Cruz Biotechnology (Dallas, TX)	sc-133207	Mouse
ALDH1/2	Santa Cruz Biotechnology	sc-166362	Mouse
ATG12	Cell Signaling Technology	2011	Rabbit
ATG7	Cell Signaling Technology	2631	Rabbit
CK19	Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA)	TROMA-III	Rat
CYP2E1	Enzo Life Sciences (Lausen, Switzerland)	BML-CR3271	Rabbit
Gapdh	Novus Biologicals (Littleton, CO)	NB 300-221	Mouse
LC3B	Sigma-Aldrich (St. Louis, MO)	L7543	Rabbit
NQO1	Abcam (Cambridge, MA)	ab34173	Rabbit
SQSTM1/p62	Abnova (Taipei, Taiwan)	H00008878-M01	Mouse
α-Smooth muscle actin	Thermo Fisher Scientific (Waltham, MA)	PA5-19465	Rabbit

Adh, alcohol dehydrogenase; Aldh, aldehyde dehydrogenase; ATG, autophagy-related protein; CK, cytokeratin; CYP, cytochrome P450; Gapdh, glyceraldehyde phosphate dehydrogenase; LC, microtubule-associated protein 1A/1B-light chain; NQO, NAD(P)H quinone dehydrogenase; Sqstm, sequestosome.

Table 2 Primer List

Gene/primer name	Sequence (forward)	Sequence (reverse)
<i>Acaca</i>	5'-GCCTCTTCCTGACAAACGAG-3'	5'-TGACTGCCGAAACATCTCTG-3'
<i>Acadl</i>	5'-GGTGGAAAACGGAATGAAAGG-3'	5'-GGCAATCGGACATCTTCAAAG-3'
<i>Acadm</i>	5'-TGTTAATCGGTGAAGGAGCAG-3'	5'-CTATCCAGGGCATACTTCGTG-3'
<i>Acox1</i>	5'-CATATGACCCCAAGACCCAAG-3'	5'-CATGTAACCCGTAGCACTCC-3'
<i>Actin</i>	5'-ACTATTGGCAACGAGCGGTT-3'	5'-CAGGATTCCATACCCAAGAAGGA-3'
<i>Adh1</i>	5'-TGTTGAGAGCGTTGGAGAAG-3'	5'-CGCTTCGGCTACAAAAGTTG-3'
<i>Adh4</i>	5'-GTAGACTCTGTCCAAACCTG-3'	5'-AAGGTCAGGATTGTTTCGGATG-3'
<i>Adh5</i>	5'-AAATCTCCACTCGTCCATTCC-3'	5'-CACTCTCCACACTCTTCCATC-3'
<i>Adh7</i>	5'-GGTTGTGGAGAGTGTGGAG-3'	5'-CCTGTGAGATCGCTCCTAATG-3'
<i>Akr1d1</i>	5'-CTCATTGGGCTTGGAACCTA-3'	5'-CATTGATGGGACATGCTCTG-3'
<i>Aldh16a1</i>	5'-GAGGTTTCGAGATGGAGATGTG-3'	5'-AAAGAGAAAGGAGTCGGCAG-3'
<i>Aldh18a1</i>	5'-CAGACATCGTGGAGGGAAAG-3'	5'-CTGTTTCAGGCTCTAAGGTAGC-3'
<i>Aldh1a1</i>	5'-ATCACTGTGTCATCTGCTCTG-3'	5'-CCCAGTTCTCTTCCATTTCCAG-3'
<i>Aldh1a2</i>	5'-ATGGATGCGTCTGAAAGAGG-3'	5'-TGAATCCCCTGCAATCGATG-3'
<i>Aldh1a3</i>	5'-AACAAAGATAGCCTTCACCGG-3'	5'-CCAAGTCCAAGTCAGCATCT-3'
<i>Aldh1b1</i>	5'-GGAGTCTTATGTCTTGATCTGG-3'	5'-TGTCGGGTGAAGCAGAAATG-3'
<i>Aldh1l1</i>	5'-CATCCAGACCTTCCGATACTTC-3'	5'-ACAATACCACAGACCCCAAC-3'
<i>Aldh1l2</i>	5'-CATTGACAGCCCAAAGCATG-3'	5'-CCCAGAAAACAGAAAACCCAG-3'
<i>Aldh2</i>	5'-TGCAGGAGAATGTGTATGACG-3'	5'-CGATTTGATGTAGCCGAGGATC-3'
<i>Aldh3a1</i>	5'-GGCGTGGTCTTGTCTATAG-3'	5'-AGGGATAAGTGTGAAAGCAGG-3'
<i>Aldh3a2</i>	5'-AGCCCTTGTTTACATTGACAGAG-3'	5'-TTCATGTACTTTCCCCAGGC-3'
<i>Aldh3b1</i>	5'-CCTTCGGTCTGGTGCTTATC-3'	5'-ACCTCAGCCAGTATCTTTTCAG-3'
<i>Aldh3b2</i>	5'-GAATCAGATGTTGGAACGCAC-3'	5'-GGAGAAGGTGTCAAAGGAGAAC-3'
<i>Aldh4a1</i>	5'-CGATAAGTCTACTGGGTCTGTG-3'	5'-GGGCTTATGAGTCTCCTTGATG-3'
<i>Aldh5a1</i>	5'-CCAAGATCATAACAGCCGAGAG-3'	5'-TCGCTTATCTTTGGCAGAGG-3'
<i>Aldh6a1</i>	5'-AAGGGAAGACTCTTGCTGATG-3'	5'-GAGGCAGACGGTAGGAATAAAG-3'
<i>Aldh7a1</i>	5'-AGATATTCTGCCCCAAAACG-3'	5'-CTGAACCTCGCCTATTCCTTC-3'
<i>Aldh8a1</i>	5'-GTATGCATTACACCGTTTCGC-3'	5'-AAGTCACTCTCACTGGGCTTG-3'
<i>Aldh9a1</i>	5'-CTGGAATACTATGCAGGGCTG-3'	5'-GCGATCTGGAAGGGATAGTTC-3'
<i>Apob</i>	5'-ATTCGAGCACAGATGACCAG-3'	5'-GTACCTTTCCACCATCAGACTCC-3'
<i>Apoe</i>	5'-CAATTGCGAAGATGAAGGCTC-3'	5'-TAATCCCAGAAGCGGTTTCAG-3'
<i>Bsep</i>	5'-CTGCCAAGGATGCTAATGCA-3'	5'-CGATGGCTACCCTTTGCTTCT-3'
<i>Cat</i>	5'-CTCGTTTCAGGATGTGGTTTTTC-3'	5'-CTTTCCCTTGAGTATCTGGTG-3'
<i>Ccl2</i>	5'-GTCCCTGTCTATGCTTCTGG-3'	5'-GCTCTCCAGCTACTCATTG-3'
<i>Ccl3</i>	5'-TTCTCTGTACCATGACACTCTGC-3'	5'-CGTGGAATCTTCCGGCTGTAG-3'
<i>Ccr2</i>	5'-GCTCTACATTCACTCCTTCCAC-3'	5'-ACCACTGTCTTTGAGGCTTG-3'
<i>Cd36</i>	5'-GCGACATGATTAATGGCACAG-3'	5'-GATCCGAACACAGCGTAGATAG-3'
<i>Col1a1</i>	5'-ACGGCTGCACGAGTCAAC-3'	5'-GGCAGGCGGGAGGTCTT-3'
<i>Ctgf</i>	5'-GGGCCTCTTCTGCGATTTC-3'	5'-ATCCAGGCAAGTGCATTGGTA-3'
<i>Cyp27a1</i>	5'-GCCTCACCTATGGGATCTTCA-3'	5'-TCAAAGCCTGACGCAGATG-3'
<i>Cyp2e1</i>	5'-TCACTGGACATCAACTGCC-3'	5'-TGGTCTCTGTTCTCTGCAAAG-3'
<i>Cyp3a11</i>	5'-CAGAAGCACCGAGTGGATT-3'	5'-GACTGGGCTGTGATCTCCAT-3'
<i>Cyp7a1</i>	5'-AACAACTGCCAGTACTAGATAGC-3'	5'-GTGTAGAGTGAAGTCTCTTAGC-3'
<i>Cyp7b1</i>	5'-CAGCTATGTTCTGGGCAATG-3'	5'-TCGGATGATGCTGGAGTATG-3'
<i>Cyp8b1</i>	5'-AGTACACATGGACCCGACATC-3'	5'-GGGTGCCATCCGGTTGAG-3'
<i>F4/80</i>	5'-TGCATCTAGCAATGGACAGC-3'	5'-GCCTTCTGGATCCATTTGAA-3'
<i>Fabp1</i>	5'-TCTCCGGCAAGTACCAATTG-3'	5'-TTGATGTCTTCCCTTTCTGG-3'
<i>Fasn</i>	5'-CCCTTGATGAAGAGGGATCA-3'	5'-ACTCCACAGGTGGGAACAAG-3'
<i>Fgfr4</i>	5'-CTGCCAGAGGAAGACCTCAC-3'	5'-GTAGTGGCCACGGATGACTT-3'
<i>Fxr (Nr1h4)</i>	5'-GGCCTCTGGGTACCACTACA-3'	5'-TGACACGGCGTTCTTGGA-3'
<i>Gfap</i>	5'-GAAAACCGCATCACCATTCC-3'	5'-CTTAATGACCTCACCATCCCG-3'
<i>Gstm1</i>	5'-ACTTGATTGATGGGGCTCAC-3'	5'-TCTCCAAAATGTCCACACGA-3'
<i>Hsd3b7</i>	5'-CCATCCACAAAGTCAACGTG-3'	5'-CTCCATTGACCTTCTTCCA-3'
<i>Ifng</i>	5'-GATGCATTATGATGATTGCCAAGT-3'	5'-GTGGACCATTCCGATGAGCTC-3'
<i>Il6</i>	5'-AGTTGCCTTCTTGGGACTGA-3'	5'-TCCACGATTTCCAGAGAAC-3'
<i>Lcn2</i>	5'-CTACAATGTACCTCCATCCTG-3'	5'-ACCTGTGCATATTTCCAGAG-3'
<i>Lipe (Hsl)</i>	5'-CTGAGATTGAGGTGCTGTGCG-3'	5'-CAAGGGAGGTGAGATGGTAAC-3'

(table continues)

Table 2 (continued)

Gene/primer name	Sequence (forward)	Sequence (reverse)
<i>Lpl</i>	5'-AACAAAGGTCAGAGCCAAGAG-3'	5'-CCATCCTCAGTCCCAGAAAAG-3'
<i>Ly6g</i>	5'-CACCTGAGACTTCTGCAAC-3'	5'-CTTCTATCTCCAGAGCAACGC-3'
<i>Mrp2 (Abcc2)</i>	5'-GCACTGTAGGCTCTGGGAAG-3'	5'-TGCTGAGGGACGTAGGCTAT-3'
<i>Mrp3</i>	5'-GGACTTCCAGTGTCTCAGAGG-3'	5'-AGCTGTGGCCTCGTCTAAAA-3'
<i>Mrp4</i>	5'-TGTTTGATGCACACCAGGAT-3'	5'-GACAAACATGGCACAGATGG-3'
<i>Nqo1</i>	5'-GCACTGATCGTACTGGCTCA-3'	5'-CATGGCATAGAGGTCCGACT-3'
<i>Osta</i>	5'-GTCTCAAGTGATGAAGTCCCA-3'	5'-TTGAGTGCTGAGTCCAGGTC-3'
<i>Ostb</i>	5'-GTATTTTCGTGCAGAAGATGCG-3'	5'-TTTCTGTTTGCCAGGATGCTC-3'
<i>Pnpla2 (Atgl)</i>	5'-ATATCCCACTTTAGCTCCAAGG-3'	5'-CAAGTTGTCTGAAATGCCGC-3'
<i>Pparg</i>	5'-ATGCCAGTACTGCCGTTTTC-3'	5'-GGCCTTGACCTTGTTCATGT-3'
<i>Saa2</i>	5'-CAGGATGAAGCTACTCACCAG-3'	5'-CTTCATGTCAAGTGTAGGCTCG-3'
<i>Scd1</i>	5'-TTCTTACACGACCACCACCA-3'	5'-CCGAAGAGGCAGGTGTAGAG-3'
<i>Slc10a1 (Ntcp)</i>	5'-CACCATGGAGTTCAGCAAGA-3'	5'-CCAGAAGGAAGCACTGAGG-3'
<i>Slc10a1 (Oatp1)</i>	5'-ATCCAGTGTGTGGGACAAT-3'	5'-GCAGCTGCAATTTTGAAACA-3'
<i>Tgfb1</i>	5'-CACCGGAGAGCCCTGGATA-3'	5'-TGTACAGCTGCCGCACACA-3'
<i>Timp1</i>	5'-ATTCAAGGCTGTGGGAAATG-3'	5'-CTCAGAGTACGCCAGGGAAC-3'
<i>Tlr4</i>	5'-TTCAGAACTTCAGTGGCTGG-3'	5'-TGTTAGTCCAGAGAACTTCCTG-3'

Fxr, farnesoid X receptor; Hsl, hormone-sensitive lipase; Mrp, multidrug-resistance protein.

Animal Models

Acute alcohol binge was conducted as previously described.⁸ After 6 hours of fasting, mice were given 31.5% (v/v) alcohol by gavage at a total accumulative dosage of 5 g/kg of body weight in four equally divided doses at 20-minute intervals. *Atg7*^{Δhep-ERT2} and control *Atg7*^{F/F} mice were injected with tamoxifen (6 mg/day s.c., for 2 days) 7 days before they were given acute alcohol binge as described in the previous sentence or as indicated in the figure legends. The control mice were given the same volume of water.

The chronic-plus-binge model was conducted as previously described.⁴³ Mice were acclimated with liquid control food for 5 days. The alcohol-treated groups were then given a liquid diet containing 5% (w/v) alcohol for 10 days. Pair-fed groups were given the same volume of control food. In the early morning of day 11, mice in the alcohol-treated groups were given a single dose of 31.5% (v/v) alcohol (5 g/kg of body weight) by gavage. Pair-fed mice were given an isocaloric dose of dextrin maltose by gavage. After 9 hours, the mice were euthanized, and blood and tissue samples were collected.

The protocols of all animal experiments were approved by the Institutional Animal Care and Use Committee of Indiana University (Indianapolis, Indiana).

Antibodies and Chemicals

Antibodies and PCR primers used in this study are listed in Tables 1 and 2, respectively. Tamoxifen (Sigma-Aldrich, St. Louis, MO) was diluted in corn oil. Alcohol (190 proof; Decon Labs, King of Prussia, PA) was diluted in water or added to a formulated liquid diet.⁴³ Bio-Serv Lieber-DeCarli '82 Shake and Pour alcohol liquid diet (catalog number

F1258SP; Fisher Scientific, Pittsburgh, PA) was used as the alcohol diet. Bio-Serv Lieber-DeCarli '82 Shake and Pour control liquid diet (catalog number F1259SP; Fisher Scientific) was used as the control diet.

Serum Biochemistry Analysis

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides (TG), and total cholesterol (TCHO) were measured using kits from Pointe Scientific (Canton, MI) according to the manufacturer's protocol. Serum total bile acids (TBA) were measured using a TBA assay kit from Diazyme Laboratories, Inc. (Poway, CA).

Hepatic Lipid Content Analysis

A piece of liver tissue was weighed and incubated in 1 mL of chloroform-methanol mix (2:1, v/v) with shaking for 1 hour at room temperature to extract lipids. After the addition of 200 μL of water, samples were vortexed and centrifuged at 3000 × g for 5 minutes. The lower lipid phase was collected and dried at room temperature in a chemical hood. The lipid pellet was resuspended in 60 μL of *tert*-butanol and 40 μL of a Triton X-114 methanol (2:1, v/v) mix. TG and TCHO contents were measured using respective kits from Pointe Scientific. The lipid contents hence were normalized with the tissue weight.

Hepatic TBA Content Analysis

Liver tissue samples (100 mg) were homogenized in 1 mL of 90% alcohol and incubated at 55°C overnight. The lysates were centrifuged at 9600 × g for 10 minutes. The supernatants were collected and measured for TBA

concentration using a TBA assay kit from Diazyme Laboratories, Inc.

Immunoblotting Analysis

The liver samples were homogenized in the radio immunoprecipitation assay buffer containing a protease cocktail. Supernatant was collected after centrifugation at $13,800 \times g$ for 12 minutes. Protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). The proteins were separated on SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride membranes, which were then blocked with 5% bovine serum albumin or 5% skim milk for 1 hour at room temperature. Membranes were incubated overnight with the appropriate primary antibody and then washed with Tris-buffered saline and 0.1% Tween 20 before being incubated with the horseradish peroxidase-coupled secondary antibodies at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific/Pierce). The images were taken digitally with the ChemiDoc Imaging System (Bio-Rad, Hercules, CA). Densitometry was measured using the companion software, Image Lab software version 6.0.1 (Bio-Rad), and the values were normalized to that of β -actin or glyceraldehyde phosphate dehydrogenase, which were then converted to fold changes of the control.

RNA Isolation and Real-Time Quantitative PCR Analysis

Total RNA was prepared from liver samples using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's protocols. cDNA was synthesized using oligo dT primers and an M-MLV Reverse Transcriptase System (Life Technologies, Carlsbad, CA). Real-time quantitative PCR analysis was performed on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies) using SYBR Green master mixes (Life Technologies). All real-time quantitative PCR results were normalized to the level of β -actin, and the gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method.

Histologic Study

Liver samples were harvested, rinsed with phosphate-buffered saline, and fixed in 10% formalin overnight. Samples were further fixed in 70% alcohol and processed as paraffin-embedded blocks. The paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin,

anti-F4/80, or Masson's trichrome C. Photomicrographs were taken using an Eclipse E200 light microscope (Nikon Instruments, Melville, NY) equipped with a SPOT RT Slider color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Area with positive F4/80 or Masson's trichrome C staining were quantified using ImageJ software version 1.51 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>); at least four random fields of each section from each mouse liver were used for quantification.

Immunofluorescence Microscopy

Paraffin sections were subjected to antigen retrieval treatment using the citrate buffer (0.01 mol/L, pH 6.0) after deparaffinization. Slides were blocked with 5% goat serum in phosphate-buffered saline containing 0.1% Triton X (PBS-Tx) for 1 hour and then incubated with primary antibodies diluted in 1% bovine serum albumin/PBS-Tx overnight at 4°C. Sections were washed with phosphate-buffered saline, followed by incubation with fluorochrome-conjugated secondary antibodies. Hoechst 33342 was used for nucleus staining. Images were obtained using as Eclipse TE 200 epi-immunofluorescence microscope (Nikon) and the companion NIS-Elements software version AR3.2 (Nikon). Quantification was performed using ImageJ software, at least four random fields of each section from each mouse liver were analyzed.

Alcohol Clearance Analysis

Mice were given a single dose of 31.5% (v/v) alcohol by gavage (5 g/kg of body weight). Blood was sampled from the tail vein before treatment and at 1, 3, and 9 hours after gavage. Alcohol concentration in the plasma was measured using an alcohol colorimetric/fluorometric assay kit (Bio-Vision Inc., Milpitas, CA).

Statistical Analysis

Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL) or Excel 2016 (Microsoft Corp., Redmond, WA). The *t*-test was used to determine the significance of differences between two groups. The significance of differences between more than two treatment groups was determined using one-way analysis of variance followed by the Duncan post-hoc test. Data are expressed as means $\pm$ SEM. Results were considered statistically significant at $P < 0.05$.

Figure 1 Genetic deletion of autophagy genes exacerbates liver injury induced by acute alcohol binge. **A:** Scheme of acute binge treatment. Mice were treated with a single dose of alcohol (ethanol) or the same volume of distilled water (ddH₂O) by gavage after 6 hours of fasting. Mice were euthanized 16 hours later. **B and C:** Liver weight and body weight (L/B) ratio, the serum levels of ALT, AST, ALP, and the hepatic levels of triglycerides (TG) and total cholesterol (TCHO) were measured in liver-specific *Atg5*-deficient mice 8 to 34 weeks old (**B**) and *Atg7*-deficient mice 8 to 16 weeks old (**C**). **D:** Scheme of the administration of alcohol to *Atg7* ^{Δ hep-ERT2} mice. Tamoxifen (TMX) was injected on days 1 and 2 to induce the deletion of *Atg7*. Alcohol or water was then given by oral gavage on day 8. *Atg7*^{F/F} mice were used as control. **E:** L/B weight ratio, the serum levels of ALT, AST, ALP, and the hepatic levels of TG and TCHO were measured in induced *Atg7* ^{Δ hep-ERT2} mice 8 to 15 weeks old. Data are expressed as means $\pm$ SEM. $n = 3$ to 5 (**E**); $n = 3$ to 6 (**C**); $n = 7$ to 14 (**B**). * $P < 0.05$ versus nontreated F/F control; [†] $P < 0.05$ versus nontreated Δ hep control; [‡] $P < 0.05$ versus treated F/F control.

Figure 2 Acute deletion of *Atg7* enhances alcohol-induced liver injury in a chronic-plus-binge model. **A:** Scheme of the chronic-plus-binge treatment. Mice were acclimated with liquid diet for 5 days. *Atg7^{F/F}* and *Atg7^{Δhep-ERT2}* mice were injected with tamoxifen (TMX) on days 3 and 4 during acclimation. After acclimation, mice were randomly divided into two groups and given a liquid diet with alcohol (EtOH-fed) or with maltose dextrin (Pair-fed) for 10 days, followed by a single gavage of alcohol (5 g/kg) or isocaloric maltose dextrin. Mice were analyzed 9 hours later. **B:** Representative Western blot images of protein expression in the livers of *Atg7^{F/F}* and *Atg7^{Δhep-ERT2}* mice after alcohol or pair-fed treatment. Asterisk indicates onspecific bands. **C:** Densitometry of each protein band was conducted. The density values of *Atg7*, p62, or Nad(p)h quinone dehydrogenase (Nqo)-1 were normalized to that of β-actin. The density of microtubule-associated protein 1A/1B-light chain (Lc)-3II was normalized to that of Lc3I. **D:** The mRNA levels of *Gstm1* and *Nqo1* in the livers of *Atg7^{F/F}* and *Atg7^{Δhep-ERT2}* mice after alcohol or pair-fed treatment. Expression levels were normalized to that of β-actin. **E and F:** Liver weight and body (L/B) weight ratio, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bile acids (TBA) in *Atg7^{F/F}* and *Atg7^{Δhep-ERT2}* mice after alcohol or pair-fed treatment. Mice were used at the age of 8 to 12 weeks. Data are expressed as the means ± SEM fold changes over those of pair-fed *Atg7^{F/F}* mice. *n* = 4 (A, C, and D); *n* = 4 to 11 (G). **P* < 0.05 versus nontreated F/F control; †*P* < 0.05 versus nontreated Δhep control; ‡*P* < 0.05 versus treated F/F control. *Gstm1*, glutathione S-transferase M1; and Nqo, Nad(p)h quinone dehydrogenase 1; Sqstm, sequestosome.

Results

Effects of Genetic Deletion of a Key Autophagy Gene on Liver Injury Induced by Acute Alcohol Binge

To evaluate the effects of hepatic autophagy deficiency on ALDs, one single binge-dose of alcohol was administered to the *Atg5^{Δhep}* mice and the control mice (Figure 1A). Hepatic *Atg5* deficiency was associated with significant hepatomegaly and liver injury (Figure 1B). Serum levels of ALT, AST, and ALP, and hepatic TG, but not hepatic TCHO, were further elevated in *Atg5^{Δhep}* mice after alcohol treatment (Figure 1B), suggesting that acute alcohol treatment exacerbated the liver pathology in these mice. A protective function of autophagy against hepatic steatosis induced by

acute alcohol administration has been shown in several studies.^{8,15,30,31}

Genetic deletion of *Atg7* in hepatic parenchymal cells caused a more severe liver phenotype than that seen in *Atg5^{Δhep}* mice⁴⁰ (Figure 1C). In these mice, a single binge dose of alcohol did not further lead to notable increases in serum levels of liver markers or hepatic TG level (Figure 1C). It seemed that a constitute deletion of *Atg7* in hepatic parenchymal cells caused too severe an injury so that the relative minor effects of acute alcohol treatment were blunted.

To separate the injury effect caused by constitutive *Atg7* deletion and alcohol treatment, *Atg7^{Δhep-ERT2}* mice were injected with tamoxifen to induce an acute deletion of *Atg7* in the liver before alcohol treatment (Figure 1D). The deficiency of hepatic autophagy in *Atg7^{Δhep-ERT2}* mice after

Figure 3 Constitutive deletion of hepatic *Atg7* enhances alcohol-induced liver injury in a chronic-plus-binge model. **A:** Scheme of the chronic-plus-binge treatment. *Atg7^{F/F}* and *Atg7^{Δhep}* mice were acclimated with liquid diet for 5 days. After acclimation, mice were randomly divided into two groups and given alcohol (EtOH) or control diet as in Figure 2. **B:** Representative Western blot images of protein expression in the liver from *Atg7^{F/F}* and *Atg7^{Δhep}* mice after alcohol or pair-fed treatment. **C:** The density of *Atg7*, p62, or NAD(P)H quinone dehydrogenase (Nqo)-1 was normalized to that of glyceraldehyde phosphate dehydrogenase (Gapdh). The density of microtubule-associated protein 1A/1B-light chain (Lc)-3II was normalized to that of Lc3I. **D** and **E:** Liver weight and body (L/B) weight ratio, serum levels of alanine aminotransferase (Alt), aspartate aminotransferase (Ast), alkaline phosphatase (ALP), and total bile acids (TBA) in *Atg7^{F/F}* and *Atg7^{Δhep}* mice after alcohol or pair-fed treatment. **F:** Hepatic triglycerides (TG) and total cholesterol (TCHO) levels in *Atg7^{F/F}* and *Atg7^{Δhep}* mice given alcohol or pair-fed treatment. Mice were used at the age of 8 to 15 weeks. Data are expressed as the means ± SEM fold changes over those of pair-fed *Atg7^{F/F}* mice. *n* = 3 (**C**), *n* = 3 to 5 (**F**). **P* < 0.05 versus nontreated F/F control; †*P* < 0.05 versus nontreated Δhep control; ‡*P* < 0.05 versus treated F/F control. LE, long exposure; SE, short exposure; Sqstm, sequestosome.

tamoxifen injection was confirmed by immunoblotting demonstration of decreases in hepatic microtubule-associated protein 1A/1B–light chain (LC)-3II/LC3I ratio and the accumulation of sequestosome (SQSTM1)-1/p62 protein (Supplemental Figure S1). Obvious hepatomegaly and serum elevation in liver injury markers in the *Atg7^{Δhep-ERT2}* mice were not observed at 9 days after induction; however, the serum markers and hepatic TG levels were significantly increased after the acute alcohol treatment in these mice (Figure 1E). The results were consistent with previous results in which autophagy was modified transiently and acutely using chemicals or gene knockout.^{8,9,15} Therefore, an acute deletion of *Atg7*, while not causing

liver injury alone, rendered the hepatocytes more susceptible to alcohol-induced injury.

Effects of Acute Deletion of *Atg7* Gene on Alcohol-Induced Liver Injury in a Chronic-Plus-Binge Model

To further examine the impact of hepatic autophagy–deficiency alcohol exposure, the response of these genetically altered mice to a more chronic alcohol exposure was analyzed using a recently established chronic-plus-binge model⁴³ (Figure 2A). *Atg7^{Δhep-ERT2}* and *Atg7^{F/F}* mice were given tamoxifen on days 3 and 4 in a 5-day

acclimation period, followed by an alcohol diet for 10 days plus an acute alcohol binge at the end of the feeding period. Autophagy deficiency was confirmed as indicated by reduced levels of *Atg7* and LC3II/LC3I ratio, and elevated SQSTM1/p62, in the livers of *Atg7*^{Δhep-ERT2} mice (Figure 2, B and C). Consistently, NF erythroid 2-related factor (Nrf)-2 was activated, and its targets, glutathione *S*-transferase M1 and Nad(p)h quinone dehydrogenase 1, were expressed at much higher levels in the livers of *Atg7*^{Δhep-ERT2} mice (Figure 2, B–D). Notably, alcohol treatment did not alter these changes due to *Atg7* deletion, although the LC3II/LC3I ratio was increased in the floxed normal mice (Figure 2, B–D).

A longer duration of *Atg7* deletion in the *Atg7*^{Δhep-ERT2} mice resulted in enlarged livers and liver injury in the chronic-plus-binge model (Figure 2E) as compared with the shorter duration of deletion in the binge model (Figure 1E). But as in the binge model, hepatomegaly and liver injury phenotype (Alt and Ast levels), but not ALP or TBA, were further augmented by alcohol treatment (Figure 2F). These results suggest a protective function of *Atg7*-mediated autophagy in alcohol-induced liver injury.

To determine potential factors that promote injury in autophagy deficiency, the extent of steatosis was examined. Hepatic TG (Figure 2G), but not TCHO (Supplemental Figure S2A), level was altered after alcohol treatment, indicating the presence of steatosis in the livers of both *Atg7*^{F/F} and *Atg7*^{Δhep-ERT2} mice, but the difference was not significant between the two types of mice. Consistently, alcohol-induced elevation of fatty acid oxidation, as measured by the serum level of β-hydroxybutyrate, was not altered in the absence of *Atg7* (Figure 2G).

Histologic examination suggested no obvious differences in general structure and fibrosis, but an elevated presence of Kupffer cells in autophagy-deficient livers (Supplemental Figure S2, B–E). Two acute-phase proteins, lipocalin-2 and serum amyloid A2, which are secreted by hepatocytes and other extrahepatic tissues in response to stress or injury, have been recently found to promote alcoholic liver pathology.^{44,45} Indeed, the expression levels of both lipocalin-2 and serum amyloid A2 were elevated in *Atg7*^{Δhep-ERT2} livers, likely in response to autophagy deficiency–induced stress (Supplemental Figure S3A). After the 10-day chronic-plus-binge alcohol treatment, the expression levels of lipocalin-2 and serum amyloid A1 were elevated in floxed-control mice, but were not higher than the already

elevated level in the *Atg7*-deficient livers (Supplemental Figure S3A). In addition, the mRNA levels of several inflammatory cytokines and inflammatory cell markers were higher in alcohol-treated *Atg7*^{Δhep-ERT2} livers than in the same treated floxed mice (Supplemental Figure S3, B and C). But the levels of several of these markers, such as C-C motif chemokine ligand 2 and Toll-like receptor 4, were also elevated in *Atg7*^{Δhep-ERT2} livers without alcohol treatment. These findings suggest that the protective effect of autophagy may not be entirely related to modulation of inflammation.

Effects of Constitutive Deletion of Hepatic *Atg7* on Alcohol-Induced Liver Injury in a Chronic-Plus-Binge Model

Chronic alcohol treatment caused more injury than did acute binge treatment (Figure 2E versus Figure 1E), which could allow the detection of *Atg7*-mediated protection even in the constitutive deletion model. Thus, in the *Atg7*^{Δhep} and the control mice, alcohol diet was administered using the chronic-plus-binge scheme (Figure 3A). As in the inducible *Atg7*^{Δhep-ERT2} mice, the reduced *Atg7* level and LC3II/LC3I ratio, and the accumulation of SQSTM1 and Nad(p)h quinone dehydrogenase 1 were not significantly altered in the livers of *Atg7*^{Δhep} mice with an alcohol diet (Figure 3, B and C). Alcohol diet was associated with enhanced hepatomegaly (Figure 3D) and liver injury, based on increased serum levels of Alt and Ast (Figure 3E). There were no significant differences in the serum levels of ALP or TBA with or without alcohol treatment (Figure 3E). In addition, total hepatic TG and TCHO levels were increased in *Atg7*-deficient liver with alcohol treatment (Figure 3F). These results indicate that constitutive loss of *Atg7* may exacerbate injury caused by chronic alcohol treatment as well.

Effects of Constitutive Deletion of Hepatic *Atg5* on Liver Injury in a Chronic-Plus-Binge Model

A single dose of alcohol-binge treatment was associated with enhanced liver injury in *Atg5*^{Δhep} mice (Figure 1B). Chronic alcohol treatment in *Atg5*^{Δhep} mice (Figure 4A) was not associated with significant alterations in the repression of autophagy in these mice (Figure 4, B–D). Alcohol

Figure 4 Constitutive deletion of hepatic *Atg5* does not enhance liver injury in a chronic-plus-binge model. **A:** Scheme of the chronic-plus-binge alcohol treatment. *Atg7*^{F/F} and *Atg7*^{Δhep-ERT2} mice were injected with tamoxifen on days 3 and 4 during acclimation. After acclimation, mice were randomly divided into two groups and given a liquid diet with alcohol (EtOH-fed) or with maltose dextrin (Pair-fed) for 10 days, followed by a single gavage of alcohol (5 g/kg) or isocaloric maltose dextrin. Mice were analyzed 9 hours later. **B:** Representative Western blot images of protein expression in the liver from *Atg5*^{F/F} and *Atg5*^{Δhep} mice after alcohol or pair-fed treatment. Anti-*Agt12* antibody was used to detect the *Atg12-Atg5* conjugate. **C:** The density of *Atg5-Atg12*, p62, or Nad(p)h quinone dehydrogenase (Nqo)-1 was normalized to that of β-actin. The density of microtubule-associated protein 1A/1B-light chain (Lc)-3II was normalized to that of Lc3I. **D:** mRNA levels of *Gstm1* and *Nqo1* in livers of *Atg5*^{F/F} and *Atg5*^{Δhep} mice after alcohol or pair-fed treatment. Expression levels were normalized to that of β-actin. **E–H:** Liver weight and body (L/B) weight ratio, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in *Atg5*^{F/F} and *Atg5*^{Δhep} mice after alcohol or pair-fed treatment. **I–L:** Hepatic triglycerides (TG) and total cholesterol (TCHO) levels in *Atg5*^{F/F} and *Atg5*^{Δhep} mice given alcohol or pair-fed treatment. Data are expressed as the means ± SEM fold changes over pair-fed *Atg5*^{F/F} mice. *n* = 4 (C); *n* = 6 (D); *n* = 7 to 18 (E–H). **P* < 0.05 versus nontreated F/F control; [†]*P* < 0.05 versus nontreated Δhep control; [‡]*P* < 0.05 versus treated F/F control.

Figure 5 Reduced inflammation and fibrosis in constitutive *Atg5*—deficient livers after chronic-plus-binge alcohol treatment. **A–E:** *Atg5*^{F/F} and *Atg5*^{ΔHep} mice (18 to 24 weeks old) were injected with tamoxifen on days 3 and 4 during acclimation. After acclimation, mice were randomly divided into two groups and given a liquid diet with alcohol (EtOH-fed) or with maltose dextrin (Pair-fed) for 10 days, followed by a single gavage of alcohol (5 g/kg) or isocaloric maltose dextrin. Mice were analyzed 9 hours later. Liver sections were subjected to hematoxylin and eosin (H&E) staining (**A**), F4/80 stain and quantified (**B** and **C**), and Masson's trichrome stain and quantified (**D** and **E**). **F:** The hepatic hydroxyproline level was determined. **G:** Representative Western blot images of α -smooth muscle actin (Sma) protein and densitometric analysis. The density of α -Sma protein was normalized to that of β -actin. **H:** The mRNA expression of fibrosis-related genes in the livers of *Atg5*^{F/F} and *Atg5*^{ΔHep} mice given alcohol or control diet. Expression levels of indicated genes were determined by real-time quantitative -PCR and were normalized to that of β -actin. Data are expressed as the means $\pm$ SEM fold-changes over those of *Atg5*^{F/F} mice given the pair-fed diet. $n = 4$ (**G**); $n = 5$ to 6 (**F**); $n = 6$ (**H**). * $P < 0.05$ versus nontreated F/F control; † $P < 0.05$ versus nontreated Δ hep control; ‡ $P < 0.05$ versus treated F/F control. Original magnification, $\times 200$ (**A**, **B**, and **D**). Ctgf, connective tissue growth factor; Gfap, glial fibrillary acidic protein; Tgf, transforming growth factor; Timp1, tissue inhibitor of metalloproteinase 1.

treatment was associated with slightly increased hepatomegaly in younger *Atg5*^{ΔHep} mice (Figure 4E), and with significant decreases in the serum levels of Alt, Ast, and ALP in both younger and older *Atg5*^{ΔHep} mice (Figure 4, F–H). The reduced injury was observed in both male and female mice (Supplemental Figures S4A and S5A). Total hepatic TG level but not TCHO level was still increased in

Atg5^{ΔHep} and in *Atg5*^{F/F} mice after alcohol treatment (Figure 4, I–L, and Supplemental Figures S4B and S5B), indicating an expected hepatic response to alcohol. Consistent with a higher level of total hepatic TG in *Atg5*^{ΔHep} mice after alcohol treatment, hematoxylin and eosin staining of the liver sections also showed a more prominent hepatic steatosis in these mice (Figure 5A).

It is well established that elevation of hepatic TG level is an immediate consequence of alcohol stimulation, and that steatosis is also a major pathologic phenomenon in AFLD.^{46,47} Generally, alcohol can affect hepatic lipid metabolism by accelerating lipogenesis, decelerating lipid breakdown, and causing defective hepatic lipid export.⁴⁷ The mRNA expression levels of several lipid metabolism related—genes in the *Atg5^{ΔHep}* mice were altered from those in the *Atg5^{F/F}* mice before and/or after alcohol treatment (Supplemental Figure S6, A–E). The expression levels of genes involved in lipid secretion, such as apolipoprotein B (*Apob*) and *Apoe*, were reduced, whereas the expression levels of genes involved in lipid uptake, such as cluster of differentiation 36 (*Cd36*) and lipoprotein lipase (*Lpl*), were increased before or after alcohol treatment. Although the expression levels of the genes involved in lipogenesis, except stearoyl-Coenzyme (Co)-A desaturase 1 (*Scd1*), were not elevated, the expression levels of the genes involved in lipolysis, such as adipose triglyceride lipase (*Atgl*) and hormone-sensitive lipase E (*Lipec*), and the genes in lipid oxidation, such as acyl-CoA dehydrogenase, long-chain (*Acadl*), acyl-CoA dehydrogenase medium chain (*Acadm*), peroxisomal acyl-CoA oxidase 1 (*Acox1*), and peroxisome proliferator-activated receptor α (*Ppara*), were repressed. Moreover, the expression levels of the genes involved in lipid oxidation were overall reduced in *Atg5^{ΔHep}* mice (Supplemental Figure S6E); however, the serum level of β -hydroxybutyrate remained elevated in response to alcohol stimulation (Supplemental Figure S6F). These changes may overall favor the accumulation of lipids in the cells and decrease lipid use.

The number of F4/80⁺-staining macrophages was significantly increased in *Atg5^{ΔHep}* livers, similar to what was observed in *Atg7^{ΔHep}* livers.⁴⁸ The level of macrophage expansion was noticeably reduced with alcohol treatment (Figure 5, B and C). The expression levels of many inflammation-related genes were not significantly altered in *Atg5*-deficient livers, except C-C motif chemokine ligand 2, which was increased in *Atg5*-deficient livers (Supplemental Figure S7). Alcohol treatment was associated with reduce levels of C-C motif chemokine ligand 2 and several other cytokines and inflammatory cell markers in these mice

Figure 6 Reduced cholestasis in constitutive *Atg5*-deficient livers after chronic-plus-binge alcohol treatment. *Atg5^{F/F}* and *Atg5^{ΔHep-ERT2}* mice (18 to 24 weeks old) were injected with tamoxifen on days 3 and 4 during acclimation. After acclimation, mice were randomly divided into two groups and given a liquid diet with alcohol (EtOH-fed) or with maltose dextrin (Pair-fed) for 10 days, followed by a single gavage of alcohol (5 g/kg) or isocaloric maltose dextrin. Mice were analyzed 9 hours later. **A**: Liver sections were immunostained with anti-cytokeratin (Ck)-19. **B**: Ck19-positive areas were quantified. **C**: Serum levels of total bile acids (TBA). **D** and **E**: The levels of TBA in each gram of liver (**D**) or in the whole liver (**E**) were determined. Data are expressed as means $\pm$ SEM. $n = 3$ (**D** and **E**); $n = 4$ to 5 (**B**); $n = 4$ to 7 (**A**, female); $n = 6$ to 12 (**A**, male). * $P < 0.05$ versus nontreated *F/F* control; † $P < 0.05$ versus nontreated *ΔHep* control; ‡ $P < 0.05$ versus treated *F/F* control. Scale bar = 0.1 mm.

Figure 7 Constitutive deletion of hepatic *Atg5* alters the expression of genes related to alcohol metabolism and serum alcohol clearance. *Atg5*^{F/F} and *Atg5* ^{Δ hep-ERT2} mice (18 to 24 weeks old) were injected with tamoxifen on days 3 and 4 during acclimation. After acclimation, mice were randomly divided into two groups and given a liquid diet with alcohol (EtOH-fed) or with maltose dextrin (Pair-fed) for 10 days, followed by a single gavage of alcohol (5 g/kg) or isocaloric maltose dextrin. Mice were analyzed 9 hours later. **A** and **B**: The mRNA levels of genes related to the oxidation of alcohol (**A**) or acetaldehyde (**B**) in the liver. **C**: The protein levels of key alcohol-metabolizing enzymes in the liver. β -Actin [for cytochrome P450 (Cyp)-2e1] and glyceraldehyde phosphate dehydrogenase (Gapdh) [for aldehyde dehydrogenase (Aldh)-1/2 and alcohol dehydrogenase (Adh)] were used as loading control. **D**: Densitometry was conducted, and the density levels were normalized to that of loading control. **E**: *Atg5*^{F/F} and *Atg5* ^{Δ hep} mice at the age of 13 to 20 weeks were given a single dose of alcohol binge (5 g/kg). Plasma alcohol concentrations were then measured at 1, 3, and 9 hours later. Data are expressed as the means $\pm$ SEM fold changes over *Atg5*^{F/F} mice given pair-fed treatment. $n = 3$ to 5 (**E**); $n = 4$ (**D**); $n = 6$ (**A** and **B**). * $P < 0.05$ versus nontreated F/F control; $^{\dagger}P < 0.05$ versus nontreated Δ hep control; $^{\ddagger}P < 0.05$ versus treated F/F control; $^{\S\S\S}P < 0.001$. Atg, autophagy-related protein; Cat, catalase.

(Supplemental Figure S7, A and B). On the other hand, the expression levels of lipocalin-2 and serum amyloid A2 remained higher (Supplemental Figure S7C). Inflammation plays important roles in the pathogenesis of ALFD.⁴⁹ In particular, C-C motif chemokine ligand 2 is associated with the severity of, and neutrophil infiltrates in, ALD.⁵⁰ Thus, the reduction of inflammation in chronic alcohol treatment condition may contribute to the reduced injury level in *Atg5* ^{Δ hep} livers.

Interestingly, the significantly elevated fibrosis in *Atg5*-deficient livers was also decreased by chronic alcohol treatment, as shown by Masson's trichrome staining (Figure 5, D and E), hydroxyproline level (Figure 5F), and the expression of α -smooth muscle actin, at the protein (Figure 5G) and the mRNA levels (Figure 5H). The levels of other fibrosis-related genes, such as connective tissue growth factor (*Ctgf*), transforming growth factor β 1 (*Tgfb1*), tissue inhibitor of metalloproteinase 1 (*Timp1*), and glial

fibrillary acidic protein (*Gfap*), were not significantly different between alcohol treatment and control diet (Figure 5H). This improving effect of alcohol is reminiscent of findings from an earlier study showing a potentially protective effect of light alcohol consumption on hepatocellular injury and liver fibrosis in patients with NAFLD.⁵¹ It seems that alcohol may have variable effects on hepatic fibrosis in different pathologic conditions, which may partially explain the improvement in liver fibrosis in *Atg5*-deficient livers after alcohol treatment.

Reduction in Cholestatic Injury in *Atg5*-Deficient Livers after Alcohol Treatment

Autophagy deficiency in the liver can cause cholestatic injury, which is accompanied by a significant ductular reaction.^{48,52} Alcohol treatment was associated with a reduction in this pathologic change in *Atg5*^{Δhep} livers, as measured by the level of cytokeratin-19—positive ductular cell (Figure 6, A and B). Consistently, the serum level of TBA was also decreased after alcohol treatment in *Atg5*^{Δhep} mice if both sexes and at both the older (Figure 6C) and younger (Supplemental Figure S8A) ages. Similar chronic alcohol treatment in *Atg7*^{Δhep-ERT2} (Figure 2F) or *Atg7*^{Δhep} (Figure 3E) mice was not associated with reductions in the elevated serum TBA levels in these mice. Unexpectedly, the hepatic TBA load remained high in *Atg5*^{Δhep} mice even after alcohol treatment, regardless of age (Figure 6, D and E, and Supplemental Figure S8, B and C). Interestingly, *Atg5*^{Δhep} mice given one dose of alcohol binge also showed a reduced serum bile acid level, in contrast to *Atg7*^{Δhep} and *Atg7*^{Δhep-ERT2} mice given the same acute treatment (Supplemental Figure S8D).

The mRNA levels of genes related to bile acid metabolism were analyzed to determine any potential effects of alcohol. The expression levels of many of these genes were decreased in *Atg5*-deficient livers, as previously reported⁵² (Supplemental Figure S9). However, overall, the mRNA levels of genes related to bile acid synthesis were not further significantly altered in *Atg5*-deficient liver after alcohol treatment (Supplemental Figure S9, A and B). In addition, the expression of genes related to farnesoid X receptor (FXR) signaling remained low in *Atg5*-deficient livers (Supplemental Figure S9C). The impact of autophagy deficiency on the expression of the bile acid transporters, most of which were not further significantly affected by the alcohol (Supplemental Figure S9, D–F), has been reported before.⁵² Exceptions were the expression levels of organic solute transporter β in *Atg5*^{Δhep} liver (Supplemental Figure S9E) and organic anion transporting polypeptide 1 (OATP1) in *Atg5*^{F/F} liver (Supplemental Figure S9F), which were significantly decreased after alcohol treatment. Gene-expression results indicated that the influence of alcohol on bile acid metabolism in *Atg5*-deficient livers would likely be limited.

Hepatic *Atg5* Deficiency and the Expression of Genes Related to Alcohol Metabolism and the Plasma Alcohol Clearance Rate in Mice

Acetaldehyde along with other alcohol metabolites are considered to be involved in ALDs.⁵³ Since alcohol-induced liver injury is affected by alcohol metabolism, it was tested whether alcohol metabolism was affected in *Atg5*-deficient livers. The expression levels of genes related to alcohol metabolism were analyzed. Notably, the mRNA levels of several alcohol dehydrogenase (*Adh*)-related genes, including *Adh* classes I to IV (*Adh1*, -4, -5, and -7, respectively), were decreased in *Atg5*-deficient livers (Figure 7A). The expression levels of cytochrome P450 (*Cyp*) family 2 subfamily E member 1 and catalase were also decreased in *Atg5*-deficient livers (Figure 7A). Interestingly, the mRNA levels of *Adh1*, *Adh4*, *Adh5*, *Cyp2e1*, and *Cat* were also decreased in the livers of *Atg5*^{F/F} mice after alcohol treatment.

The mRNA levels of several aldehyde dehydrogenases (*Aldh*) in the liver were analyzed. Significant decreases in the mRNA expression levels of *Aldh2*, -4a1, -5a1, -7a1, -8a1, and -9a1 were observed in the *Atg5*-deficient livers (Figure 7B). Although alcohol did not seem to cause further changes in the expression levels of most of these genes, it was associated with elevated *Aldh1a3* gene expression in *Atg5*-deficient livers, and elevated *Aldh18a1* gene expression in *Atg5*^{F/F} livers (Figure 7B). The expression levels of several other *Aldh* family members, including *Aldh1a1*, -1a2, -1b1, -11l, -112, -3a2, -3b1, and -6a1, were not significantly changed in the livers of *Atg5*-deficient mice or with alcohol treatment (Supplemental Figure S10).

The protein levels of *Adh*, *Cyp2e1*, and *Aldh1/2* were further analyzed. Protein stability of CYP2E1 is crucial to its induction by alcohol.⁵⁴ The protein levels of *Cyp2e1* and *Adh* were decreased in older (18 to 24 weeks) (Figure 7, C and D) and younger (8 to 11 weeks) (Supplemental Figure S11) *Atg5*-deficient livers. However, consistent with findings from a previous study,⁵⁴ the protein level of *Cyp2e1* was significantly increased after alcohol treatment in the livers of both *Atg5*^{Δhep} and *Atg5*^{F/F} mice (Figure 7, C and D, and Supplemental Figure S9). No consistent changes in *Adh* or *Aldh1/2* in either older (18 to 24 weeks) or younger (8 to 1 week) mice were observed (Figure 7, C and D, and Supplemental Figure S9). The gene-expression and protein-expression data indicate that *Atg5* deficiency in the liver alters the expression of genes related to alcohol metabolism; however, alcohol itself did not seem to have affected the mRNA levels of most of these genes. These changes could result in a lower metabolism of alcohol and therefore lower levels of metabolites of alcohol that are known to be involved in its toxicity.

The lower metabolism of alcohol might cause a higher plasma alcohol level in the *Atg5*-deficient mice. To examine this possibility, *Atg5*^{Δhep} and *Atg5*^{F/F} mice were given a single dose of alcohol by gavage, and the plasma levels of alcohol were

measured at different time points (Figure 7E). The plasma alcohol in nontreated mice was undetectable. The plasma levels of alcohol at 1 hour after gavage were comparable between *Atg5^{Δhep}* and *Atg5^{F/F}* mice, suggesting that there was no difference in the intestinal absorption of alcohol (Figure 7E). Interestingly, plasma levels of alcohol were slightly lower at 3 hours and dramatically lower at 9 hours in *Atg5^{Δhep}* mice than those in *Atg5^{F/F}* mice (Figure 7E). These results indicate an increased plasma alcohol clearance in *Atg5^{Δhep}* mice despite reductions in the expression of genes in the alcohol-metabolism pathway. Other mechanisms may therefore account for the alcohol elimination in the autophagy-deficient mice.

Discussion

Effects of How Autophagy Is Inhibited and How Alcohol Is Administered on Alcohol-Induced Liver Injury

This is the first study in which the role of autophagy in alcohol-induced liver injury was assessed through a genetic approach to disrupt the formation of autophagosomes using either inducible or constitutive knockout of two different autophagy genes, *Atg7* and *Atg5*. These models differ in the duration of autophagy deficiency, the specific gene being affected, and the severity of endogenous liver injury. This study thus allows an extensive analysis of the interaction of alcohol administration and autophagy function in the context of acute and chronic liver injury.

Previous studies have examined the role of autophagy by using siRNA-mediated knockdown of *Atg7*, or by interference of the lysosomal functions with chloroquine or transcription factor EB.^{8,9,12,15,31} In these studies, the protective role of autophagy in alcohol-induced liver injury had been shown in the models of an acute binge, a chronic 4-week Liber-DeCarli feeding, and a chronic-plus-binge feeding. The protective effects of autophagy in the gastric-infusion model had also been observed with the use of chloroquine (J.Z. and X.-M.Y., unpublished data). In the infusion model, alcohol (up to 37.1% of calorie uptake) was given in either an 8% low-fat or 40% high-fat base over a period of 28 days.⁵⁵ The administration of chloroquine led to increased hepatic TG and blood ALT.

Here we have confirmed the protective effect of autophagy in the inducible *Atg7*-deficient livers (*Atg7^{Δhep-ERT2}*) using both the acute binge model and a new chronic-plus-binge model. In both models, autophagy deficiency via *Atg7* deletion was induced only shortly before alcohol treatment. The inducible approach minimized the liver injury caused by autophagy deficiency alone, so that the impact of autophagy deficiency on alcohol-induced injury could be directly examined. However, mice harboring constitutive deletion of *Atg7* (*Atg7^{Δhep}*) had different responses to alcohol treatment in the acute binge and chronic-plus-binge models. Liver injury was noticeably enhanced in *Atg7^{Δhep}* mice after the chronic-plus-binge treatment, but not after acute binge treatment. The

difference in the liver injury after alcohol treatments between the two *Atg7*-deletion models may have been due to the difference in the durations of gene deletion, which would have resulted in varying levels of autophagy dysfunction and liver injury. It is possible that a higher level of detrimental SQSTM1/p62 accumulation and NRF2 activation in the *Atg7^{Δhep}* mice (Figure 3, B and C, and Figure 2, B and C) may have accounted for this resistance.

In mice with constitutive *Atg5* deletion in the liver (*Atg5^{Δhep}*), liver injury was noticeably enhanced after a single dose of binge treatment, but paradoxically improved after the chronic-plus-binge treatment. In the latter, the improved presentation included lower blood levels of liver enzymes and TBA and reduced hepatic inflammation, ductular reaction, and fibrosis. It is possible that the level of NRF2 activation could have been in part involved in the difference between the *Atg5^{Δhep}* and *Atg7^{Δhep}* mice. Thus the levels of SQSTM1/p62 and Nad(p)h quinone dehydrogenase 1 were higher in the *Atg7^{Δhep}* livers than in the *Atg5^{Δhep}* livers (Figure 3, B and C, and Figure 4, B and C). The extent of Nrf2 activation may determine the severity of endogenous injury,^{41,48,56} which in turn may affect the response to alcohol treatment.

These results demonstrate that the outcomes of the interaction of alcohol and hepatic autophagy function could depend on how autophagy function is disabled (constitutively or acutely), which autophagy gene is deleted (*Atg7* or *Atg5*), and how alcohol is administered. However, published and current results clearly demonstrate that acute inactivation of autophagy or lysosome function, via a genetic, chemical (siRNA), or pharmacologic approach, promotes alcohol-induced liver injury in a variety of alcohol-administration models with different dosages and durations. But the more complex findings of alcohol-induced phenotypes in chronically autophagy-deficient mice perhaps more realistically reflect clinical scenarios in which autophagy functions could be compromised through a more chronic process by alcohol and other coexisting factors such as viral infection^{16–19} or a high-fat diet.^{13–15}

Variable Liver Pathology in *Atg5^{Δhep}* and *Atg7^{Δhep}* Mice and Their Differential Responses to Alcohol Given through Different Regimens

Embryonic deletion of *Atg5* or *Atg7* in the parenchymal cells under the albumin promotor leads to severe liver pathology.^{48,56} Both *Atg7* and *Atg5* are crucial for LC3 to conjugate to phosphatidylalcoholamine on the autophagosomal membrane, which is required for autophagosome formation. However, the hepatic phenotypes of *Atg5^{Δhep}* and *Atg7^{Δhep}* mice are interestingly different in the degree and progression of the injury. Overall, *Atg5^{Δhep}* mice seemed to present a less severe phenotype of liver injury (Figure 1, B and C). Similar observations could be found in earlier studies.^{48,56} In addition, *Atg5*-deficient mice seem to undergo a spontaneous improvement when they became older (4 months and beyond) (Figure 4, F–H).⁵⁶ Also, the improvement was mainly contributed by the male mice

(Supplemental Figure S4A), not the female mice (Supplemental Figure S5A). The mechanisms for the more severe phenotype of *Atg7*^{Δhep} mice are not clear, but may be related to the more crucial function of *Atg7* being the only E1-like molecule involved in both *Atg5-Atg12* conjugation and LC3-phosphatidylalcoholamine conjugation.⁵ Alternatively, the possibility that *Atg7* might have additional functions cannot be ruled out.

The mild liver injury associated with *Atg5* deficiency could have allowed the detection of the added alcohol-induced insults in the acute binge model (Figure 1B). On the other hand, *Atg7*^{Δhep} mice might have had a blunted response to an acute alcohol binge and failed to show an increased injury. Since the elevation of hepatic TG level is an immediate consequence of alcohol stimulation and steatosis is a major pathologic phenomenon in AFLD,^{46,47} the change in the hepatic total TG level was examined in *Atg7*^{Δhep} and *Atg5*^{Δhep} mice. Indeed, TG level was not elevated in *Atg7*^{Δhep} mice (Figure 1C) but was elevated in *Atg5*^{Δhep} (Figure 1B) mice receiving an acute binge treatment. This observation supports that *Atg7*^{Δhep} mice had a blunted response to acute alcohol stimulation. In the chronic-plus-binge regimen, *Atg7*^{Δhep} mice responded to the prolonged alcohol stimulation since the total TG level was elevated (Figure 3E), and consequently an elevated injury was observed (Figure 3D).

The response of *Atg5*^{Δhep} mice to the chronic-plus-binge treatment was unexpected. The liver injury phenotype was paradoxically reduced in these mice despite that they seemed to respond to alcohol as evidenced by elevated hepatic TG level (Figure 4, I and J). The improvement in liver injury despite an elevation in hepatic TG was evident in both sexes and regardless of age (Supplemental Figures S4 and S5). An earlier study had also shown that *Atg5*^{Δhep} mice were paradoxically resistant to acetaminophen-induced liver injury.⁵⁷ A common feature of alcohol- and acetaminophen-induced liver injury is the induction of oxidative stress. Thus it is possible that *Atg5*^{Δhep} mice had pathologic changes that rendered them resistant to oxidative stress.

The deletion of either *Atg5* or *Atg7* in the liver causes SQSTM1/p62 accumulation in the liver, which in turn activates NRF2.^{40,41,56,58} The latter may create a somewhat reduced environment to resist damage caused by oxidative stress.⁵⁷ However, the elevated NRF2 activation in autophagy deficiency is likely correlated with the pathology, as shown by the co-deletion of *Nrf2* and the ensuing blockage of most of the liver phenotypes in *Atg7*^{Δhep} and *Atg5*^{Δhep} mice.^{41,48,56} Thus the improved hepatic presentation of *Atg5*^{Δhep} mice given chronic-plus-binge alcohol treatment may have been due to other factors.

The effect of alcohol in mice with chronic liver injury due to autophagy deficiency is thus complicated by the baseline liver changes in the autophagy-deficient condition. Caution must be exercised in reaching conclusions on the potential mechanisms that are involved. Notably, clinically ALD is often complicated by other conditions such as viral

infection^{16–19} and NAFLD,^{13–15} both of which could compromise autophagy function. Long-term alcohol use can also suppress autophagy by itself.^{9–12,15} The findings from this study demonstrate that the effects of alcohol can be quite diverse in livers with varying degrees of autophagy deficiency.

Potential Multiple Factors in Improvement of Liver Injury in *Atg5*^{Δhep} Mice by Chronic Alcohol Treatment

Other factors to consider include the potential role of inflammation, which is known to play important roles in the pathogenesis of ALFD.^{49,50} The reduced injury in *Atg5*^{Δhep} livers by the chronic alcohol treatment may have been related to reduced inflammation. In addition, changes related to the metabolism of bile acids and alcohol itself may deserve attention as well.

As previously published,^{52,56} *Atg5*^{Δhep} and *Atg7*^{Δhep} mice develop cholestatic injury, which is accompanied by an increased level of cytokeratin-19–positive ductular cells (ductular reaction), altered mRNA levels of genes in the bile acid–metabolism pathways, and an increase in circulating TBA level. Interestingly, several studies have shown a relationship between alcohol administration and bile acid metabolism.^{59–61} Indeed, serum levels of TBA were decreased by alcohol treatment in both *Atg5*^{F/F} and *Atg5*^{Δhep} mice. However, the improving effect in *Atg5*^{Δhep} mice by alcohol may not have been related to intrahepatic bile acid metabolism since the hepatic bile acid level in *Atg5*^{Δhep} remained high (Figure 6, D and E) and the expression of genes that are related to bile acid metabolism did not change significantly (Supplemental Figure S9). Nonetheless, this reduction in serum TBA may still be beneficial, as suggested by the decrease in ductular reaction (Figure 6A) and serum ALT/AST (Figure 4, F–G). How chronic alcohol treatment and *Atg5* deficiency lead to improved cholestatic outcome is an interesting topic for future studies.

Alcohol is generally metabolized in hepatic parenchymal cells, which express the highest levels of alcohol-oxidizing enzymes, including ADHs, CYP2E1, and catalase.⁴⁶ The first step of alcohol breakdown in the liver by these enzymes generates toxic metabolites, including acetaldehyde and other potentially damaging molecules.⁵³ Interestingly, the mRNA levels of the *Adh*, *Cyp2e1*, and *Cat* genes were decreased in *Atg5*-deficient livers. Reduced levels of these alcohol-oxidizing enzymes may reduce the generation of toxic metabolites, and thus less or no alcohol-mediated toxicity. However, there was no consistent difference in the expression of these genes between *Atg5*^{Δhep} livers and *Atg7*^{Δhep} livers (data not shown), which may thus not account for the difference in how these mice responded to alcohol.

The toxic acetaldehyde can be enzymatically removed by ALDH. *Aldh2* is one of the most important *Aldh* family of genes for alcohol metabolism. In other studies in mice, the pharmacologic activation of either *Aldh2* or global *Aldh2* overexpression seemed to ameliorate chronic alcohol-

induced hepatic steatosis and improved ALD,^{62,63} and *Aldh2* deficiency paradoxically resulted in improved alcoholic fatty livers, although worse liver inflammation and fibrosis.⁶⁴ In the present study, the expression levels of several *Aldh* genes, including *Aldh2*, were decreased in *Atg5*-deficient livers, although the protein levels remained unchanged. The significance of these changes is not known, but the overall findings would suggest that alcohol metabolism might have been altered in *Atg5*^{Δhep} livers, which may have contributed to the observed phenotypes.

A lower expression of these enzymes may result in a slower blood alcohol clearance, as in *Adh1* and *Adh4* knockout mice.⁶⁵ Surprisingly, a faster clearance rate of plasma alcohol in *Atg5*^{Δhep} mice was observed. It is notable that alcohol could be cleared from circulation by other mechanisms independent of these enzymes, such as through breath. The potential involvement of these independent mechanisms needs to be investigated in the future.

In summary, the findings from this study demonstrate a protective role of autophagy in alcohol-induced liver injury, which was best illustrated in models in which an autophagy gene *Atg7* was acutely deleted. In mice with constitutive deletion of *Atg7* or *Atg5*, the effects of autophagy deficiency on alcohol-induced liver phenotype become complicated, in large part due to the coexistence of liver pathology caused by autophagy deficiency. Collectively, the findings from this study indicate the complexity of the relationship between autophagy status and alcohol-induced liver injury. This relationship may be dictated by the timing of the autophagy inhibition and alcohol exposure, the regimen of alcohol administration, and the particular autophagy-related gene being inactivated. These conditions may draw some similarities to the clinical ALDs, which are often complicated by additional confounding factors, such as viral infection^{16–19} and NAFLD,^{13–15} in which autophagy function can be compromised. This study may provide a model to understand the complex interactions of alcohol with autophagy deficiency at various degrees caused by other underlying conditions.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.05.011>.

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