# H₂O₂-mediated autophagy during ethanol metabolism

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ABSTRACT

*Background:* Alcoholic liver disease (ALD) is the most common liver disease worldwide and its underlying molecular mechanisms are still poorly understood. Moreover, conflicting data have been reported on potentially protective autophagy, the exact role of ethanol-metabolizing enzymes and ROS.
*Methods:* Expression of LC3B, CYP2E1, and NOX4 was studied in a mouse model of acute ethanol exposure by immunoblotting and immunohistochemistry. Autophagy was further studied in primary mouse hepatocytes and huh7 cells in response to ethanol and its major intermediator acetaldehyde. Experiments were carried out in cells overexpressing CYP2E1 and knock down of NOX4 using siRNA. The response to external H₂O₂ was studied by using the GOX/CAT system. Autophagic flux was monitored using the mRFP-GFP-LC3 plasmid, while rapamycin and chloroquine served as positive and negative controls.
*Results:* Acute ethanol exposure of mice over 24 h significantly induced autophagy as measured by LC3B expression but also induced the ROS-generating CYP2E1 and NOX4 enzymes. Notably, ethanol but not its downstream metabolite acetaldehyde induced autophagy in primary mouse hepatocytes. In contrast, autophagy could only be induced in huh7 cells in the presence of overexpressed CYP2E1. In addition, overexpression of NOX4 also significantly increased autophagy, which could be blocked by siRNA mediated knock down. The antioxidant N-acetylcysteine (NAC) also efficiently blocked CYP2E1-and NOX4-mediated induction of autophagy. Finally, specific and non-toxic production of H₂O₂ by the GOX/CAT system as evidenced by elevated peroxiredoxin (Prx-2) also induced LC3B which was efficiently blocked by NAC. H₂O₂ strongly increased the autophagic flux as measured by mRFP-GFP-LC3 plasmid.
*Conclusion:* We here provide evidence that short-term ethanol exposure induces autophagy in hepatocytes both *in vivo* and *in vitro* through the generation of ROS. These data suggest that suppression of autophagy by ethanol is most likely due to longer alcohol exposure during chronic alcohol consumption with the accumulation of e.g. misfolded proteins.

## 1. Introduction

Alcoholic liver disease (ALD), due to excess alcohol consumption is a major health problem worldwide [1]. ALD spans from hepatic steatosis over inflammation to fibrosis and typically develops over 10–15 years of heavy drinking. Most critical and life-threatening end points are decompensated alcoholic liver cirrhosis and the rare and clinically defined alcoholic hepatitis (AH) which typically affects younger patients with a shorter drinking history [1]. The exact underlying mechanisms

for ALD and specifically for AH, however, are still incompletely understood, and alcohol abstinence remains the most effective treatment option in drinkers during all disease stages. Many pathophysiological pathways have been unraveled in the past four decades and the formation of acetaldehyde and reactive oxygen species (ROS) are considered a cornerstone for ethanol-mediated liver damage that early on causes mitochondrial damage [2]. The role of ROS is further underlined by the fact that, apart from steroids [3], the antioxidant N-acetylcysteine (NAC) has proven effectivity for the treatment of AH in a randomized

controlled trial [4].

Recently, evidence has been provided that autophagy plays an important role in the pathology of ALD [5], next to well established hallmarks such as liver necrosis and apoptosis [1]. It is long known that liver enlargement in ALD arises both from the accumulation of proteins and lipids [6]. Similar to hepatic steatosis, protein accumulation during alcohol metabolism is due to protein modifications by reactive molecules generated from ethanol metabolism such as ROS [2]. Autophagy (formerly also known as macro-autophagy) is an evolutionarily conserved catabolic process in response to stress to eliminate proteins and cytoplasmic debris through lysosomal degradation [7]. Autophagy is vital in developmental biology [8] and in immunity against microbial infections [9]. It is also associated with a variety of pathological conditions in humans but starvation or ischemia are among the best understood conditions that lead to autophagy [10].

Based on studies on starvation-mediated autophagy, it has been shown that the mechanistic target of rapamycin (mTOR), a serine/threonine-specific protein kinase that belongs to the family of phosphatidylinositol-3 kinase (PI3K) related kinases (PIKKs) plays a central role in suppressing autophagy. Autophagosome generation, autophagosome-lysosomal fusion (autolysosome) and degradation are controlled by the evolutionary conserved autophagy-related (Atg) gene family [11,12]. To date, among known Atg-encoded proteins, only microtubule-associated protein light chain 3 (LC3) is known to exist in all types of autophagic membranes [13,14]. Nascent LC3 (proLC3) is cleaved by Atg4-family proteins into LC3-I immediately after synthesis, and cleaved LC3-I is further conjugated to phosphatidylethanolamine to become autophagosomal-membrane bound LC3-II [14]. The lipid modified form of LC3, referred to as LC3-II, is believed to be involved in autophagosome membrane expansion and fusion events. To some extent, the expression of LC3 (LC3-II/LC3-I turnover ratio) can be used to precisely reflect the activated autophagy. Among the four isoforms of LC3, LC3B protein is widely used as biomarker for monitoring autophagy [15–17]. Nevertheless, the exact role of LC3 in the autophagic pathway still requires further clarification [18].

Similar to ethanol-mediated suppression of apoptosis and regeneration [19], it is generally believed that ethanol blocks autophagy [20] and numerous studies indicate that pharmacological or genetic inhibition of autophagy greatly enhances cell death [21–23]. Thus, in the absence of autophagy, cell death is significantly increased. Several mechanisms have been proposed to mediate the suppression of autophagy by ethanol including elevation of lysosomal pH with subsequent decreased proteolytic capacity or lowered cathepsin content by disrupted trafficking of these enzymes to lysosomes [10]. It has been generally thought that ethanol oxidation enhances autophagosome content by suppressed mTOR activity through CYP2E1 metabolites such as acetaldehyde or other reactive metabolites [24]. Moreover, ethanol is not able to induce autophagy in HepG2 cells due to the absence of functional CYP2E1 [25]. Ethanol is also known to inhibit the AMP kinase, an important mTOR suppressor and regulator of catabolic pathways. Finally, ethanol disrupts functional microtubule cytoskeleton in hepatocytes which is required for intracellular movement of autophagic cargo [26].

Conflicting results, however, have been reported from various laboratories regarding the role of autophagy during ethanol exposure [24, 27–32]. This may be due to the rather heterogenous molecular and cellular response of liver tissue to ethanol and its modulation by genetic and non-genetic factors. Nevertheless, it remains poorly understood at which step and by which ethanol modulates autophagy. In mice, acute gavage of ethanol caused ca. 35% increase of LC3-II protein after 12 h and this increased autophagosome content is found in parallel with indirect signs of enhanced oxidative stress such as reduced glutathione levels and increased lipid peroxides [27]. In addition, in livers of transgenic GFP-LC3 mice, an increased number of GFP-LC3 puncta as indicator for an enrichment of autophagosomes in the LAMP1-positive heavy membrane, lysosome containing fraction has been observed [5,

33] and, likewise, electron microscopic studies also demonstrated an accumulation of autophagosomes in response to ethanol which could be blocked by knockdown of Atg7 [24]. Ding et al. demonstrated that autophagy protected cells from the toxic effects of ethanol in livers of mice [24]. Findings from another detailed study suggest that the effect of acute ethanol gavage on hepatic autophagy differs significantly from that after chronic ethanol feeding [27]. Similar, seemingly paradox findings have been recently reported on the central iron master switch hepcidin [34].

At lower physiological levels, ROS and namely $H_2O_2$ can act as a classical intracellular signaling molecule regulating kinase-driven targets involved in proliferation, migration, survival, and autophagy [35, 36]. The specific role of $H_2O_2$ in autophagy, however, is poorly understood. There are recent observations of spontaneous $H_2O_2$ release during autophagy induced by rapamycin and $H_2O_2$ signaling may be part of the autophagic process [37]. More complex, $H_2O_2$ can induce upstream proteins of autophagy but inhibited the autophagic flux [38]. The important but still poorly understood role of $H_2O_2$ in cell death or regeneration further complicates autophagy studies [39]. Moreover, ROS studies are often prone to artificial conditions. For instance, to explore the physiological role of $H_2O_2$, bolus treatments are commonly used which do not mimic the typical continuous release during (patho) physiological processes [40]. In addition, far too high $H_2O_2$ concentrations ($>50$ μM) are generally used as compared to the *in vivo* situation [40,41]. Finally, the exact role of how ROS are generated during ethanol metabolism is also complex and partly not clear. Thus, hepatic CYP2E1 which is strongly induced during the ethanol metabolism in liver, has been shown to generate ROS and enhance the progression of liver disease and cancer in ALD [42,43]. Likewise, NADPH-dependent oxidases (NOX), namely liver-expressed NOX4 also play a crucial role in the process of liver injury [1,44] and preliminary immunochemistry data indicate that NOX4 expression is increased in hepatocytes [34]. Moreover, in cardiomyocytes, NOX4 which directly produces $H_2O_2$ was shown to trigger autophagy [45].

Therefore, we here study in detail the role of ROS, namely $H_2O_2$, during ethanol metabolism and its effect on hepatocellular autophagy. We first confirm that *in vivo* markers of autophagy and the two important ROS-generating enzymes CYP2E1 and NOX4 are induced in a mice model of acute ethanol exposure. We also demonstrate that ethanol only induces autophagy in hepatocytes in the presence of functional CYP2E1. In addition, both overexpression of NOX4 or exposure to external $H_2O_2$ using the enzymatic glucose oxidase/catalase (GOX/CAT) system induce autophagy. In conclusion, our data indicate that ethanol metabolism efficiently modulates autophagy through the generation of ROS and that low nontoxic levels of $H_2O_2$ both from intra- and extracellular sources can induce hepatocyte autophagy.

## 2. Materials and methods

### 2.1. Animal experiment

All animals received humane care according to the rules of the local committee for Animal Welfare of the Regierungspräsidium Baden-Württemberg. 14 male C57BL/6 mice were randomly divided into control and ethanol treatment group. ethanol binge was conducted in the ethanol treatment group 12 h before the sacrificed, and each mouse was shortly given 33% (vol/vol) ethanol at a total accumulative dosage of 4.5 g/kg body weight by gavages. Control mice received the same energy contained maltodextrin solution. For autophagy inhibition, CQ (100 mg/kg) [46,47] was given intraperitoneally to one mouse in each group 30 min before the administration of ethanol.

### 2.2. Cell culture

Huh7 cells from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan) were grown under standard conditions using

Dulbecco's modified Eagle medium (Sigma-Aldrich), 4500 mg/L glucose, and 10% fetal calf serum at 37 °C under 5% $CO_2$ [48]. Murine primary hepatocytes kindly provided by Dr. Sai Wang (University of Heidelberg, Germany) were grown under standard conditions ($1*10^5$/well in 12-well plate) using Williams' medium (Sigma-Aldrich, Taufkirchen, Germany), 10% fetal bovine serum, 1% P/S (Penicillin and Streptomycin), 1% L-Glutamine, 0.5% ITS (Insulin--Transferrin-Selenium), 0.1% Dexamethasone. ethanol (50/100 mmol/L), acetaldehyde (100 µmol/L), and the following chemicals were applied as indicated in the figure legends: Rapa (20 nmol/L), CQ (20 µmol/L), NAC (10 mmol/L). Considering the high evaporation ability of acetaldehyde, we refreshed the medium every 6 h.

### 2.3. Reagents

The following reagents were used in this study: Glucose Oxidase (GOX, G0543), Catalase (CAT, C3155), Rapamycin (Rapa, R8781), Chloroquine (CQ, C6628), N-Acetyl-L-Cysteine (NAC, A8199), Methanethiosulfonate (MMTS, 64306), ethanol (ethanol, 32205-M), and Acetaldehyde solution (W200379) were purchased from Sigma-Aldrich.

### 2.4. Exposure of cells to steady-state $H_2O_2$

Steady-state $H_2O_2$ treatment was performed using the glucose oxidase and catalase system (GOX/CAT system) as described previously [40,49]. Briefly, to avoid starvation induced autophagy, only $5*10^4$ Huh7 cells were seeded with 10% FCS medium in 12-well plate for 24 h, following with 24 h of $H_2O_2$ incubation controlled by GOX/CAT system. The GOX was always kept in dilution of 1:1600,000 in PBS, and CAT was diluted to 1: 300,000 in PBS.

### 2.5. Transfection experiments

Huh7 cells ($5.5*10^4$/well in 12-well plate) were transfected with mRFP-GFP-LC3 plasmid, CYP2E1 plasmid, or/and NOX4 plasmid constructs using 3 µl Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the construction of mRFP-GFP-LC3 plasmid, mRFP cDNA was exogenously added with restriction site of NheI and AgeIto its 5′ end and 3′ end respectively, and removed of the termination codon by PCR. The PCR product was digested by each restriction enzyme and inserted into the NheI-AgeI site of pEGF-mRFP plasmid. CYP2E1 cDNA was cloned into the XhoI and MluI restriction sites of a pCI-neo vector, resulting in antisense orientation with respect to the cytomegalovirus (CMV) promoter. mRFP-GFP-LC3 or CYP2E1 or/and NOX4 plasmid [49] construct transfections were carried out for 24 h, after 6 h transfection, the medium was refreshed. GFP plasmid was performed as a control. For $H_2O_2$ and ethanol incubation, the transfected cells were kept under different treatments for additional 24 h.

### 2.6. RNA silencing

Huh7 cells ($5.5*10^4$/well in 12-well plate) were transfected using 3 µl Lipofectamine 2000 and 10 nM siRNA against NOX4 [49]. NOX4 was purchased from Ambion (Foster City, CA, USA). Equimolar concentrations of universal negative siRNA (Sigma-Aldrich, Taufkirchen, Germany) were used as control.

### 2.7. Immunoblotting

Cells were washed in ice-cold 1 × PBS and harvested in RIPA buffer plus 1 × Complete ® protease inhibitor with EDTA (Roche Applied Sciences) on ice. Frozen liver tissue (about 10 mg) was homogenized in 200 µl of RIPA buffer using a tight-fitting plastic homogenizer on ice. For Prx2 Western blotting, the cells were incubated with the thiol-blocking agent MMTS at 80 mM prepared in ice-cold PBS for 10 min before harvesting, to avoid lysis-induced oxidation of Prx2 [41]. Prx2 samples were subjected to non-reducing SDS-PAGE, and the Western Blotting was performed as described previously [48]. Equal protein loading was confirmed by protein staining with Ponceau-S solution as well as β-actin/GAPDH. Primary and secondary antibodies are listed in Suppl. Table 1. Immune-reactive bands were detected by chemiluminescence (Rotilumin, Carl Roth, Karlsruhe, Germany) and exposed to autoradiography film or after incubation with fluorescent secondary antibody. The membranes were scanned using an infrared imaging system (Odyssey CLx; LI-COR, Inc., Lincoln, NE, USA). Band intensities were quantified using ImageJ for further statistical analysis.

### 2.8. RNA isolation, cDNA synthesis, and real-time quantitative PCR analysis

RNA was isolated with Trifast (Peqlab Biotechnology GmbH, Erlangen, Germany) according to the manufacturer specifications. Reverse transcription and the real-time quantitative PCR reactions were performed as previously described [49]. Primers and probes were designed using the Probefinder software (Roche, Mannheim, Germany), and the sequences are provided in Suppl. Table 2

### 2.9. Fluorescence microscopy

Cells expressing fluorescent proteins were fixed for 15 min with 4% PFA, washed, and embedded directly with mountant solution (ThermoFisher, Lot: 2181004) containing DAPI for staining of nuclei. After covered with cover slips overnight, cells were imaged using a 60× oil immersion objective mounted on an Olympus B×41 equipped with a F-view II CCD camera controlled by the cell^D software.

### 2.10. Immunohistochemistry and immunofluorescence staining

Paraffin-embedded liver tissues were prepared from sacrificed mice. Briefly, after dewaxing, liver sections were submitted to antigen retrieval at 99 °C for 25 min in a Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 9) solution. After cooling and washing with PBS (3 × 5 min), the samples were blocked for 5 min using a blocking solution (ZUC007; Zytomed Systems), washed with PBS, and incubated overnight at 4 °C with the primary antibody diluted in antibody diluent (ZUC025) 1:200. Following washing in PBS (3 × 5 min), the samples were incubated with ZytoChem Plus (AP) Polymer anti-Rabbit (ZUC031) for 30 min and shortly washed with PBS after incubation. Liver sections were finally incubated with ImmPACT® Vector® Red AP Substrate (SK-5105; Vector Laboratories) for 10 min, washed with PBS to stop the reaction, and counterstained with hematoxylin for 5 min. After washing and clearing with xylene, the samples were mounted and further visualized using a Nikon Ni-E microscope equipped with a DS-Ri2 color camera (Nikon, Minato, Japan). Immunofluorescence staining was conducted to analyze the expression of NOX4. In brief, the frozen liver sections were incubated with NOX4 (1:100 dilution) antibody overnight at 4 °C. After washing three times with PBS, the slides were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody for 1 h in the dark. Finally, the slides were rinsed with PBS and analyzed under a fluorescent microscope (Olympus FV500; Olympus, Tokyo, Japan). DAPI (Sigma, St Loise, MO, USA) was used as counterstain.

### 2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Comparisons between two groups were performed by using Student's $t$-test. All the data are expressed as mean $\pm$ SD. A value of $p < 0.05$ was considered statistically significant.

## 3. Results

Ethanol induces CYP2E1, NOX4, and autophagy in a mouse model of
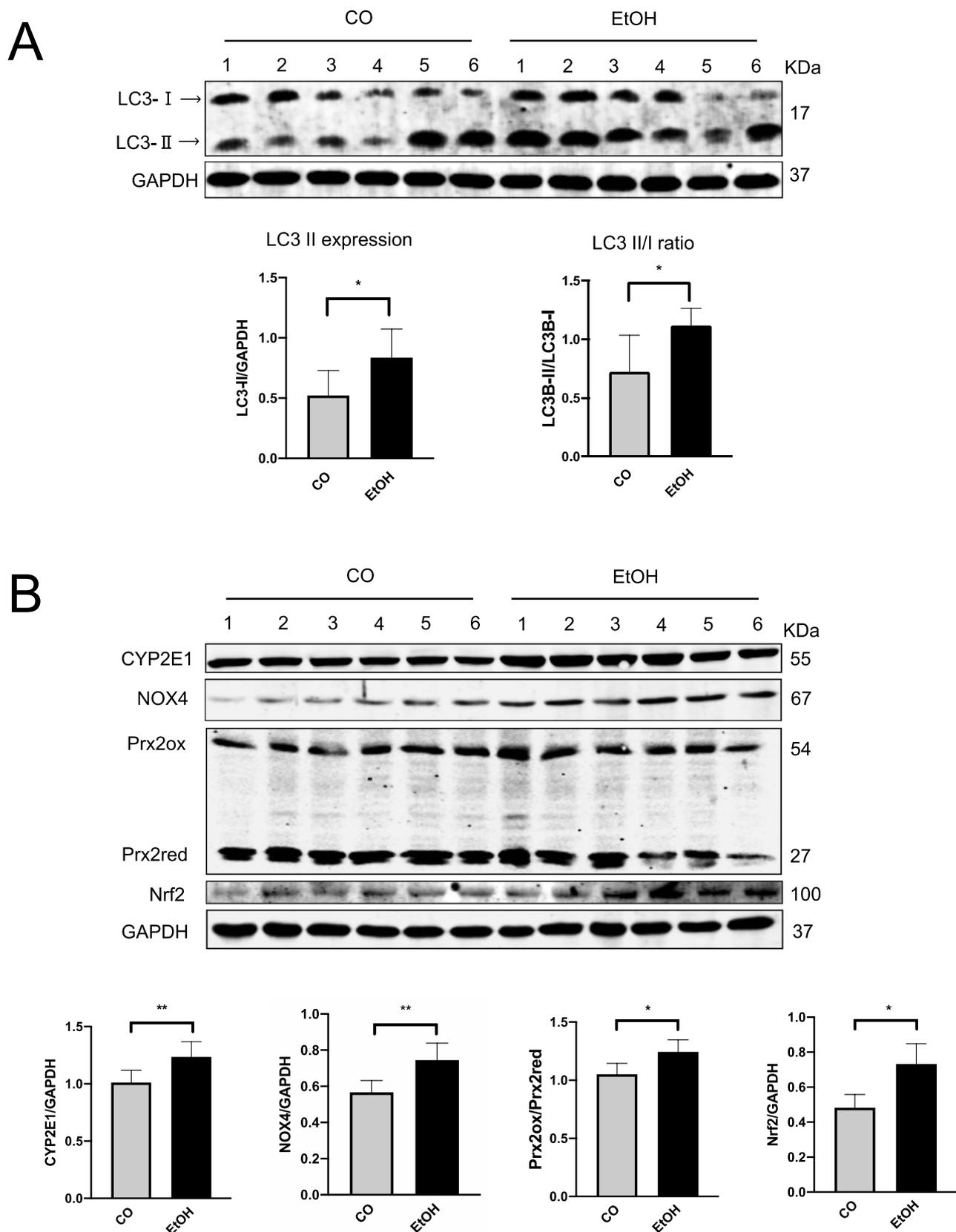
**Fig. 1. Acute ethanol treatment activates autophagy, CYP2E1, and NOX4 in mice.** (A) Six mice in the ethanol group were shortly given 33% (vol/vol) ethanol at a total accumulative dosage of 4.5 g/kg body weight by gavages. Western blot analysis of LC3B in mice liver total extracts (35 μg per lane, $n = 6$ mice per group). (B) Western blot analysis of CYP2E1, NOX4, Prx2, and Nrf2 in mice liver total extracts (35 μg per lane, $n = 6$ mice per group). (C) Immunohistochemistry staining of CYP2E1 in the paraffin-embedded liver section. Immunofluorescence staining of NOX4 in the frozen liver section. All target protein levels were quantified and normalized to those of GAPDH. *, $P < 0.05$, **, $P < 0.01$.
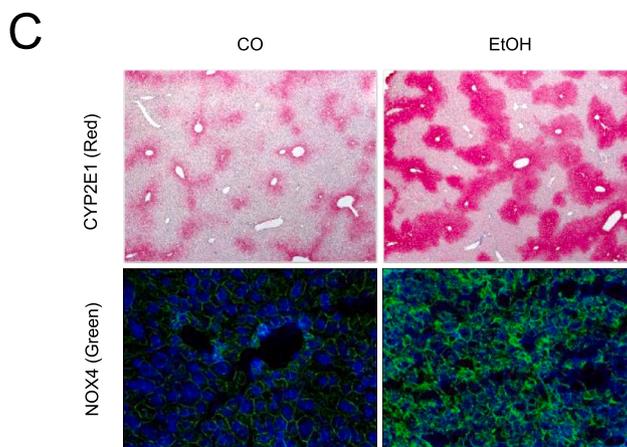
**Fig. 1.** (*continued*).

acute ethanol exposure.

To confirm whether autophagy could be regulated during acute alcohol exposure, mice were treated by binge ethanol using gavage. Expression of LC3-II and conversion to LC3-I were used to assess autophagy. As shown in Fig. 1A, acute ethanol exposure significantly increased hepatic LC3-II expression as well as LC3B turnover ratio (LC3-II/LC3-I). Ethanol also strongly induced CYP2E1 and NOX4 expression in ALD mice liver (Fig. 1B). Spearman Rho correlation between densitometric CYP2E1 and NOX4 expression levels showed a significant positive association (r = 0.692, $p < 0.05$) (not shown). Prx2 and nuclear factor erythroid-derived 2-like 2 (Nrf-2) [50,51], a transcription factor that plays a key role in the activation of cellular antioxidant enzymes in response to oxidative stress [52,53], were also significantly induced by ethanol suggesting elevated ROS, especially $H_2O_2$ generation existed in mice liver tissue (Fig. 1B). Nrf2 downstream target heme-oxygenase-1 (HO-1) was also upregulated (Suppl. Fig. 1A). Of note, both mTOR activation, related major upstream AMP kinase (Suppl. Fig. 1B) were not changed under these conditions. In addition, in line with previous data [54], CYP2E1 was drastically induced in the centrilobular area of liver tissues (Fig. 1C). NOX4 expression was also strongly induced (Fig. 1C). To confirm whether increased LC3B expression is due to activated autophagic flux and to monitor autophagic flux, Chloroquine (CQ) was intraperitoneally injected into one mouse 30 min before the ethanol gavage. Since autophagy is blocked by CQ, any elevated autophagy flux will result in further accumulation of total LC3B expression [24]. As shown in Fig. 2A, CQ caused further accumulation of LC3B, suggesting an increased flux. In line with *in vivo* data, similar results were observed in murine primary hepatocytes (Fig. 2B). Exposure of 100 mM ethanol for 24 h significantly increased LC3-II expression as well as LC3B turnover ratio (LC3-II/LC3-I) in these cells.

In conclusion, acute ethanol significantly induces autophagy, CYP2E1, NOX4, and ROS in mice liver and autophagy as well as autophagic flux are induced by ethanol both *in vivo* but also in primary mouse hepatocytes.

CYP2E1 overexpression re-establishes ethanol mediated induction of autophagy in huh7 cells.

We next set up a series of experiments to study ethanol-mediated autophagy in hepatoma huh7 cells, a widely used *in vitro* model for liver studies. Notably, neither 100 mM ethanol nor 100 μM of its major metabolite acetaldehyde was able to induce autophagy in hepatoma huh7 cells (Fig. 3A and B). Detailed time response studies during 3–24 h of acetaldehyde exposure also did not show any effects on autophagy (not shown). In order to further validate the autophagic flux *in vitro*, we treated Huh7 cells with ethanol or acetaldehyde in the presence of CQ which did not result in further accumulation of LC3B (Suppl. Fig. 2A and 2B). In addition, a tandem mRFP-GFP-LC3 plasmid transfection experiment, widely used to monitor autophagy flux [55,56] was explored.

Briefly, the increased formation of LC3 protein can be traced with increased mRFP (red) or GFP (green) -tagged LC3 dots [15,57]. Huh7 cells were transfected with two fluorescence proteins labeled LC3 plasmids and treated with ethanol. However, neither the number of RFP and RFP/GFP merged autophagosomes were significantly induced (Suppl. Fig. 2C). Since some hepatoma cells are known to lack functional CYP2E1 [25] and CYP2E1 overexpression is required to recapitulate ethanol metabolism [1,58,59], we repeated the studies with huh7 cells transiently transfected with CYP2E1-overexpressing plasmids. CYP2E1 overexpression was successfully confirmed by Western blotting (Fig. 3C) (Suppl. Fig. 3A) and treatment of these cells with 100 mM ethanol significantly induced autophagy (Fig. 3D). Transfection efficiency was confirmed by GFP (Suppl. Fig. 3B). These findings highly suggest that CYP2E1 and potentially CYP2E1-generated ROS seem to be essential for ethanol-induced autophagy.

*3.1. Induction of autophagy by CYP2E1-and NOX4-mediated generation of intracellular ROS*

To further confirm whether known CYP2E1-mediated ROS production is involved in autophagy, we co-treated CYP2E1-overexpressing huh7 in addition to ethanol with 10 mM N-Acetyl-L-cysteine (NAC). As shown in Fig. 4A, NAC was able to efficiently block autophagy, suggesting the involvement of ROS in this model. We next explored NOX4, an additional important ROS source involved in hepatocyte signaling and induced during ethanol abuse [49] and autophagy [45,60]. Transfection NOX4 plasmid into Huh7 cells drastically increased NOX4 mRNA (Suppl. Fig. 4A) and markedly elevated NOX4 protein followed by LC3-II induction (Fig. 4B) (Suppl. Fig. 4B). Moreover, and in line with CYP2E1 experiments, NOX4-mediated autophagy could be efficiently blocked by 10 mM NAC (Suppl. Fig. 4C). *Vice versa*, siRNA-mediated knockdown of NOX4 (Suppl. Fig. 4D and 4E) reduced LC3-II expression in Huh7 cells (Fig. 4C). As demonstrated in Fig. 4D and E, co-transfection with CYP2E1 and NOX4 further enhanced autophagy. As compared with CQ treatment alone, the co-transfection caused further LC3-II accumulation, when the degradation was blocked by CQ. Furthermore, Huh7 cells co-transfected with CYP2E1 and NOX4 plasmid showed stronger LC3B expression in the presence of ethanol, which attributes to a robust ROS generation.

In summary, we here show that intracellular ROS generation by both CYP2E1 and NOX4 are able to induce autophagy in huh7 cells in an additive manner.

*3.2. External non-toxic $H_2O_2$ significantly increases LC3-II expression without mTOR suppression*

We next set up several experiments to analyze the role of the external
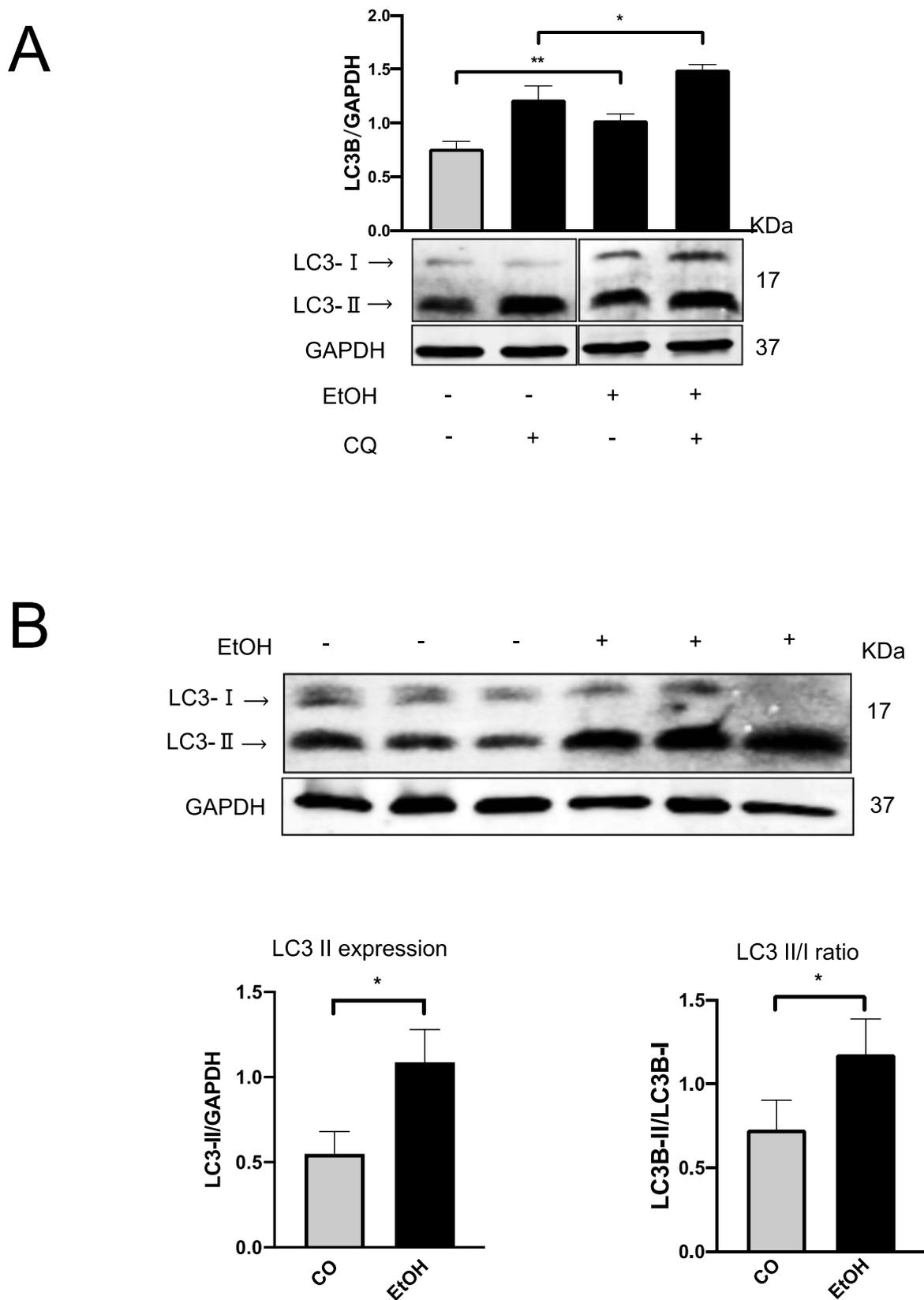
**Fig. 2. Ethanol induces autophagy flux in mice liver and triggers autophagy in primary murine hepatocytes.** (A) Western blot analysis of LC3B in mice liver total extracts. One mouse in the control (CO) and ethanol group was injected with CQ (100 mg/kg) 0.5 h before getting the ethanol gavage. (B) The murine primary hepatocytes were incubated with 100 mM ethanol for 24 h, the LC3B expression was detected by Western blot. LC3-II levels were quantified and normalized to those of GAPDH. *, $P < 0.05$.
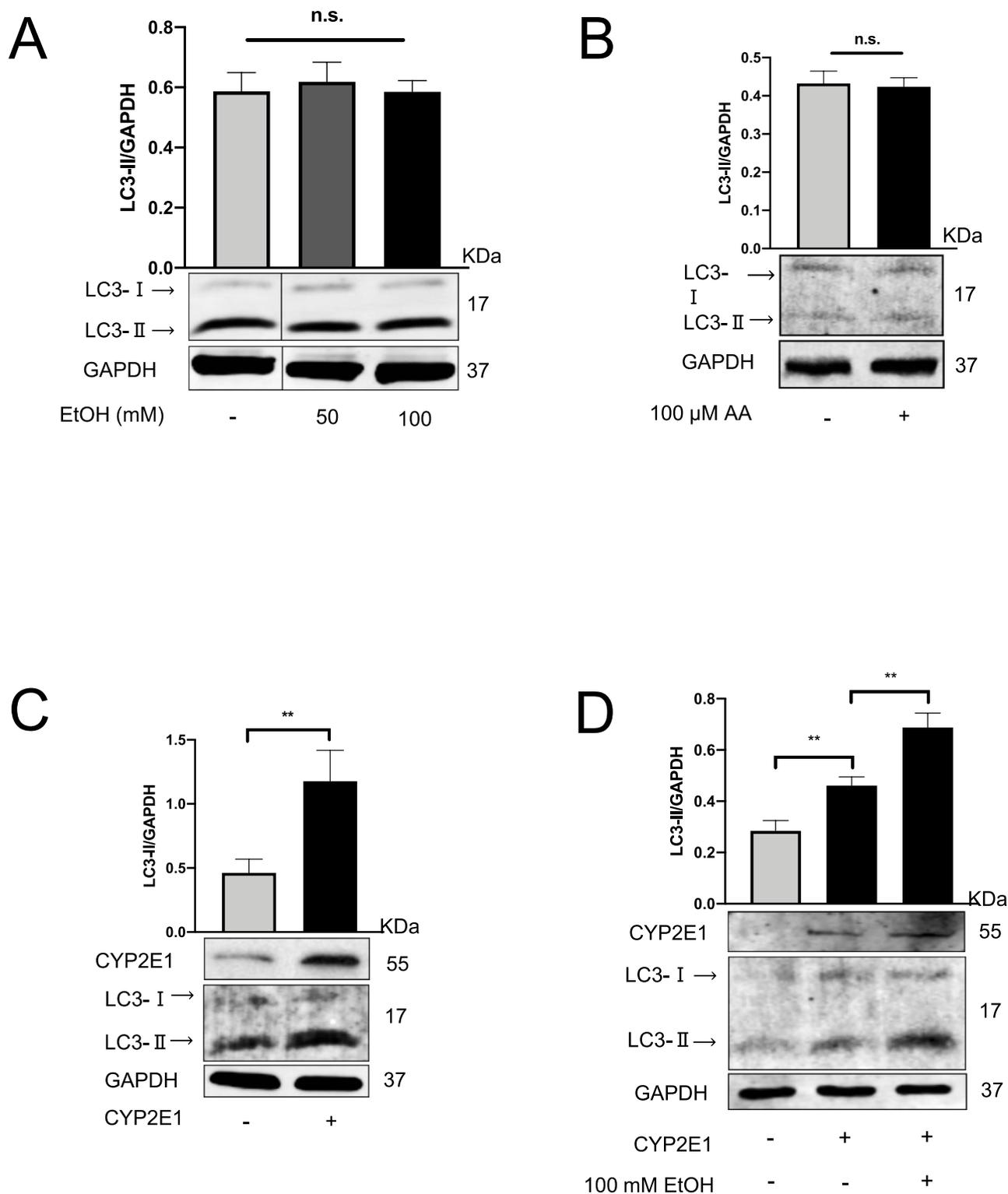
**Fig. 3. Transfection of huh7 cells with CYP2E1 recapitulates ethanol-mediated induction of autophagy.** (A) Huh7 cells were exposed to 50 and 100 mM ethanol for 24 h. The expression levels of LC3-II were analyzed by the western bolt. (B) Huh7 cells were exposed to 100 μM acetaldehyde (AA) for 24 h. (C) Huh7 cells were transfected with the CYP2E1 plasmids for 24 h. The expression levels of CYP2E1 and LC3-II were analyzed by the western bolt. (D) After 24 h of CYP2E1 transfection, the cells were further treated with 100 mM ethanol for another 24 h. LC3-II levels were quantified and normalized to those of GAPDH. Representative data of at least three independent experiments are shown for protein data. **, $P < 0.01$, n.s.: not significant.

release of $H_2O_2$ on autophagy using the enzymatic GOX/CAT system [40], which allows the specific formation of steady-state $H_2O_2$ (ss$H_2O_2$) level over a huge concentration range and time period [40]. Cell toxicity studies confirmed not toxic conditions (not shown) but effective elevation of cytosolic $H_2O_2$ levels were confirmed by Prx2 Western blotting [41,49]. Indeed and as shown in Fig. 5A, $H_2O_2$ induced oxidized Prx2 (Prx2$_{ox}$). These low and non-toxic ss$H_2O_2$ levels (1–2 μM) were also able to induce LC3B (Fig. 5B), which could be efficiently blocked by 10
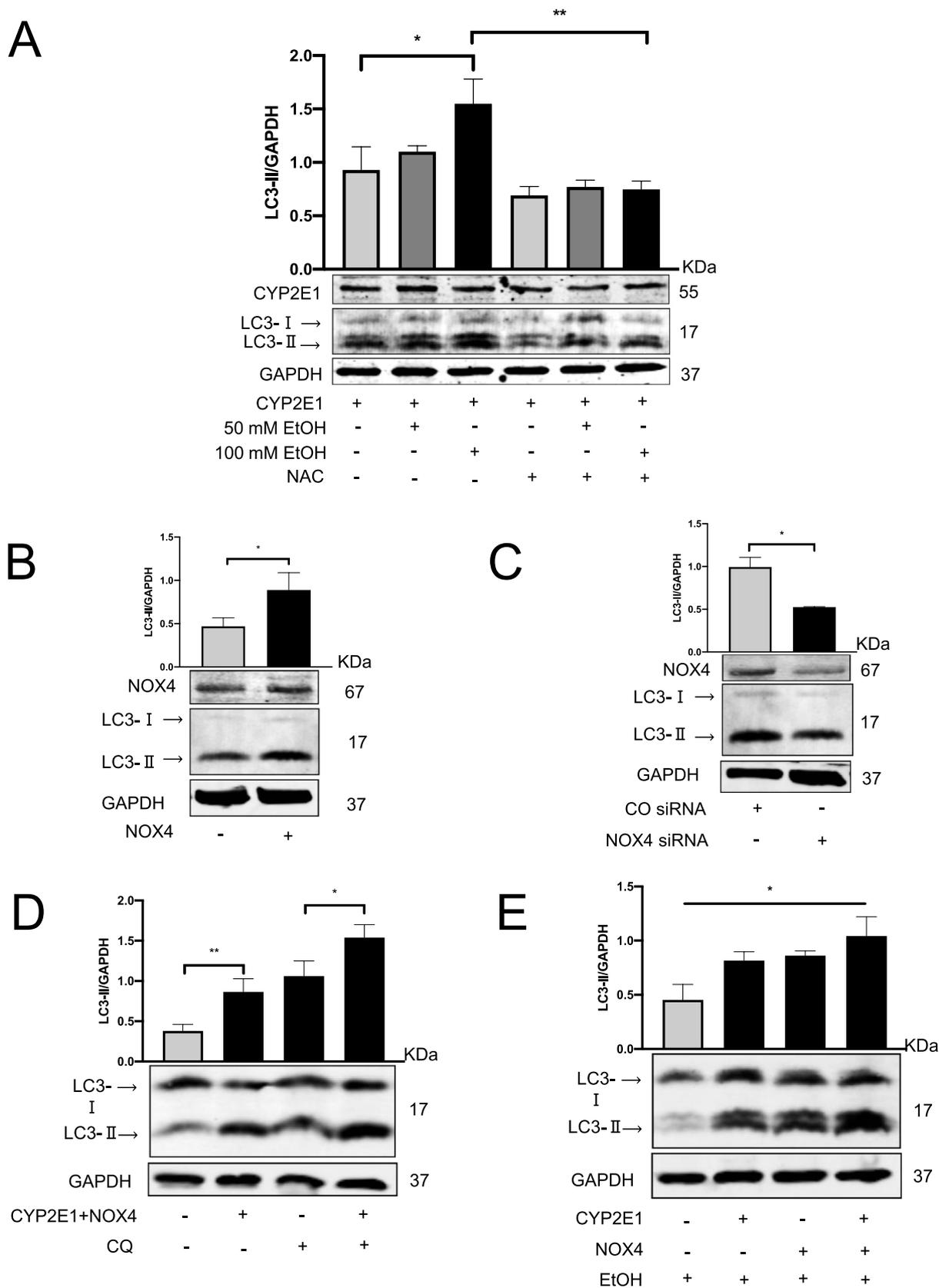
**Fig. 4. NOX4 cooperates with CYP2E1 and further up-regulated autophagy. NAC impaired the autophagy induction.** (A) CYP2E1 transfected Huh7 cells were further incubated with 50/100 mM ethanol separately or in the presence of 10 mM NAC. LC3-II and CYP2E1 expression were analyzed by Western blot. (B) Huh7 cells were transfected with the NOX4 plasmids or (C) NOX4 siRNA for 48 h. LC3-II and NOX4 expression were analyzed by Western blot. (D) Huh7 cells were co-transfected with CYP2E1 and NOX4 and further incubated within the presence or absence of 20 μM CQ for 6 h. LC3-II expression was analyzed by Western blot. (E) CYP2E1, NOX4, or co-transfection were performed 24 h, followed by 24 h 100 mM ethanol incubation. LC3-II expression was analyzed by Western blot. *, $P < 0.05$, **, $P < 0.01$.
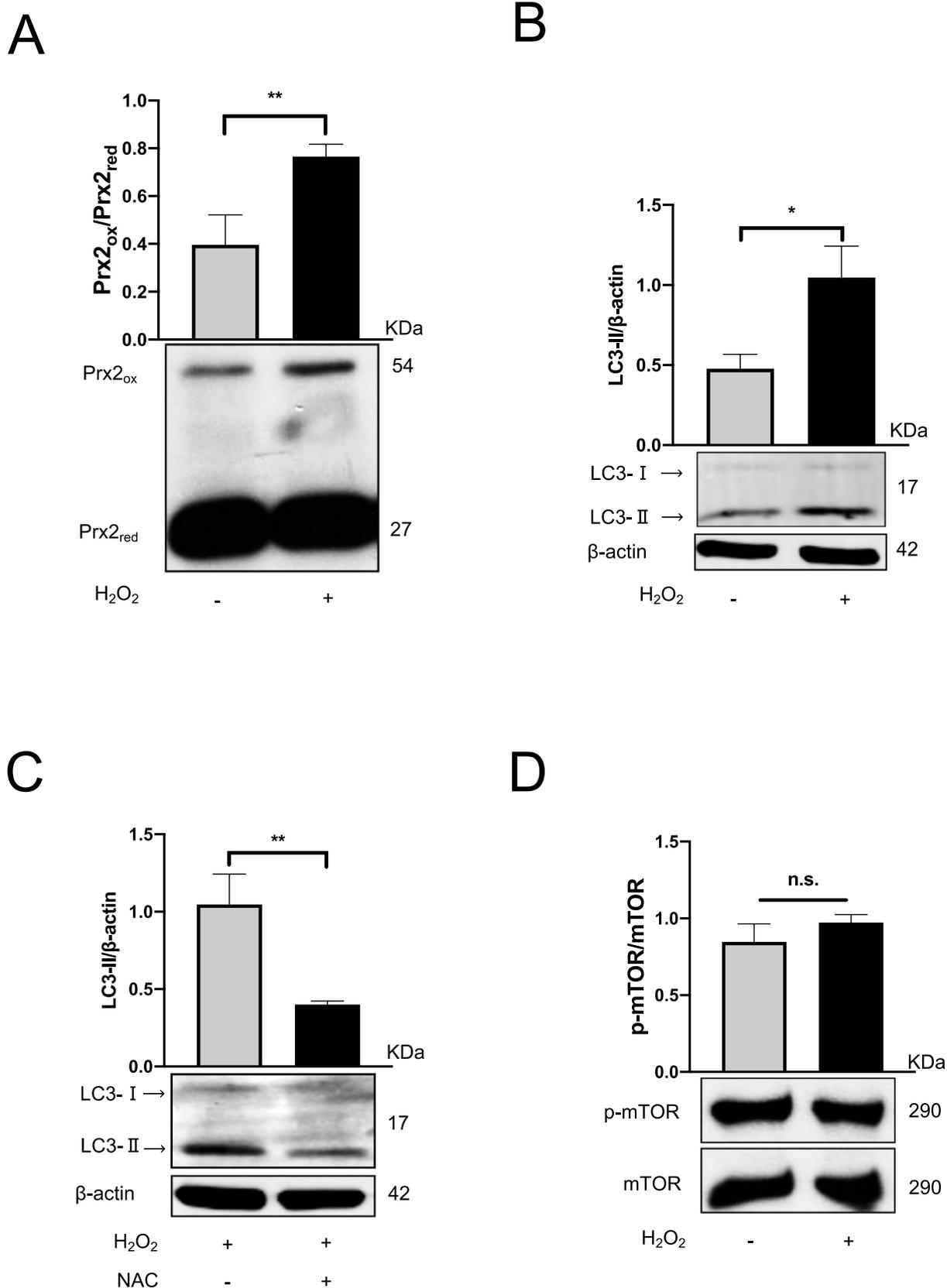
**Fig. 5. $H_2O_2$ activates autophagy in Huh7 cells.**
Huh7 cells were exposed to $H_2O_2$, which was independently controlled by GOX/CAT system. (A) $H_2O_2$ generation was confirmed by $Prx2_{ox}$ expression. (B) The expression levels of LC3-II were analyzed by the western bolt. LC3-II levels were quantified and normalized to those of β-actin. (C) Huh7 cells were exposed to $H_2O_2$ in the presence or absence of 10 mM NAC for 24 h. The expression levels of LC3-II and (D) $p$-mTOR/mTOR were analyzed by Western blot. Representative data of at least three independent experiments are shown for protein data. *, $P < 0.05$, **, $P < 0.01$, n.s.: not significant.

mM NAC (Fig. 5C). Although ROS have been shown to suppress *p*-mTOR [24,43,61], no suppression was observed under conditions of non-toxic $ssH_2O_2$ (Fig. 5D). In contrast, based on the dose-dependent disappearance of total LC3B with the co-incubation of $H_2O_2$ and rapamycin (Rapa), the specific inhibitor of mTOR, $H_2O_2$ highly contributed to autophagy degradation process, which is the final stage of autophagy flux. Notably, enhanced degradation was observed without a further mTOR impairment (Suppl. Fig. 5A and 5B). Although mTOR was completely blocked by rapamycin, $H_2O_2$ was still able to modulate autophagy ruling out any major mTOR involvement under these experimental. In line with unchanged mTOR expression, no changes of major upper-stream proteins of mTOR, such as PI3K/Akt and *p*-AMP-K/AMPK, were observed (data not shown). Collectively, low level $H_2O_2$ induces LC3-II expression in Huh7 cells in the absence of mTOR suppression.

### 3.3. $H_2O_2$ activates the autophagic flux in Huh7 cells

We now studied $H_2O_2$-mediated modulation of autophagic flux in Huh7 cells by tandem fluorescent-tagged mRFP-GFP-LC3 transfection. Rapamycin and CQ were used as the positive and negative control. In this assay, GFP fluorescence (green) is rapidly quenched in the acidic environment, e.g. in autolysosome, while RFP fluorescence (red) remains stable and serves as a more specific marker of LC3B expressed in autolysosome. After transfection, mRFP-LC3 visualizes autolysosomes while most GFP-LC3 signals do not colocalize with autolysosome [15, 62]. As shown in Fig. 6A, $H_2O_2$ exposure led to significantly increased numbers of RFP tagged LC3 protein puncta, while the number of GFP puncta did not increase in line with positive control rapamycin expression. Compared with the control group, there are significantly increased RFP puncta, but no GFP puncta after $H_2O_2$ incubation (Fig. 6B). This observation highly suggests that $H_2O_2$ improves the fusion between the lysosome and the autophagosome to successfully form the autolysosome and activates the autophagic flux in Huh7 cells. On the contrary, after CQ treatment, more yellow labeled puncta (overlay between RFP and GFP puncta) was detected since the GFP fluorescence was not quenched due to impaired acidification of the autolysosome compartment. Subsequently, the induced autophagic flux was also confirmed using western blotting. The $H_2O_2$ treatment in the presence of CQ and rapamycin showed the most elevated LC3-II levels as compared to any other treatment group (Fig. 6C). p62, a cargo protein responsible for delivering the damaged organelles to autophagosomes for further degradation [56], typically serving as a negative-related marker to study autophagic flux [63], was likewise induced in the three-treatment group. In addition, $H_2O_2$ and rapamycin co-treatment further increased LC3-II accumulation in a synergistic effect, and a weaker p62 protein expression indicated a functional autophagic flux. Thus, these data suggest that $H_2O_2$ is an mTOR-independent inducer of autophagy that can activate autophagic flux in Huh7 cells (Fig. 7).

### 4. Discussion

We here study the role of ethanol and its specific intermediate acetaldehyde on hepatocellular autophagy *in vivo* and *in vitro*, namely the role for important ALD-associated ROS-producing enzymes such as CYP2E1 and NOX4 under non-toxic conditions. We first confirm in a murine model of acute ethanol exposure that CYP2E and NOX4 are both induced, leading to enhanced autophagy as measured by LC3B levels. While these findings could be recapitulated in primary mouse hepatocytes, commonly used hepatoma cells huh7 required overexpression of CYP2E1 to restore ethanol-mediated autophagy. Moreover, overexpression and knock down of NOX4 could also strongly modulate autophagy in these cells and both CYP2E1-and NOX4-mediated autophagy was efficiently suppressed by the potent antioxidant NAC. Finally, using an enzymatic model of the specific, continuous and non-toxic release of steady-state $H_2O_2$ mimicking an extracellular e.g.

inflammatory ROS source, we demonstrate that $H_2O_2$ is a potent modulator of autophagic flux and, surprisingly, without the involvement of mTOR under these non-toxic conditions.

Our data shed new light on the role of ROS in autophagy, especially $H_2O_2$. Oxidation of ethanol by the microsomal ethanol oxidizing system (MEOS) namely CYP2E1 has long been established as an important pathway of ethanol metabolism and it has been implicated in the progression of disease [64]. Deletion of CYP2E1 in mice has been shown to decrease ROS and DNA adduct formation [65] and also reduced diethylnitrosamine (DEN)-induced hepatic tumour formation [66]. However, the mechanisms by which CYP2E1 generates ROS are still complex and not completely understood although being studied using active-site mutants designed to alter the coupling of ethylbenzene hydroxylation in former reports [67]. In addition to CYP2E1, we therefore studied here two other enzymatic ROS sources: First, overexpression and knock down of NOX4, an important NADPH oxidase expressed in hepatocytes, and induced by ethanol [34] and, second, external and specific generation of non-toxic dosages of $H_2O_2$ by the GOX/CAT system [40] mimicking an extracellular $H_2O_2$ source in patients with ALD e.g., activated neutrophils.

In confirmation of previous data [27], we demonstrate the induction of LC3B and CYP2E1 in a mouse model of acute ethanol exposure. In addition, we also show an induction of NOX4 and indirect signs of oxidative stress through increased levels of the redox-sensitive transcription factor Nrf2 and peroxiredoxin 2 [41]. We were then able to demonstrate in various *in vitro* experiments using LC3B to monitor autophagy that both, the presence of CYP2E1 and NOX4, can induce autophagy. Notably, the important ethanol intermediate acetaldehyde was not able to induce autophagy while external and specific exposure to $H_2O_2$ at non-toxic concentrations or incubation with NAC potently modulated autophagy. We conclude that ROS such as $H_2O_2$ seems to be an important effector of ethanol metabolism rather than acetaldehyde which most likely enhances the need for autophagy through irreversible protein modifications. We also confirm that $H_2O_2$ at these conditions is indeed able to enhance the autophagic flux using well established tandem mRFP-GFP-LC3 plasmid transfection experiments. To our surprise, no direct impact of $H_2O_2$ on mTOR or upstream regulators such as AMP kinase was observed. This is in contrast to previous reports, where almost 500 times higher bolus levels of peroxide were used to observe mTOR changes [68]. Even the mTOR signaling pathway inhibited by ROS generation was reported as an underlying mechanism [24]. We have demonstrated in the past that even opposite effects can be observed with redox sensitive signaling molecules e.g. during hepcidin signal transdurction if non-physiological $H_2O_2$ concentrations are used [48]. While $H_2O_2$ levels in the 100 μM range were able to block hepcidin through unspecific inhibition of the transcription machinery, low continuous levels induced hepcidin and the effect was even potentiated under conditions of mild hypoxia [48,49]. However, it remains unclear how $H_2O_2$ induces autophagy. The recent *in vivo* observation of $H_2O_2$ release during autophagy could suggest that $H_2O_2$ actively participates in the autophagic process as a signaling molecule [37].

Moreover, our data clearly point to ROS-mediated induction of autophagy by ethanol at these non-toxic conditions. First, we demonstrate that CYP2E1 is required for autophagy induction in huh7 cells in the presence of ethanol and NAC completely blocked this induction. Second, although another mandatory end-product of CYP2E1-mediated ethanol metabolism, acetaldehyde in various concentrations and time exposure had no effect on autophagy. Third, autophagy was also induced by other ROS/$H_2O_2$ sources such as NOX4 [49] or $H_2O_2$ release by the GOX/CAT system. Thus, the induction of autophagy by ROS could explain some of the conflicting data on how alcohol affects autophagy [24,27–32] namely whether exposed to acutely or chronically as discussed recently [27]. On the one side, ROS formation and degradation depends on the mode of ethanol application (chronic versus acute) and on the other side, we suggest to use more standardized ROS conditions in cell culture experiments. For instance, bolus addition of $H_2O_2$ requires
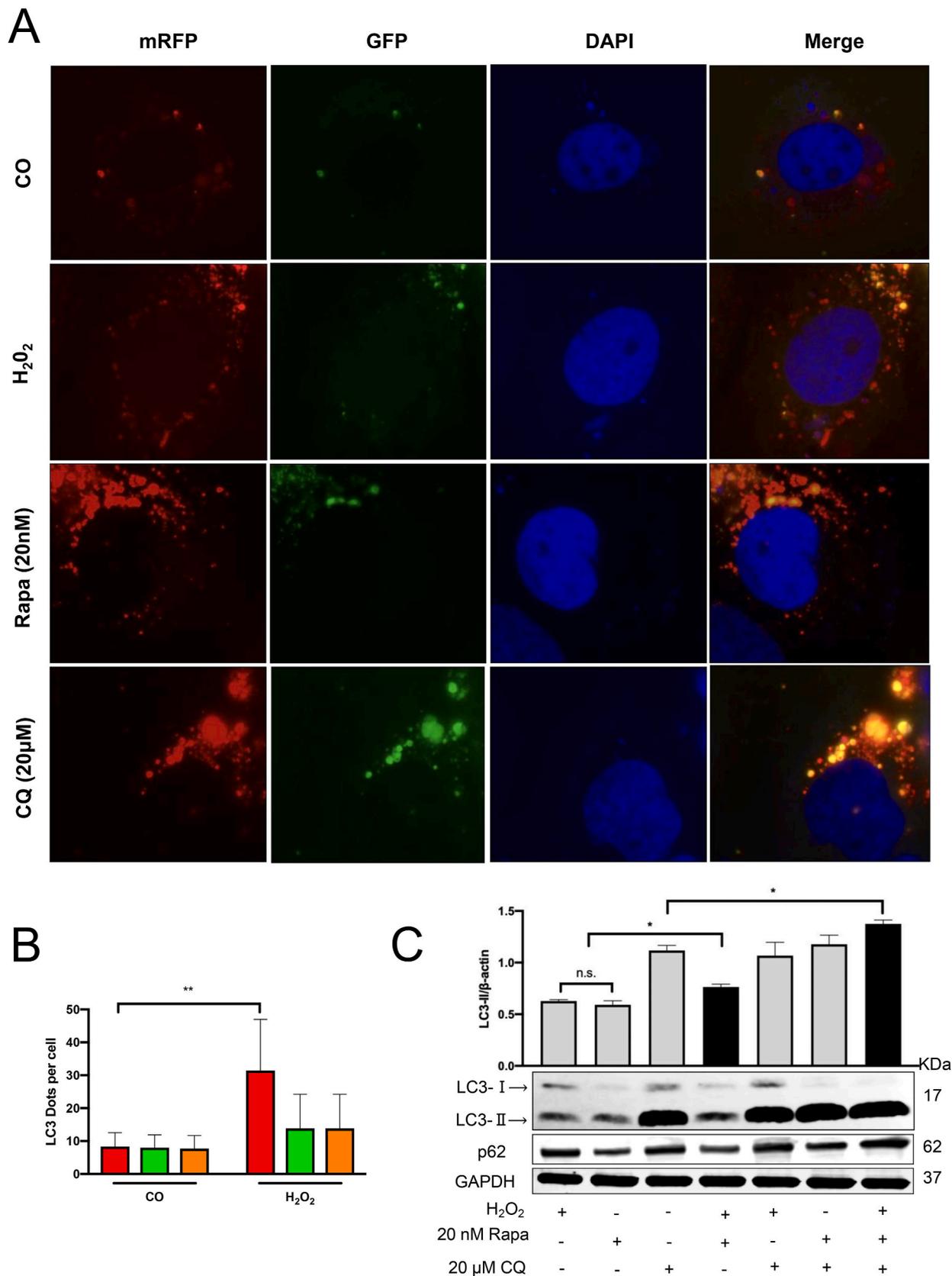
**Fig. 6. Effect of H$_2$O$_2$ on autophagic flux in Huh7 cells by mRFP-GFP-LC3 and immunoblotting analysis.** (A) Huh7 cells were transfected with the tandem mRFP-GFP-LC3 plasmids. 24 h after the transfection, the cells were treated with H$_2$O$_2$ for another 24 h 6 h CQ (20 μM) and Rapa (20 nM) treatment group for negative and positive control. (B) More than 9 cells were counted in the control and the H$_2$O$_2$ group, and data (mean ± SD) are representative of two independent experiments. (C) Huh7 cells were treated with H$_2$O$_2$ for 24 h in the presence or absence of 6 h CQ or/and Rapa treatment. The expression levels of LC3-II and p62 were analyzed by Western blot. *, $P < 0.05$, **, $P < 0.01$, n.s.: not significant.
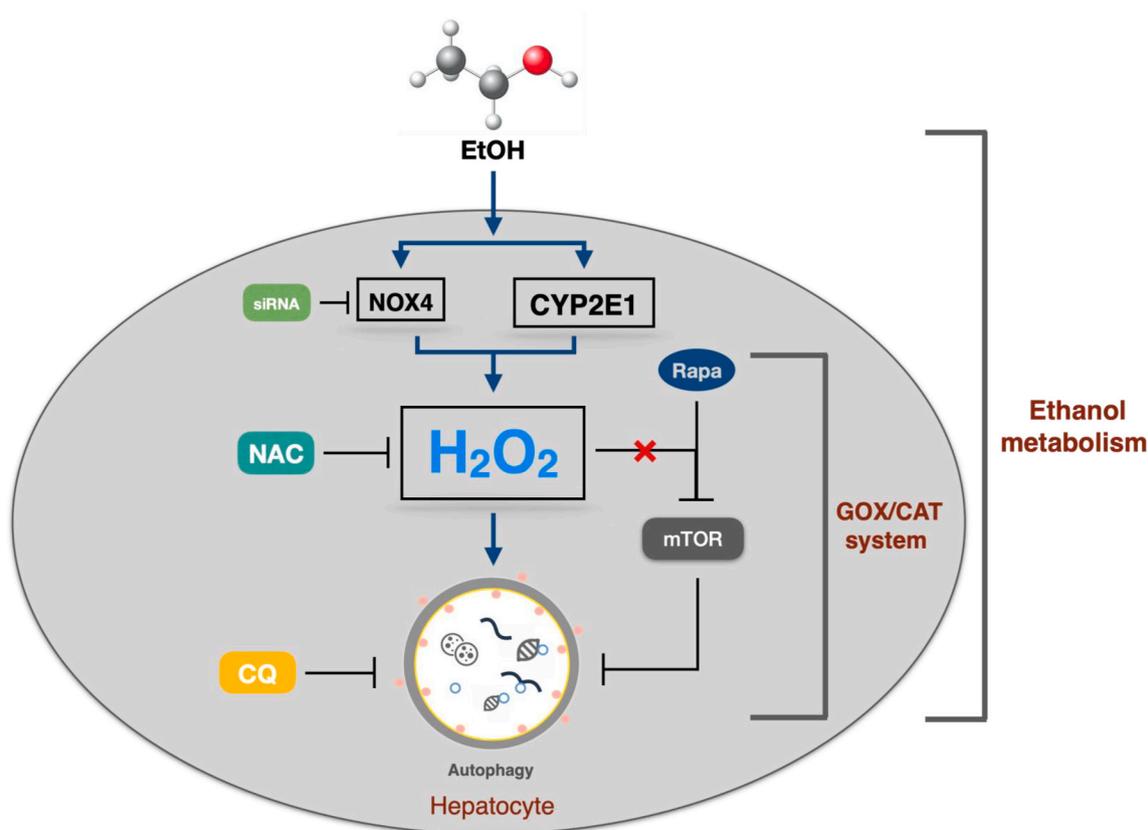
**Fig. 7. Scheme depicting the role of ROS on autophagy.** Especially $H_2O_2$ is generated by CYP2E1 and NOX4 during alcohol metabolism in the liver. Note that mTOR seems not to be involved.

the usage of unphysiologically high $H_2O_2$ dosages since $H_2O_2$ is rapidly removed within minutes from cultured cells [40]. In such settings, cells will be exposed for a too short time to too high $H_2O_2$ levels that may cause unspecific oxidation of various cellular proteins [69].

Our data on the role of $H_2O_2$ on autophagic activity also point to the complex but essential relation between autophagy and programmed cell death. $H_2O_2$ is known to be a traditional inducer of apoptosis e.g. in lymphocytes [35] and it also stimulates proliferation and regeneration and low levels [36]. In the context with our findings, $H_2O_2$ at non-toxic low levels could stimulate both apoptosis and autophagy. On the other side, however, numerous studies indicate that pharmacological or genetic inhibition of autophagy greatly enhances cell death [21–23]. It is generally agreed that autophagy is a pro-survival mechanism by not only providing nutrients for cell survival during starvation but also selectively removing damaged organelles, including damaged mitochondria [70]. Liver-specific knockout of Atg7 leads to hepatomegaly and severe liver injury [23] and suppression of autophagy exacerbates alcohol-induced liver injury by increasing alcohol induced apoptosis [24]. A recent human study on heavy drinkers undergoing alcohol detoxification clearly demonstrated that ethanol primarily suppresses apoptosis and regeneration. In this study, it was shown that levels of caspase 3 cleaved CK18 fragments (M30) in serum were highly correlated with its expression in liver tissue and M30 levels could be used as rather specific marker of liver apoptosis [19]. In addition, detoxication from ethanol caused significant induction of M30 levels and markers of regeneration while signs of necrosis rapidly normalized [19]. We therefore feel that future studies on ethanol-mediated liver damage should better dissect the various biochemical effects of ethanol (e.g. ROS versus acetaldehyde) and should also take into consideration that some of the metabolites such as $H_2O_2$ could be part of the physiological signaling machinery e.g. autophagy or apoptosis. Moreover, as has been already conceived in human [1] and animal studies [27], the drinking

pattern (acute versus chronic) is critical for the understanding of ethanol-mediated organ damage and may explain some of the seemingly contradictive findings in the literature. In this concept, a continuous exposure to ethanol may eventually block these physiological processes due to accumulation of misfolded or modified proteins and congested lysosomes as is typically seen in livers from ALD patients with the formation of Mallory-Denk bodies.

In summary, we here demonstrate that ethanol primarily stimulates autophagy by induction of enzyme systems such as CYP2E1 or NOX4 and enhanced ROS generation such as $H_2O_2$, a condition that is able to induce autophagy at non-toxic conditions. Of note, such non-toxic conditions are not able to suppress mTOR and it remains to be clarified in future studies whether $H_2O_2$ is an essential mediator of autophagy and what specific molecular steps are involved in this regulatory process.

### Author contributions

CC, VR, and SM conceived the study, designed the experiments, and analyzed the data. CC, SJ performed the experiments. CC and SM wrote the manuscript. All authors critically discussed the experimental designs and data.

### Declaration of competing interest

The authors declare no potential conflicts of interest.

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## Appendix A. Supplementary data

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