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Highlights

- Quercetin alleviates alcoholic liver disease
- Quercetin inhibits ethanol-induced exosomes release
- Synergetic effects of autophagy and exosomes in quercetin's hepaprotective activity
- Quercetin regulates autophagy and exosomes via restoring lysosome function

Title page

Autophagy and exosomes coordinately mediate quercetin's protective effects on alcoholic liver disease

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Running title
Synergistic effects of autophagy and exosomes

Key words
Alcoholic liver disease, autophagy, exosomes, lysosome, quercetin

Autophagy and exosomes coordinately mediate quercetin's protective effects on alcoholic liver disease

Abstract

Alcoholic liver disease (ALD), a spectrum of liver abnormalities induced by chronic alcohol abuse, continues to be the major cause of life-threatening liver disease in developed countries. Autophagy and exosomes were individually confirmed to be involved in the pathogenesis of ALD. Here, we sought to identify the role of autophagy and exosomes in the liver protective effects of quercetin. We observed decreased hepatic LC3II/LC3I and increased p62 level in ethanol-fed mice, and these changes were alleviated by quercetin. Meanwhile, nanoparticle tracking analysis (NTA) showed elevated serum exosomes numbers in ethanol-fed mice, which was combated by quercetin. Ethanol induced elevated LDH, ALT, and AST in HepG2 supernatant, which was alleviated by cytochalasin D (exosomes uptake inhibitor). Moreover, quercetin reduced ethanol-induced LDH and ALT elevation in vitro, and the effects of quercetin were reversed by Rab27a overexpression (induce exosomes release) or wortmannin treatment (autophagy inhibitor). Transcriptomic analysis supported that quercetin reversed the change of lysosome related genes disturbed by ethanol. Meanwhile, western blot analysis exhibited decreased hepatic expression of LAMP2 and ATPA6V1B2, and active Cathepsin B/Cathepsin B by quercetin treatment, indicating quercetin alleviated lysosome dysfunction in ethanol-fed mice. Baf A treatment or transfection of siTFEB offset quercetin's effects in ethanol-induced LDH and ALT elevation, exosomes release, and autophagy inhibition (LC3II/I and p62 accumulation). Taken together, quercetin coordinately activates autophagy and combats exosomes release by restoring lysosome function, and further mitigates ethanol-induced liver damage.

Keywords: Alcoholic liver disease, autophagy, exosomes, lysosome, quercetin

1 Introduction

Quercetin, which possess antioxidant and anti-inflammatory activities, is a dietary flavonoid widespread distributed in fruits and leafy vegetables[1, 2]. This flavonoid is widely utilized as a nutritional supplement and as a phytochemical remedy for the prevention and therapy to various diseases such as alcoholic liver disease (ALD)[1, 3]. It is a major kind of chronic liver disease caused by long-term hazardous drinking, which contributes to a substantial liver disease burden globally, with a wide range of prevalence from 14.8% to 53.8%[4-6].

Mechanically, hepatic cells are metabolic dyshomeostasis on account of organelle injury or metabolite accumulation after chronic ethanol intake[7, 8]. Numerous researches suggested that autophagy is a pivotal process for maintaining cellular homeostasis through cellular waste clearance[9-11]. In ethanol-fed mice and ethanol-treated hepatocytes, autophagy dysfunction occurs gradually and losses its clearance ability[12, 13]. We previously observed quercetin alleviated ethanol-induced mitophagy and lipophagy dysfunction in mice fed with ethanol for 12

or 15 weeks[3, 14], while whether autophagy mediate the quercetin's protective effects of ALD is still unknown. In chronic ethanol-fed mice, autophagy agonist carbamazepine or rapamycin treatment alleviated but not entirely abrogated alcohol-induced liver damage in mice[15], indicating other mechanisms are potentially involved in the pathogenesis of ALD. Exosome, a kind of bilipid vesicles with size from 40 to 160 nm, is an alternative pathway as it is loaded with intracellular cargo and is released out of cells[16, 17]. During last decades, exosomes were proved to be increased in ALD patients and Gao-Binge modeled mice, which further induced hepatic injury as indicated by elevated ALT[18]. Additionally, number of trypan blue–positive cells (cell death) was increased in IL-1β/TNF-treated siAtg5 AML12 cells, and exosomes isolated from the medium induced inflammation[19], indicating the potential synergistic effects of autophagy and exosomes in liver disease. Importantly, quercetin was reported to alleviate ethanol-induced hepatic damage by chelating excess free iron[20]. Furthermore, iron overload was proved to spark exosomes release[21]. These studies indicated the potential effects of quercetin on exosomes production. Based on the crosstalk between autophagy and exosomes, we were interested to investigate whether autophagy and exosome coordinately mediate quercetin's protective effects on ALD.

Lysosome function is critical for maintaining the autophagy flux. Previous report showed that ethanol impaired lysosomal biogenesis by inhibiting hepatic transcription factor EB (TFEB) expression, and further induced autophagy inhibition and liver injury in Gao-Binge modeled mice[8]. To our knowledge, lysosome function is also crucial to exosomes production by regulating the degradation of multicellular vesicular bodies (MVBs)[17]. Exosomes are released when MVBs fuse with cellular plasma membrane. While the excretion of exosomes is reduced following the degradation of MVBs by fusing with lysosome[22]. Interestingly, recent studies suggested that the crosstalk between autophagy and exosomes production is associated with lysosome function in the pathogenesis of ALD[23, 24]. Moreover, quercetin enhanced cellular degradation by activating TFEB-mediated lysosome function in ARPE-19 cells[25]. Our previous work illustrated that quercetin recover the number of lysosomes in ALD mice[14, 26]. What's the role of lysosome in quercetin's protective effects?

Thus, we aimed to investigate the effects of quercetin on exosomes release and autophagy, as well as elucidate the underlying mediation of lysosome on the hepatoprotective effects of quercetin. Taken together, our findings highlighted that quercetin coordinately regulates autophagy and exosomes release to prevent hepatocytes from ethanol-induced damage, and this process may be mediated by the regulation of lysosome function. Our work may shed new light on the novel molecular mechanisms of quercetin's liver beneficial effects.

2 Materials and methods

2.1 Animal Model of ALD

Seventy 8-9 weeks old female C57BL/6N mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a temperature-controlled room (20–24 °C) with a 12-h light: dark cycle. Lieber-DeCarli (TP4030D and TP4030C) were purchased from Trophic Animal Feed High-Tech Co., Ltd. (Nantong, China). Mice were randomly assigned to control (CT, n=15), ethanol (ET, n=20), ethanol plus quercetin (EQ, n=20), and quercetin (Q,

n=15) group. In this report, a modified protocol based on the method reported by Gao Bin et al was used[27]. Briefly, after 5 days acclimation feeding, all mice were fed with control (TP4030C) or ethanol diet with or without quercetin supplementation (100 mg/kg·BW), followed by an intragastric administration of ethanol 5 g/kg·BW or maltose dextran 9 g/kg·BW, and finally sacrificed 9 hours later. ET and EQ were allowed free access to the ethanol containing Lieber-DeCarli diet, and CT and Q were pair-fed with isocaloric control diet. All the procedures were approved by the Institutional Animal Care and Use Committee at Huazhong University of Science and Technology.

2.2 Histological Analysis of Liver Tissue

Liver tissue were sectioned into consecutive 7-mm thick sections for hematoxylin and eosin (H&E), oil red O staining. The protocol was as previously described[28].

2.3 Determination of ALT and AST Levels in Cell Culture Supernatants

ALT and AST levels of serum or culture medium were detected according to the instruction of the commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4 Cytotoxicity Assay

LDH activity in the medium was assessed to investigate the toxic effect of ethanol incubation. Serum or culture supernatants were collected and centrifuged 2000 ×g for 5 min at 4 ℃. LDH assay purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) was performed according to the manufacturer's instructions. The absorbance was measured in a microplate reader (HITACHI, Japan) at 490 nm with subsequent blank at 620 nm.

2.5 Real-Time PCR

Total RNA was isolated from 20mg liver tissue using TRIzol® (Invitrogen, USA). The concentration of RNA was measured by using a Nanodrop (Thermo Fisher Scientific, USA). cDNA was obtained according to the manufacturer's instructions (Takara, Japan). The following sequences were used: IL-6 forward 5'-TTCTTGGGACTGATGCTGGTG-3', reverse 5'-GCCATTGCACAACTCTTTTCTC-3', 5'-GCATCCAGCTTCAAATCTCGC-3', reverse 5'-TGTTCATCTCGGAGCCTGTAGTG-3'. PCRs were performed in triplicate using a QuantStudio 7 (Thermo Fisher Scientific, USA), and the relative amount of cDNA was calculated by the comparative CT method and normalized by GAPDH.

2.6 Exosomes Isolation

Exosomes from mice serum were extracted according to the manufacturer's instructions (Invitrogen, USA). Briefly, 200 μl serum sample were centrifuged at 2000 ×g, 4 ℃ for 30 min to remove cells and debris, and then they were transferred to a new tube and further followed by an addition of 0.2 volumes of the Total Exosome Isolation (from serum) reagent. Samples were incubated at 2 ℃ to 8 ℃ for 30 minutes and then centrifuged at 10,000 ×g for 10 min at room temperature. Finally, we obtained exosomes after aspirating and discarding the supernatant. Exosomes from culture medium were purified as previously described[29].

2.7 Exosomes Quantification by Nanoparticle Tracking Analysis (NTA)

The purified exosomes were diluted 1:500–1:1000 in PBS and subjected to NTA using NanoSight NS300 (Malvern, UK). Samples were measured in triplicates. Three 60 seconds videos were recorded of each sample and temperature was monitored throughout the measurements. Particle numbers were analyzed with NTA software version 3.3 to determine the concentration and size of measured particles with corresponding standard error.

2.8 Transmission Electron Microscopy

Exosomes were resuspended in PBS after purification, and then they were transferred to carbon-coated 200-mesh copper electron microscopy grids followed by 10 min incubation at room temperature. Next, exosomes were incubated with 2% phosphotungstic acid for 5 min at room temperature. Micrographs were observed under a transmission electron microscope.

2.9 Cell Culture and Treatments

HepG2 cells and L02 cells were purchased from the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). They were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) at 5% CO2 and 37 ℃, supplemented with 10% fetal bovine serum (Gibco, USA), 100 μg/ml streptomycin (Invitrogen, USA), and 100 U/ml penicillin (Invitrogen, USA). Cells were treated with 100 mM ethanol for 24 hours with or without 50 μM quercetin (Sigma, USA) supplementation.

2.10 Lentivirus Infection and Stable Cell Line Generation

Lentiviral particles carrying CYP2E1 cDNA were purchased from Vigene Biosciences. Inc (Jinan, China) and were used to infect HepG2 cells. After lentivirus infection, cells were selected with 2 μg/ml puromycin for 72 h, and cells that were successfully transfected survived in this selection. The sequences were listed in Table S1. All the control lentiviral particles were obtained from Vigene Biosciences. Inc (Jinan, China).

2.11 Transfection and RNA Interference

Rab27a overexpression plasmid was constructed by Qzenebio Co., Ltd (Shanghai, China) and siTFEB was purchased from RiboBio Co., Ltd (Guangzhou, China). The TFEB siRNA sequences used are as follows: siRNA-1, sense 5′-GACGAAGGUUCAACAUCAATT-3′; siRNA-2, 5′-TTGCTGCTAATAGCTCTCGAG-3′; siRNA-3, 5′-AGACGAAGGUUCAACAUCATT-3′; and negative control (NC) siRNA, 5'-AACGTACGCGGAATACTTCGA-3'. For plasmid transfection/RNA interference, cells were transfected with control or targeting plasmids/siRNA with Lipo3000 (Invitrogen, USA), and cultured for 48h before the next operation. The plasmid and siRNA sequences used in this study are shown in Table S1.

2.12 Immunofluorescence

Liver frozen sections were washed by cold PBS for 5 min 3 times, followed by cold 0.5% TX-100 incubation for 20 min. Slides were incubated with 3% BSA for 1 hour at room temperature. Primary antibodies TSG101(1:200; Abcam, ab125011), LAMP2 (1:200; Proteintech, 66301-I-Ig) and LC3 (1:200; Santa cruz, sc-271625) were incubated for overnight, corresponding secondary goat anti-rabbit antibodies (AF488, Thermo Scientific, USA) and goat anti-mouse (AF555, Thermo Scientific, USA) were incubated for 1 hour. Nuclei were stained with DAPI (Beyotime,

China) and analyzed with fluorescent microscope (Olympus, Japan). Intensity was quantified by two independent, blinded observers in three randomly picked fields of view.

2.13 Exosomes Labeling and Uptake

A total of 1×10^6 cells were seeded into dishes with a diameter of 20 mm. 100 μg purified exosomes were labeled with 5 μM DiI (Invitrogen, V-22885, USA) for 20 min at 37 °C[30]. To monitor exosomes trafficking in vitro, exosomes were added to a final concentration of 40 μg pretein/ml and incubated in the HepG2 cells for 24 h at 37 °C with or without pretreatment with 2 μg cytochalasin D/mL (Cyto D) for 4 h[31, 32]. Supernatants were removed, and cells were washed with PBS three times. Subsequently, cells were fixed using 4% paraformaldehyde (Beyotime, China) for 10 min and then washed with PBS three times. Cell nuclei was visualized with DAPI (Beyotime, China) for 10 min and imaged using confocal laser scanning microscopy (Leica, Germany). Commercial dyes DiI (λ_{ex} = 549 nm, λ_{em} = 565 nm) were assessed.

2.14 Exosomes Release Inhibition

To blockade of HepG2 generation of exosomes, 10 μM GW4869 (Sigma-Aldrich, USA) was applied in HepG2 culture for 24 h, which was initially dissolved in DMSO into a stock solution of 5 mM and diluted in culture medium. The effects of GW4869 on LDH and ALT activity were determined after wash-out procedures.

2.15 Transcriptomics Analysis

Total hepatic RNA was extracted from 15 individual mice using TRIzol® (Invitrogen, USA) and purified with the RNeasy kit (Qiagen, Germany) following the manual. The RNA integrity and purity were quantified by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and NanoPhotometer Spectrophotometer (Implen, Germany), respectively. A sequencing library was prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (Illumina, USA). The library was quantified by Qubit 2.0 Fluorometer (Invitrogen, USA), and insert size of the library was detected using Agilent 2100 Bioanalysis (Agilent Technologies, USA). Libraries were pooled according to the manual and submitted for paired-end sequencing on an Illumina HiSeq. Obtained gene counts were imported into MATLAB. Genes with ≤ 1 read at all time points were excluded from further analysis. The transcriptional data was normalized with the standard DEseq2 normalization.

2.16 Intralysosomal pH by LysoTracker

Intralysosomal pH was monitored by LysoTracker Red DND-99 (Thermo Fisher Scientific, L7528, USA) following the manufacturer's guideline. Briefly, HepG2 cells were cultured in 6-well plate (Corning, USA) for 24 h prior to treatment. LysoTracker (100 nM) was added into the culture medium and incubated at 37 °C for 30 min. Images were captured by fluorescent microscope (Olympus, Japan). Commercial dyes LysoTracker Red DND-99 (λ_{ex} = 577 nm, λ_{em} = 590 nm) were assessed.

2.17 NAGLU Activity

Alpha-N-acetyl glucosaminidase (NAGLU) activity were determined to assess lysosome function[33]. NAGLU assay kit from Nanjing Jiancheng Bioengineering Institute (China) was

used according to the manual. Enzyme activities in the lysosomal fraction and whole cell were estimated as change in absorbance or fluorescence per μg of protein. Cytosolic lysosomal enzyme activities were determined as fractions of their activities in total tissue or cell lysate per μg protein of whole cell lysate. Briefly, cells were lysed in radio immune precipitation assay buffer (RIPA, Beyotime, China) with protease inhibitor supplementation (Beyotime, China). Protein concentration was detected by BCA kit (Beyotime, China). Afterward, cell lysates containing 15 μg protein in total were adjusted to have an equal volume and measured for NAGLU assay kit. Each experiment was replicated at least 3 times.

2.18 Western Blot

Cells or tissues were lysed in RIPA buffer (Beyotime, China) supplemented with phosphatase inhibitors (Beyotime, China). Protein concentration was detected by BCA kit (Beyotime, China). Subsequently, 30 μg protein lysate were subjected to SDS-PAGE-Western blotting. Primary antibodies targeting TSG101 (Abcam, 1:1000; ab125011), CD63 (Abcam, 1:1000; ab68418), ALIX (Proteintech, 1:1000; 12422-1-AP), GRP78 (Abcam, 1:1000; ab212054), Beclin1 (CST, 1:1000; 3495S), P62 (CST, 1:1000; 5114T), LC3B (CST, 1:1000; 3868S), GAPDH (CST, 1:2000; 2118S), CYP2E1 (Proteintech, 1:1000; 19937-1-AP), LAMP2 (Santa, 1:1000; sc-19992), ATP6V1B2 (Santa, 1:1000; sc-166122), Cathepsin B (Santa, 1:1000; sc-365558), TFEB (Abcam, 1:1000; ab113372) and Rab27a (ABclonal, 1:1000; A1934) were used. Western blot data in the figures were all representative of more than three independent experiments.

2.19 Data Availability

All transcriptomics raw data that support the findings of this research are available from the corresponding author upon request.

2.20 Statistical Analysis

Graph plots and P-values were generated using GraphPad Prism 8 software, and all data are shown as the mean ± SD. For comparisons between groups for continuous variables, unpaired student's t tests or one-way analysis of variance (ANOVA) test was performed. P value < 0.05 was considered statistically significant. All experiments were repeated at least 3 times.

3 Results

3.1 Quercetin alleviates features of histological findings and inflammation in ethanol-fed mice

Female C57BL/6N mice were fed by Lieber-DeCarli diet with or without quercetin for 12 weeks, as previously described[27]. Quercetin ameliorated the reduction of weight gain and the elevation of the ratio of liver to body weight induced by ethanol intake (Supple Table1). After 12 weeks, ET mice exhibited disordered hepatic lobule and obvious hepatic lipid accumulation compared with CT group mice. These ET-induced changes were significantly abrogated by quercetin (Fig.1A-B). In addition, quercetin ameliorated elevated serum ALT, AST and LDH levels induced by ethanol intake (Fig.1C). RT-PCR analysis showed a higher level of IL-1β and IL-6 mRNA in ET group

than in CT group, and quercetin supplementation normalized these elevations (Fig.1D). These results suggest the protective effects of quercetin on ALD.

3.2 Quercetin ameliorates autophagy dysfunction in ethanol-fed mice

To evaluate the autophagy status, hepatic protein was extracted and determined by western blot. The ratio of LC3II to LC3I, and level of p62 (compared to GAPDH) were used to evaluate autophagy according to guidelines[34]. Quercetin combated the upregulation expression of Beclin1 and p62, and the reduction of LC3II/LC3I induced by ethanol intake (Fig.2A-B), whereas quercetin alone did not affect their expression. Besides, quercetin alleviated the decreased co-localization of hepatic LC3 and lysosome marker LAMP2 induced by ethanol intake (Fig.2C). Taken together, our data indicates that quercetin ameliorated ethanol-induced hepatic autophagy dysfunction.

3.3 Quercetin mitigates exosomes elevation induced by ethanol administration

In order to investigate the change of exosomes in the condition of ethanol with or without quercetin treatment, serum exosomes were isolated as previously described, and NTA was performed. The expression of exosome marker ALIX, TSG101 and CD63 was showed by western blot and the size of most exosomes was less than 100 nm detected by NTA and TEM, indicating the excellent purity of these vesicles (Fig.3A-C). Quercetin significantly blunted the increased number of circulating exosomes elevation induced by ethanol (Fig.3D). Furthermore, to demonstrate whether the change of intracellular exosomes production MVBs is consistent with exosomes, co-localization of MVBs marker TSG101 and lysosome marker LAMP2 was detected by immunofluorescence staining. Quercetin significantly induced the co-localization of TSG101 and LAMP2 in comparison with ET group mice (Fig.3E), indicating MVBs degeneration by lysosome probably was increased after quercetin supplementation.

3.4 Quercetin prevents ethanol-induced cell damage in vitro

It is known that HepG2 cell lines is CYP2E1 deficient, whereas this enzyme is a major contributor to ethanol-induced oxidant stress. Thus, we established stable transfected HepG2CYP2E1 to apply in the following experiment in vitro (Fig.4A). To identify the protective effect of quercetin on ethanol-induced cell damage, LDH, ALT and AST were detected. Ethanol treatment significantly induced LDH, ALT and AST increased in vitro, while quercetin alleviated their elevation (Fig.4C). These data demonstrate that quercetin showed positive effect on ethanol-induced cell damage in vitro.

To demonstrate the role of exosomes from cells incubated by ethanol with or without quercetin, exosomes uptake was tracked by labeling exosomes with DiI. The uptake of DiI-labeled exosomes in recipient cells was blocked by Cyto D (Fig.4F), an inhibitor of exosome uptake[32]. Importantly, Cyto D administration mitigated elevation of LDH, ALT and AST induced by exosome incubation. Similarly shown in cells incubated by exosomes derived from cell medium with ethanol and quercetin (Fig.4E). These data demonstrate that ethanol-induced exosomes caused cell damage and it was alleviated by quercetin treatment.

3.5 Quercetin alleviates ethanol-induced cell damage via coordinately regulates autophagy

and exosomes release.

Next, we intended to identify the role of autophagy and exosomes in quercetin's protective effect on ethanol-induced damage. To explore whether quercetin alleviates cell damage through combating exosomes release, Rab27a-overexpressed plasmids and GW4869 were used to promote or inhibit exosomes release, respectively[35, 36]. Rab27a overexpression abrogated quercetin's opposite effect on ethanol-induced exosomes elevation, and effects of Rab27a were offset by GW4869 administration. Similarly shown in ALT and LDH levels (Fig.4B and 4D).

We detected autophagy related proteins in vitro. Quercetin decreased the LC3II/I and p62 expression, while quercetin's effects were reversed by wortmannin treatment (Fig.5A-B). Moreover, wortmannin neutralized quercetin's beneficial effects on cell damage such as LDH and ALT levels (Fig.5D).

Several studies reported the close relationship between autophagy and exosomes, and our work exhibited the change in both of them under quercetin treatment. Thus, we next to investigate whether autophagy and exosomes coordinately regulate the process of quercetin alleviation of ethanol-induced cell damage. Either wortmannin administration or Rab27a overexpression combated quercetin's protective effect which was indicated by decreased ALT and LDH. Furthermore, wortmannin treatment plus Rab27a overexpression entirely reversed quercetin-induced reduction of LDH and ALT levels (Fig.5D), similarly shown in L02 cells (Supple Fig.2B). Our data indicates that quercetin prevented cells from ethanol-induced damage by activating autophagy and inhibiting exosomes release simultaneously.

3.6 Quercetin reverses the expression of genes involved in autophagy and vesical trafficking network disturbed by ethanol administration

We next assessed the effect of quercetin and ethanol on the expression of genes involved in autophagy and cellular vesical trafficking network. The relative mRNA expression of Atg4c, Atg2b, Myh10, etc. were increased in ET mice compared with control mice, and quercetin reversed their elevation. Besides, we observed the significant overexpression of endocytosis related and cargo sorting related genes like Actn4, Rac1, Cd151, etc. in ET mice, while quercetin significantly attenuated the change of all these genes (Fig.6A). We analyzed the protein-protein interaction network by using Cytoscape version 3.5.1, and close relationship existed in these differential genes (Fig.6B). Blue dots represent genes enriched in lysosome and autophagy pathway.

Kyoto Encyclopedia of Genes and Genomes (KEGG) didn't contain exosomes pathway. However, emerging data supports the role of lysosome in autophagy and exosomes production. We presumed lysosome is the key of quercetin's regulation on both autophagy and exosomes production. As expected, ethanol decreased the expression of LAMP2 and ATP6V1B2, and the ratio of active Cathepsin B to Cathepsin B (Fig.6C-D). We next detected lysosome function in vitro by using LysoTracker and NAGLU activity kit. Ethanol incubation decreased fluorescence intensity of LysoTracker and NAGLU activity in cells, and these changes were attenuated by quercetin supplementation (Fig.6E-F).

3.7 Quercetin combats cell damage caused by ethanol incubation through recovering lysosome function

We next investigated whether quercetin's protective effect on ethanol-induced cell damage is mediated by lysosome function through using Baf A and transfecting siTFEB. siTFEB transfection and Baf A treatment abrogated the elevation of fluorescence intensity of LysoTracker and NAGLU activity by quercetin (Fig.7A-C,). Furthermore, siTFEB transfection and Baf A treatment offset quercetin's downregulation in LDH and ALT levels (Fig.7G), similarly shown in L02 cells (Supple Fig.2C). Next, we evaluated the change of autophagy and exosomes numbers. Quercetin administration decreased LC3II/I and p62 accumulation induced by ethanol incubation, while siTFEB transfection and Baf A reversed these changes (Fig.7D-E). Similarly shown in exosomes numbers (Fig.7F).

To identify whether the coordinate effect of autophagy and exosomes is mediated by lysosome function, clioquinol (Clio Q) was used to re-acidify lysosomes as a membrane-permeable zinc ionophore[37]. The treatment of Clio Q increased the fluorescence intensity of LysoTracker and abrogated the reduction of NAGLU activity in cells that incubated with ethanol (Supple Fig.1). Similar with quercetin administration, Clio Q treatment reversed the elevation of LDH and ALT levels, and the effect of Clio Q was combated by both the treatment of wortmannin and transfection of Rab27a. Besides, wortmannin supplementation plus Rab27a overexpression entirely reversed these changes by Clio Q administration. Similarly shown in quercetin treatment. Our data demonstrates that the recovery of lysosome function mediated the cell protective effect of quercetin through mitigating autophagy impairing and exosomes release.

4 Discussion

Quercetin, a flavonoid with anti-oxidation and anti-inflammation activities, has been established to protect against ALD. In the past, several researches exhibited that quercetin inhibits ethanol-induced inflammation[38, 39], and protect hepatocyte from ethanol-induced damage by blunting excess free iron[20]. However, the underlying mechanism of its hepatoprotection effect is still unclear. In the present research, we investigated the effects of quercetin on ethanol-induced liver injury in vivo and vitro. The results showed that quercetin administration remarkably alleviated ethanol-induced hepatic damage through coordinately regulating autophagy and exosomes production in lysosome dependent pathway. These findings would be important for the potential utilization of quercetin in the treatment of ALD.

Our study indicates that quercetin treatment activated autophagy in ALD and further alleviated hepatic damage. Similarly, in our previous studies, quercetin intervention blocked chronic ethanol-induced hepatic lipophagy suppression in male mice and further alleviated hepatic lipid accumulation[14, 26]. Besides, mitophagy suppression was also alleviated by quercetin treatment in 15 weeks ethanol-fed male mice[3]. In this study, autophagy antagonist wortmannin partially reversed the liver protective effects of quercetin, further identifying the mediation of autophagy.

Except for alleviating autophagy dysfunction in ALD mice, quercetin was also determined to reverse the elevation of exosomes numbers in our study. There are barely no reports showed that

quercetin regulates exosomes production. Nonetheless, quercetin was reported to inhibit caspase-3 activity in sodium nitroprusside treated chondrocytes[40], and it was demonstrated that exosomes release from hepatocytes incubated with ethanol was caspase-3 dependent in another research[18]. In addition, quercetin decreased miR-155 level in LPS treated RAW264.7 cells[41], and this miRNA was determined to mediate ethanol-induced exosomes release in other study[42]. Recently, quercetin was reported to alleviate ethanol-induced hepatic steatosis by regulating Rab7 which plays key role in lipophagy and exosome production in other study[26, 43]. Importantly, our previous data clarified that quercetin alleviates ethanol-induced iron overload[20], and excess iron was proved to stimulate exosomes production in other study[21]. These findings supported that quercetin probably regulates exosomes release. Interestingly, in our study, it seems that quercetin affects not only the exosomes release, but also its cargo. While we focused on the effects of quercetin on the number of exosomes in this research, and further study about its effects on exosomes cargo needs to be done in future.

More importantly, our data indicates that quercetin attenuated ethanol-induced cellular damage through coordinately activating autophagy and decreasing exosomes production. The crosstalk of autophagy and exosomes has been determined previously[42, 44]. On the one hand, in our research, we observed that wortmannin insignificantly offset the reduction of exosomes induced by quercetin in cells incubated with ethanol. This result supports that autophagy probably affects exosome release. Similarly, disrupted autophagy flux caused exosomes release in ALD mice modeled by ethanol feeding for 5 weeks[42]. On the other hand, GW4869 blocked the effects of adipose-derived mesenchymal stem cells-derived exosomes on target neurons[45], supporting the regulation of exosomes on autophagy. Most importantly, our study exhibited the synergies of autophagy activation and exosomes downregulation in the liver protection of quercetin. Similarly, the synergies of them was also determined by other researches[44, 46]. For instance, sulforaphane, a phytochemical occurring in large amounts in Brassica genus plants, induced senescence of human esophageal cancer cells via coordinately inhibiting autophagy and stimulating exosomes release[46]. Moreover, previous study reported that chronic ethanol feeding caused autophagy flux disruption and exosome elevation mice[42], supporting the potential synergetic effects of autophagy and exosomes release in our research. We further investigated how quercetin concurrently regulates autophagy and exosome release in ALD.

In our study, quercetin alleviated ethanol-induced lysosome dysfunction in vivo and vitro. We further investigated whether lysosome mediates the coordinate regulation of quercetin on autophagy and exosomes release. Our data showed that Baf A treatment or siTFEB transfection offset downregulation of LDH and ALT downregulation the effects by quercetin via coordinately disrupting autophagy activation and exosome reduction. The treatment of Clio Q showed similar effects with quercetin, which was reversed by wortmannin treatment plus Rab27a overexpression. Lysosome is the key organelle involved in autophagosome and MVBs degradation[47], and its dysfunction is one of critical features and central pathophysiological components underpinning ALD[8]. Consistent with our research, quercetin reversed lipophagy inhibition and the downregulation of lysosome marker LAMP1 and LAMP2 in ethanol-fed mice[14, 26], indicating that lysosome probably mediates quercetin's effects on ALD. Although the role of lysosome in quercetin's effects on exosome in ALD has not been investigated, the synergetic effects of autophagy and exosome mediated by lysosome could still be supported by other studies. The nuclear translocation of TFEB was increased and further induced expression of genes related to

lysosome production and acidification after quercetin treatment in ARPE-19 cells[25]. In Gao-Binge modeled ALD mice, impaired TFEB-mediated lysosome biogenesis promoted chronic ethanol-induced liver injury and steatosis via inhibiting autophagic flux[8], and disrupted autophagy stimulated exosome release in another NIAAA model[42]. Additionally, quercetin alleviated LPS induced miR-155 elevation in murine RAW264.7 cells[41]. This microRNA was reported to inhibit the expression of LAMP1 and LAMP2, and further inhibited autophagy flux and stimulated exosome release in ALD mice[42]. Collectively, our data demonstrates that quercetin coordinately activated autophagy flux and decreases exosomes release, further alleviated ethanol-induced liver injury. Importantly, these results confirmed the essential role of lysosome function in quercetin's protective effects.

5 Conclusions

In conclusion, our study indicates that quercetin coordinately activates autophagy flux and rescues exosomes release to attenuate ethanol-induced injury, and this process was mediated by lysosome activation. These data further clarified the mechanisms of quercetin's protective effects in ALD. Thus, quercetin warrants further investigation in clinical ALD therapy.

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7 Authors' contributions

Yuhan Tang conceived the research. Huimin Chen performed most experiments, analyzed the data, and drafted the manuscript. Shufen Peng and Guang Yang helped for mice feeding. Jingjing Liu, Xueer Cheng, Li Chen, Han Zhang, Ying Zhao, and Ping Yao contributed to manuscript revision. All authors read and approved the final manuscript.

8 Conflict of interest

All authors have no conflict of interest to declare.

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Figure legend

Figure 1 Quercetin alleviates pathological score of liver and inflammation in ALD mice. **A** H&E staining and the pathological score of liver in control and ethanol containing Lieber-DeCarli diet-fed (12 weeks) ALD mice with or without quercetin administration. **B** Oil Red O staining of liver tissue frozen section and analysis of area of lipid droplets. **C** Serum ALT and AST levels and LDH activity. **D** Expression of IL-1β and IL6 mRNA in liver by RT-PCR. *p < 0.05, **p < 0.01.

Figure 2 Quercetin ameliorates autophagy dysfunction in ALD mice. **A-B** Western blot analysis for the protein expression of Beclin1, LC3 and P62 in the liver of control and ethanol containing Lieber-DeCarli diet-fed (12 weeks) ALD mice with or without quercetin administration. **C** Immunofluorescence staining of liver section to examine the abundance of LC3 and LAMP2, and co-localization of them. *p < 0.05, **p < 0.01.

Figure 3 Quercetin decreases the elevation of serum exosomes induced by ethanol intake in ALD mice. **A** Western blot analysis for the expression of ALIX, TSG101, CD63 and GRP78 in exosomes isolated from serum and culture medium. **B** Transmission electron microscope determination for the morphological features of exosomes. **C** Size distribution of exosomes detected by NanoSight NS300. **D** Number of serum exosomes by NanoSight NS300. **E** Immunofluorescence staining of liver section to examine the abundance of TSG101 and LAMP2, and co-localization of them. *p < 0.05, **p < 0.01.

Figure 4 Quercetin prevents ethanol induced cell damage, and exosomes induced by ethanol cause cell damage in vitro. **A-B** Western blot analysis for the protein expression of CYP2E1 and Rab27a in HepG2 cells. **C** ALT and AST levels and LDH activity of supernatants from HepG2 after 24 h ethanol (100 mM) treatment with or without quercetin (50 μM). **D-E** Numbers of exosomes derived from HepG2 cells detected by NanoSight NS300, and supernatants ALT, AST and LDH activity. **F** Confocal scanning images to examine exosomes uptake by HepG2. *p < 0.05, **p < 0.01.

Figure 5 Quercetin alleviates ethanol induced cell damage via coordinately regulates autophagy and exosome release. **A-B** Western blot analysis for the protein expression of LC3 and P62 in HepG2. **C** Numbers of exosomes derived from HepG2 cells detected by NanoSight NS300. **D** Supernatants ALT and LDH activity. **F** Confocal scanning images of exosomes uptake by HepG2. *p < 0.05, **p < 0.01.

Figure 6 Quercetin reverses the change of autophagy related, endocytosis related, vesicular cargo sorting related and lysosome related gene expression under ethanol administration. **A** Heatmap of differential genes in the liver of control and ethanol containing Lieber-DeCarli diet-fed (12 weeks) ALD mice with or without quercetin administration. **B** Protein-protein interaction network by Cytoscape version 3.5.1. **C-D** Western blot analysis for the protein expression of LAMP2, ATP6V1B2 and CTSB in mice. **E** LysoTracker for detecting lysosomal pH in HepG2 after 24 h ethanol (100 mM) treatment with or without quercetin (50 μM). **F** NAGLU activity in HepG2. *p < 0.05, **p < 0.01.

Figure 7 Quercetin combats cell damage caused by ethanol incubation through restoring lysosome function. **A** Western blot for the expression of TFEB in HepG2 transfected with or without siTFEB. **B** NAGLU activity of HepG2 after 24 h ethanol (100 mM) treatment with or without quercetin (50 μM). **C** LysoTracker for detecting lysosomal pH in HepG2 cells. **D-E** Western blot analysis for the protein expression of LC3 and P62 of HepG2 cells. **F** Numbers of serum exosomes derived HepG2 cells detected by NanoSight NS300. **G** ALT and LDH activity of HepG2 cells. *$p < 0.05$, **$p < 0.01$.

Figure 1

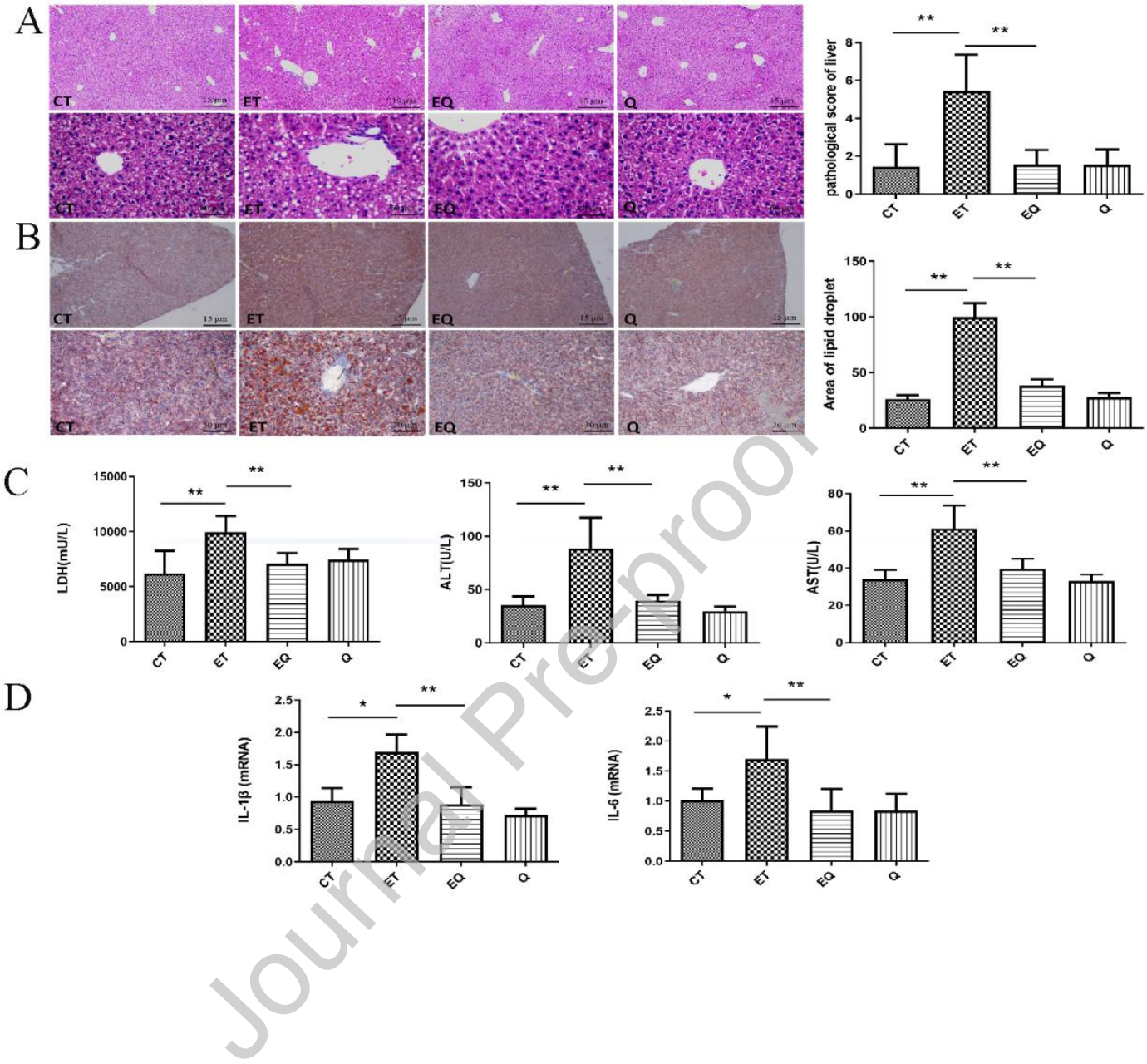

Figure 2

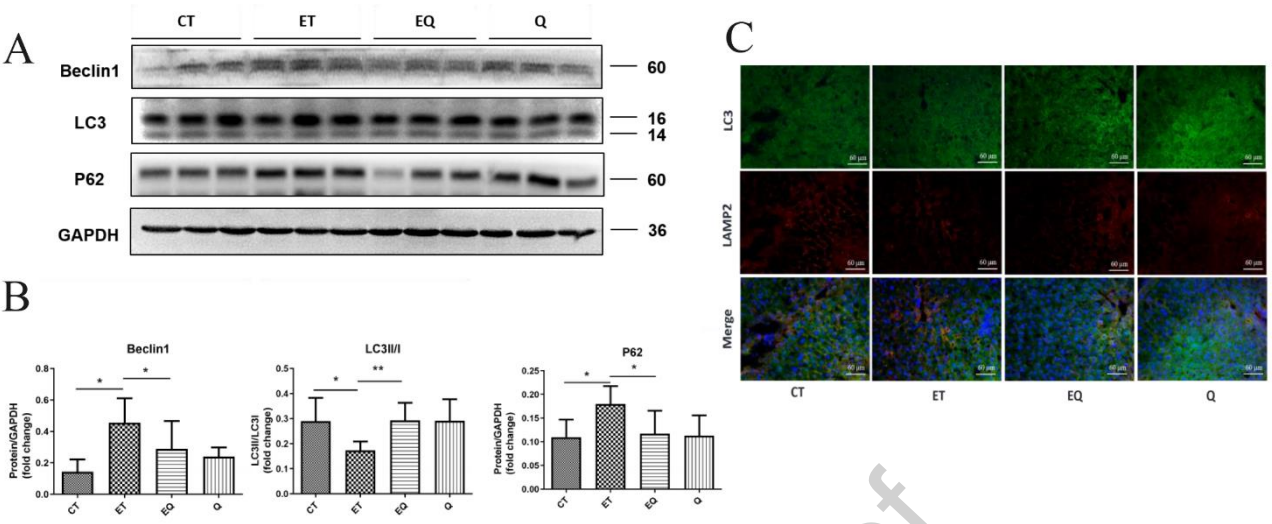

Figure 3

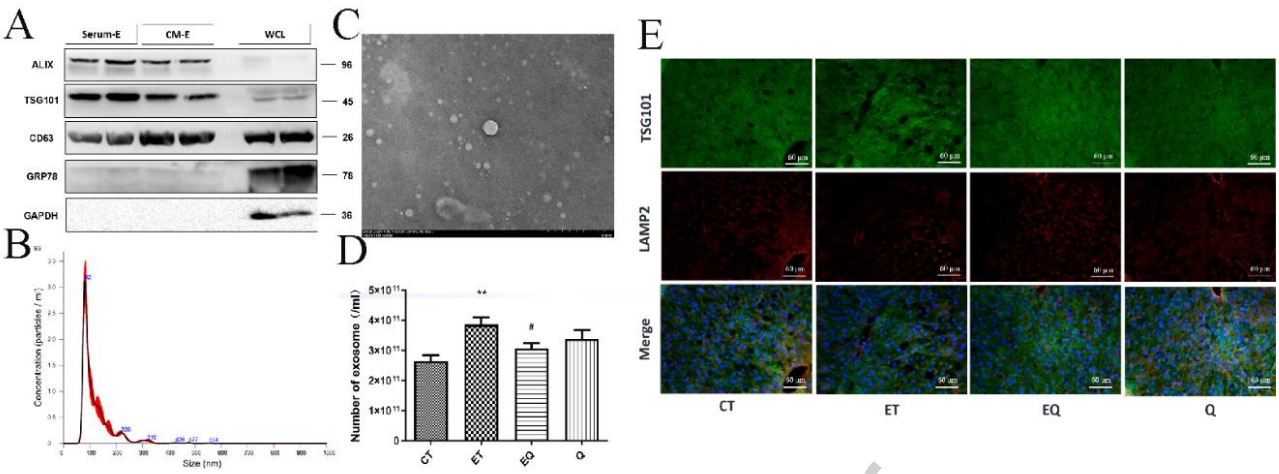

Figure 4

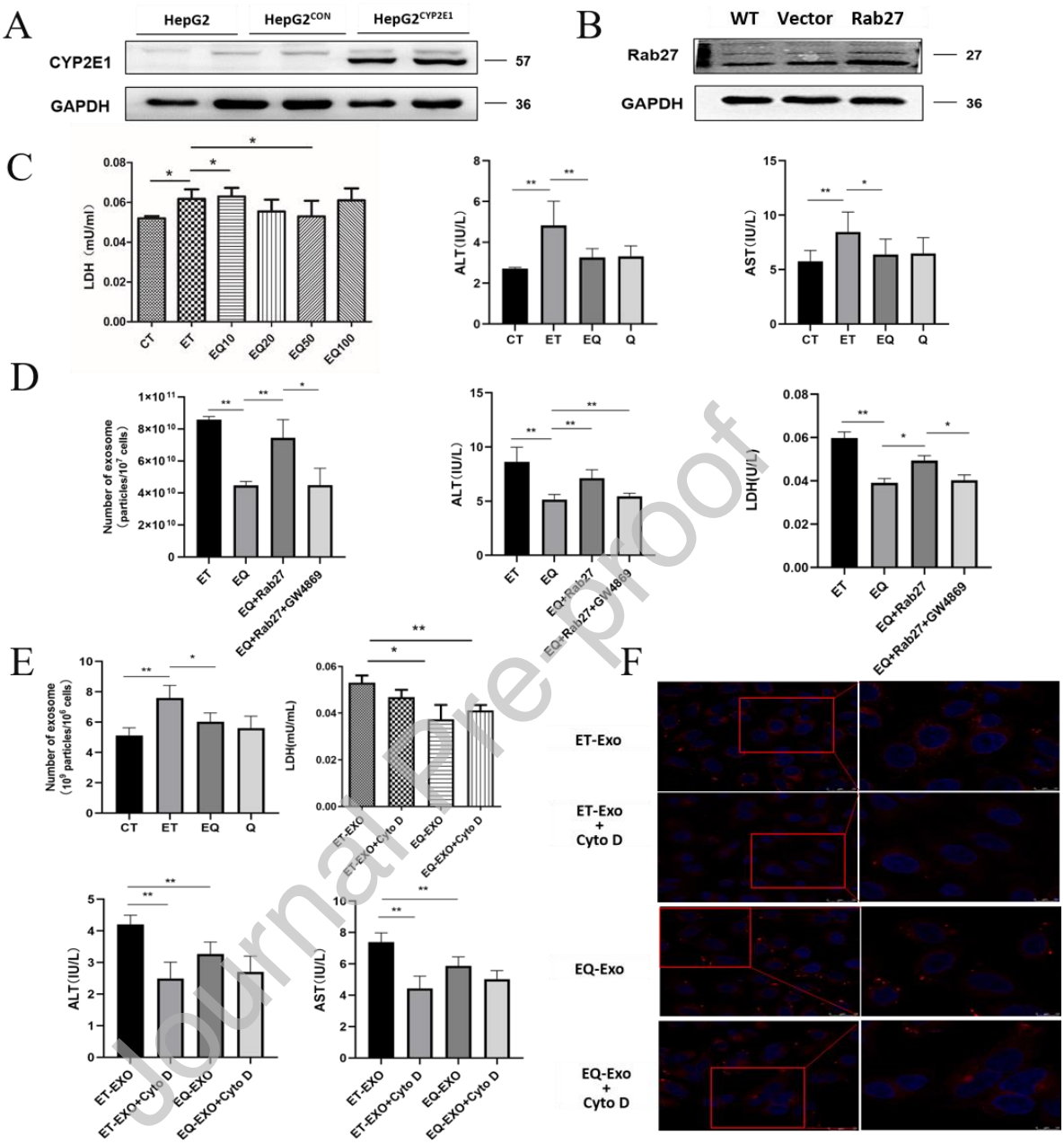

Figure 5

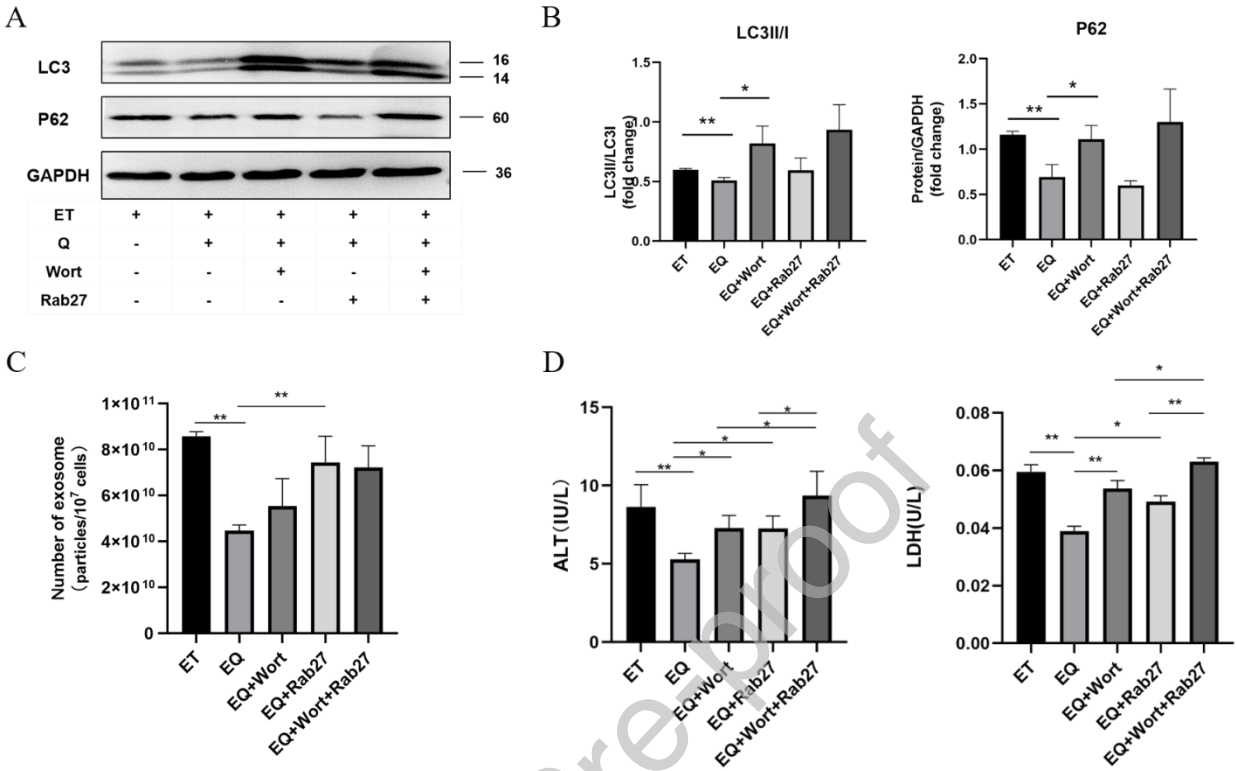

Figure 6

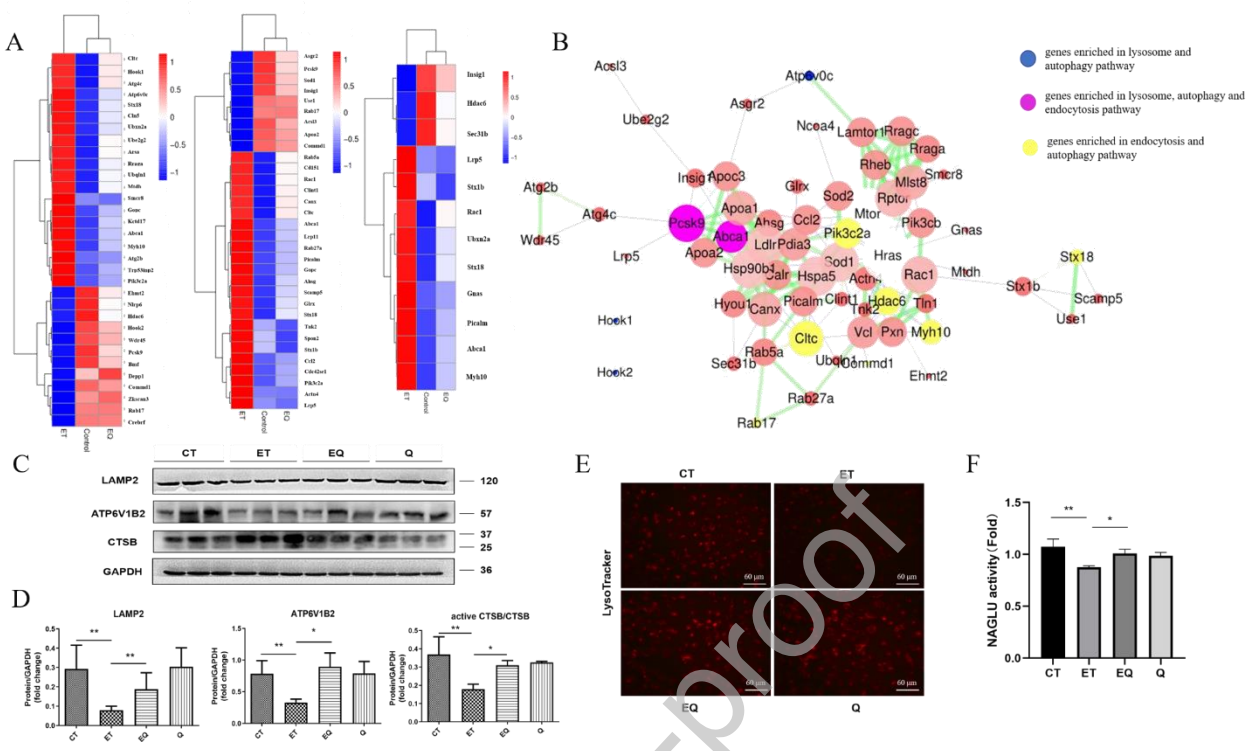

Figure 7