

Activation of TFEB-Dependent Autophagy Underlies the Protective Effects of Polygoni Cuspidati Rhizoma et Radix Extract Against Hepatic Steatosis

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ABSTRACT

Hepatic steatosis, a pathological condition defined by the abnormal buildup of lipids within liver tissue, can be mitigated through the upregulation of autophagy. Polygoni Cuspidati Rhizoma et Radix (PCRR), a botanical remedy conventionally prescribed for the management of atherosclerosis, hepatitis, and cholelithiasis, has recently been shown to exert anti-steatotic properties in hepatic environments. Nevertheless, the precise bioactive components and the molecular pathways driving these effects have not yet been fully elucidated. The present study explored whether an aqueous extract of PCRR attenuates steatosis by regulating hepatic autophagic flux. Our results demonstrate that the PCRR aqueous extract stimulated autophagic flux, accelerated lysosomal biogenesis, and reduced intracellular lipid deposition in both hepatic cell lines and the liver tissue of steatotic rat models. From a mechanistic standpoint, the PCRR aqueous extract suppressed the activity of mechanistic target of rapamycin complex 1 (mTORC1), which induced the dephosphorylation and subsequent nuclear translocation of transcription factor EB (TFEB)—a pivotal master regulator of lipophagy. Conversely, the silencing of TFEB via knockdown experiments diminished the PCRR-induced enhancement of lipophagy in hepatic cell lines. Additionally, interrupting the autophagic pathway using chloroquine (CQ) neutralized the protective efficacy of PCRR against liver steatosis in rats maintained on a high-fat diet (HFD). Collectively, these data indicate that the aqueous extract of PCRR functions as an innovative autophagy promoter and represents a promising therapeutic agent for the treatment of hepatic steatosis.

Keywords: Polygoni Cuspidati Rhizoma et Radix extract, Steatosis, Autophagy, TFEB

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Introduction

When a patient exhibits hepatic steatosis alongside type 2 diabetes, clinical overweight/obesity, or distinct metabolic abnormalities, they are diagnosed with metabolic dysfunction-associated fatty liver disease (MAFLD)—a condition previously termed non-alcoholic fatty liver disease (NAFLD) [1]. Though intrahepatic lipid accumulation characterizes the benign baseline of MAFLD, it serves as a pathological gateway to high-acuity disorders, including nonalcoholic steatohepatitis (NASH), advanced liver cirrhosis, and hepatocellular carcinoma [2, 3]. Halting initial lipid accumulation is therefore the primary clinical line of defense against MAFLD progression. While nutritional interventions and routine physical exercise can successfully reverse steatosis, long-term patient adherence to these lifestyle shifts is notoriously poor.

To bridge this gap, several anti-obesity and anti-diabetic therapeutics (including pioglitazone, pan-peroxisome proliferator-activated receptor agonists, and glucagon-like peptide-1 receptor agonists) have been deployed to mitigate NASH and fatty infiltration [4, 5]. Furthermore, adult NASH treatment recently advanced with the FDA approval of resmetirom, a liver-specific thyroid hormone receptor beta (THR-β) selective agonist [6]. This agent

limits steatosis, secondary inflammation, and subsequent fibrosis by accelerating hepatic fatty acid oxidation, minimizing very-low-density lipoprotein export, and upregulating lipoprotein receptor expression.

Unfortunately, these pharmacological options present a distinct burden of side effects. For example, the GLP-1 receptor agonist semaglutide frequently triggers transient gastrointestinal distress, namely nausea, constipation, vomiting, and diarrhea [7]. Similarly, patients undergoing resmetirom therapy most frequently report adverse episodes of nausea and diarrhea [8], and the drug's long-term safety profile remains unestablished. Given these clinical barriers, traditional Chinese medicine has emerged as an appealing alternative strategy for MAFLD management owing to its established safety history, affordability, and therapeutic efficacy [9].

Data collected over the last decade indicates that preventing fatty liver progression depends heavily on balancing intrahepatic lipid accumulation against disposal pathways. A critical component of this homeostatic machinery is autophagy—a tightly orchestrated cellular mechanism that isolates and degrades dysfunctional proteins and organelles. Specifically, the autophagic breakdown of intracellular lipid droplets (known as lipophagy) directly modulates systemic lipid balance, whereas a failing autophagic system correlates strongly with MAFLD onset [10].

Consequently, manipulating the autophagy-lysosomal pathway via genetic or pharmacological means presents a viable therapeutic target; doing so accelerates lipid droplet clearance and boosts hepatic insulin sensitivity [11–13]. In murine models, genetically overexpressing transcription factor EB (TFEB) protected against high-fat diet (HFD)-induced fatty liver and rescued metabolic impairments via autophagic upregulation [14]. Similarly, lipid-reducing pharmaceuticals like ezetimibe [15] and nuciferine (a natural alkaloid derived from lotus leaves) [12] alleviate NASH and steatosis by triggering TFEB-driven autophagy. These collective insights suggest that TFEB counteracts MAFLD primarily by driving autophagy-dependent lipid clearance.

In traditional Chinese medicine, *Polygoni Cuspidati Rhizoma et Radix* (PCRR) is commonly prescribed to combat metabolic syndrome due to its broad anti-inflammatory, insulin-sensitizing, antihyperlipidemic, and antioxidant profile [16–18]. Extracted butanol fractions and key phytochemicals isolated from the herb, such as polydatin and resveratrol, have demonstrated clear efficacy in protecting liver metabolism and improving hepatic lipid profiles [19–22].

While our previous work proved that an aqueous extract of PCRR successfully mitigates fatty liver in HFD-fed rats [23], the exact molecular mechanics—specifically whether this extract regulates lipophagy and lipid clearance via TFEB—remain unknown. This investigation was designed to uncover that pathway. Our current data show that the aqueous PCRR extract lowers hepatic steatosis by suppressing mechanistic target of rapamycin complex 1 (MTORC1) activity, which in turn dephosphorylates TFEB and triggers downstream autophagy.

Materials and Methods

Plant extraction procedures and analytic method analysis

To prepare the extract, 600 g of dehydrated PCRR roots sourced from an authentic traditional Chinese medicine provider (Wai Yuen Tong Medicine, Hong Kong, China) were boiled in 6 L of distilled water for 1 h. The resulting solution was separated via centrifugation and the supernatant was subsequently lyophilized following established methodologies [23]. This protocol yielded 72.6 g of dried extract, translating to a 12.1 % extraction efficiency. The lyophilized aqueous PCRR extract was kept under refrigeration at 4 °C until use.

Analytical reference compounds (purity grade HPLC > 98 %) for emodin 8-O- β -D-glucopyranoside, polydatin, emodin, resveratrol, and physcion 8-O- β -D-glucopyranoside were obtained from Shanghai ChemPartner Co., Ltd. (Shanghai, China). High-performance liquid chromatography profiling was executed using an Agilent 1100 series HPLC-diode array detector (HPLC-DAD) platform fitted with an Alltima HP C18 column (4.6 mm $\times$ 25 cm, 5 μ m). Eluted compounds were tracked at a wavelength of 290 nm using a dynamic gradient of mobile phase A (water) and mobile phase B (acetonitrile) set as follows: 20 % B at 0–1 min, 40 % B at 6 min, 50 % B at 10 min, 60 % B at 28 min, and 100 % B at 45 min. A constant flow rate of 0.8 mL/min was maintained throughout the run.

Cell culture

Experimental models utilized the human cell lines HeLa, HepG2, LO2, and HEK293T, which were maintained in Dulbecco's Modified Eagle Medium (Cat. No. 12800–017, ThermoFisher Scientific, Waltham, MA, USA) under environmental conditions of 5 % CO₂ at 37 °C. The culture medium was supplemented with 10 % fetal bovine

serum (FBS) (Cat. No. 10270106, ThermoFisher Scientific) along with an antibiotic cocktail of 100 µg/mL streptomycin and 100 U/mL penicillin (Cat. No. 15140-122, ThermoFisher Scientific).

To induce cellular steatosis, a 2:1 molar ratio mixture of oleic acid and palmitic acid (free fatty acids, or FFA) was introduced into the HepG2 and LO2 cultures. These individual fatty elements—oleic acid (OA) (Cat. No. O1008) and palmitic acid (PA) (Cat. No. P5585)—were sourced from Sigma-Aldrich. For a 24 h window, HepG2 cells were exposed to 0.15 mM FFA and LO2 cells to 0.3 mM FFA, during which they were concurrently treated with or without the aqueous PCRR extract. Control cohorts were incubated exclusively with fatty acid-free bovine serum albumin (BSA) (Cat. No. A8806, Sigma-Aldrich).

Lipid accumulation was visualized by staining the cells for 15 min at room temperature using either 3 µM Nile Red (Cat. No. 72485, Sigma-Aldrich) or a 5 µM BODIPY 493/503 lipid probe (Cat. No. D3922, ThermoFisher Scientific), followed by a 15-min immobilization in 4 % paraformaldehyde. Intracellular fluorescence emissions were captured via a Leica TCS SP8 Multiphoton confocal microscope or recorded on a CLARIOstar spectrophotometer, with Image J software (National Institutes of Health, Bethesda, MD, USA) handling the quantification of the BODIPY signals. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (G3581, Promega, USA) was deployed to track overall cell viability.

Western blot assay

Immunoblotting operations were executed according to the experimental methodologies outlined in our prior literature [24].

Immunoprecipitation

Protein extraction from liver tissues and cultured cells matched the protocols used for Western blotting. To isolate target proteins, cell lysates were mixed with FLAG Magnetic Beads (Cat. No. M8823, Sigma-Aldrich) at 4 °C for 12 h. Under identical temperature and time parameters, liver tissue homogenates were incubated with a TFEB-specific antibody (Cat. No. A303-673A, ThermoFisher Scientific) complexed with Dynabeads Protein G (Cat. No. 10004D, ThermoFisher Scientific).

The protein-bound beads were subsequently cleaned via a three-step washing sequence: twice with immunoprecipitation wash buffer (Cat. No. 26149, ThermoFisher Scientific) and a final rinse with UltraPure DNase/RNase-Free Distilled Water (Cat. No. 10977015, ThermoFisher Scientific). Elution was achieved by boiling the bead complexes within a 1 × SDS-PAGE sample loading buffer (Cat. No. P0015A, Beyotime), followed by molecular weight separation on SDS-PAGE gels.

Cell transfection and gene knockdown assay

Transient genetic overexpression was achieved in HeLa cells by introducing plasmids for GFP-tagged wild-type TFEB or FLAG-tagged wild-type TFEB (Integrated Biotech Solutions Company, Shanghai, China) via Lipofectamine™ 3000 Transfection reagent (Cat. No. L3000150, ThermoFisher Scientific). PCRR exposure was initiated for 4 h after a 48 h post-transfection incubation period.

To generate stable reporter cell lines, LO2 or HeLa cultures were infected with 5×10^6 PFU of lentiviral particles containing GFP/RFP-LC3 or GFP-RFP-LC3. Selection was carried out using puromycin for 24 h, starting 48 h after the initial viral infection.

For targeted gene silencing experiments, Lipofectamine RNAiMAX (Cat. No. 13778150, ThermoFisher Scientific) was used to transfect LO2 cells with 50 nM of either non-targeting control small interfering RNAs (siCtrl) or specific TFEB-targeting duplexes (siTFEB-1 or siTFEB-2). Following a 24 h post-transfection window, cells were incubated with the aqueous PCRR extract for 8 h.

Immunofluorescence assay and immunohistochemistry assay

The protocols for immunohistochemistry (IHC) and immunofluorescence aligned with the methods detailed in our previous study [24]. Briefly, following cellular fixation and blocking steps, HeLa or LO2 cells were incubated at 4 °C overnight with a primary anti-lysosomal-associated membrane protein 1 antibody (anti-LAMP1, Cat. No. 9091, Cell Signaling Technology, MA, USA).

After washing away unbound primary antibodies, a 1 h room-temperature incubation was performed using secondary antibodies conjugated to Alexa Fluor 647 (Cat. No. A21244, ThermoFisher Scientific) or Alexa Fluor 555 (Cat. No. A32732, ThermoFisher Scientific). Nuclei were visualized via DAPI counterstaining (Cat. No.

H1399, ThermoFisher Scientific), and micrographs were captured on a Leica TCS SP8 Multiphoton confocal microscope. For the tissue-level IHC assays, harvested rat liver sections were probed with an LC3B antibody (Cat. No. NB100-2220, Novus Biologicals) overnight at 4 °C.

Measurement of lysosomal pH

Lysosomal pH shifts were monitored through both qualitative and quantitative approaches based on our previously described methods [25]. In short, LO2 cells underwent a 6 h incubation with PCRR. For qualitative evaluation, the cells were labeled with 1 μ M LysoSensor Green DND-189 (Cat. No. 40767ES50, Yeasen, Shanghai, China). Quantitative assessments were performed by staining the LO2 cells with 2 μ M LysoSensor Yellow/Blue DND-160 (Cat. No. 40768ES50, Yeasen).

Reverse transcription-quantitative PCR assay

Gene transcription levels were quantified through reverse transcription-quantitative PCR (RT-qPCR) assays in accordance with our previous report [23].

Animal studies

The Animal Ethics Committee of the Hong Kong Polytechnic University reviewed and approved all in vivo procedures under institutional permit number 21-22/214-ABCT-R-GRF. Male Sprague-Dawley (SD) rats (n = 32, initial weight 90–110 g) were obtained from Bestest Biotech (Zhuhai, China).

Following a 1-week acclimation period, the rats were distributed into four separate experimental groups (n = 8 animals per cohort) and maintained on specified protocols for 4 weeks: a normal control diet (Cat. No. D12450B, Research Diet, New Brunswick, NJ, USA); a high-fat diet (HFD, Cat. No. D12492, Research Diet); an HFD + PCRR group (HFD combined with an oral dose of 80 mg/kg PCRR); and an HFD + PCRR + CQ group (HFD plus 80 mg/kg PCRR, combined with an intraperitoneal injection of chloroquine at 10 mg/kg body weight/day administered 16 h before sacrifice).

Biochemical analysis

Circulating serum profiles for total cholesterol (TC, Cat. No. 1010), triglycerides (TG, Cat. No. 2100-430), alanine transaminase (ALT, Cat. No. 2930-430), and aspartate aminotransferase (AST, Cat. No. 2920-430) were determined using enzymatic colorimetric assay kits from Stanbio Laboratory (Texas, USA). Serum low-density lipoprotein cholesterol levels (LDL-c, Cat. No. A113-2-1) were quantified using a commercial assay kit purchased from Nanjing Jiancheng (Nanjing, China).

Hepatic lipid content, TG and TC content, histological analysis of liver tissues

Our previous investigations [23, 26] outline the methodologies utilized for histological evaluation (via Oil Red O and hematoxylin and eosin [H&E] staining), alongside the determination of total lipids, TG, and TC within liver tissues. To gauge overall hepatic lipid storage capacity and monitor fat accumulation dynamics, total intrahepatic lipids—comprising diacylglycerol, free cholesterol, cholesterol esters, phospholipids, fatty acids, and glycerides—were quantified.

For this total lipid assessment, a cordless motor homogenizer (Kimble, 749540-0000, USA) was used to process ~150 mg of hepatic tissue in a 2:1 (v/v) chloroform-methanol mixture to yield a 1:20 (w/v) final dilution. Next, 3 mL of this tissue homogenate was mixed thoroughly with 0.6 mL of water by vortexing, and then spun down at 900 g for 20 min. The lipid-containing chloroform phase was isolated and left to air-dry. Weighing the remaining desiccated mass yielded the total intrahepatic lipid content, normalized to the initial tissue sample mass (expressed as weight of lipid per gram of liver tissue).

To precisely distinguish between the two primary lipid classes involved in fatty liver pathology, individual assays for hepatic TG and TC were conducted. Approximately 25 mg of liver tissue was disrupted in 500 μ L of PBS and then left overnight at 4 °C in 6 mL of a 2:1 (v/v) chloroform-methanol solution. Following this, the mixture received 1.2 mL of a 0.7 % NaCl solution and was left to sit for a second overnight incubation at 4 °C. The bottom chloroform layer was subsequently isolated, evaporated to dryness, and reconstituted using 150 μ L of isopropanol. Hepatic concentrations of TG and TC were then quantified using a commercial TG kit (Cat. No. 2100-430; Stanbio Laboratory) and a TC kit (Cat. No. 1010; Stanbio Laboratory) according to the operational guides provided by the manufacturer.

Transmission electron microscopy assay

Ultrastructural cellular characteristics were evaluated via transmission electron microscopy (TEM) in accordance with our previously established protocol [24]. In brief, LO2 cell suspensions and harvested rat liver specimens were fixed for a 3-h window in 2.5 % (v/v) glutaraldehyde (G1102; Servicebio). After rinsing the fixed specimens three times with phosphate buffer, a 15-min step-by-step dehydration process was carried out using a graduated series of alcohol and acetone concentrations. Infiltration was executed by exposing the dehydrated specimens to a 1:1 blend of embedding medium and 100 % acetone for 3 h, followed by a 6-h treatment in pure embedding medium to achieve complete matrix permeabilization.

To ensure optimal cross-linking and polymerization, samples were kept at 40 °C for 12 h and then transferred to 60 °C for two days. Ultra-thin sections of 60–80 nm were sliced using a Leica UC7 Ultramicrotome (Leica, Wetzlar, Germany). Section staining took place in the dark for 8 min using a 2 % uranium acetate solution prepared in saturated alcohol, followed by three sequential rinses in 70 % ethanol and three rinses in ultrapure water. To prevent artifactual staining from interacting with ambient CO₂, sections were treated for 8 min with a 2.6 % lead citrate solution and given a final three-step wash in ultrapure water. Visualization and image capture were completed on a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan).

Statistical analysis

All experimental values are reported as mean $\pm$ SEM. For datasets demonstrating equal variance and normal distribution profiles, statistical differences were evaluated through either a two-tailed Student's t-test, a One-way ANOVA, or a Two-way ANOVA, utilizing Tukey's post-hoc correction for multiple endpoint comparisons as noted in the respective Figure descriptions. Data analysis was managed via Prism 9 software (GraphPad Software, CA, USA), and an alpha level of P less than 0.05 was chosen to denote statistical significance.

Results and Discussion

Quantitative analysis of primary constituents in the PCRR aqueous extract sample

The characteristic chromatograms representing both the standard reference mixture and the aqueous extract of PCRR are displayed in **Figure 1**. Relative to the total mass of the desiccated aqueous extract, the measured proportions of the 5 identified constituents were determined to be: polydatin (5.69 %), resveratrol (0.67 %), physcion 8-O- β -D-glucopyranoside (0.61 %), emodin 8-O- β -D-glucopyranoside (0.28 %), and emodin (0.32 %).

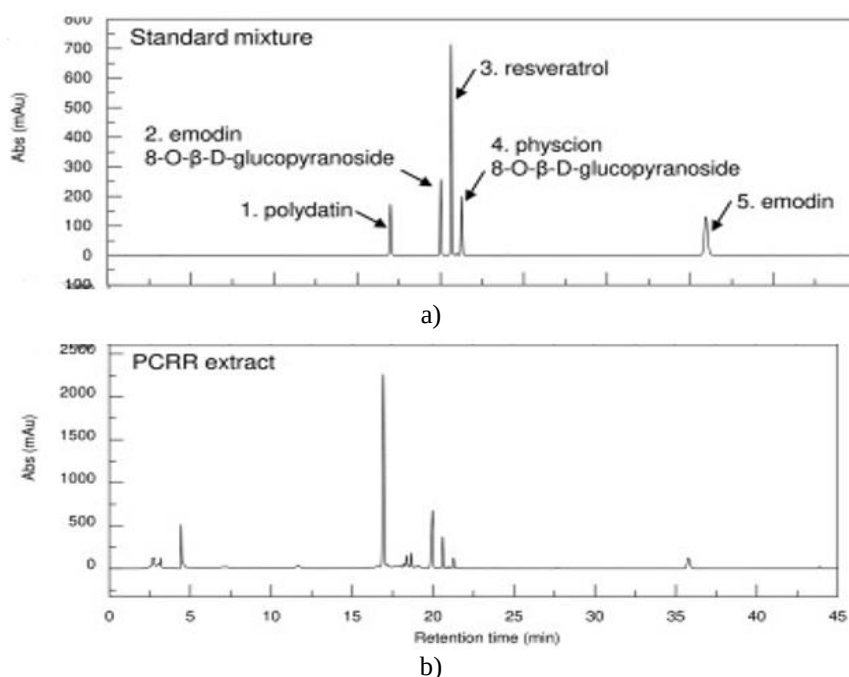


Figure 1. Characteristic chromatograms of the reference standards (a) and the aqueous extract of PCRR (b) obtained via HPLC-DAD/ Peak 1: polydatin, 2: emodin 8-O- β -D-glucopyranoside, 3: resveratrol, 4: physcion 8-O- β -D-glucopyranoside, and 5: emodin.

PCRR accelerates autophagic flux in vitro

The objective of this analysis was to determine whether autophagy plays a role in driving the anti-steatotic properties of PCRR. Initially, light microscopy was employed to observe the appearance of substantial, semi-transparent vacuoles within LO2 cells exposed to the PCRR aqueous extract (**Figure 2a**). This morphological feature is likely a consequence of alterations in autophagic flux, aligning with observations from prior literature [27]. Furthermore, ultrastructural examination via electron microscopy confirmed the presence of numerous autophagic vacuoles within the PCRR-exposed LO2 cells (**Figure 2b**). To characterize how PCRR modulates autophagic processes, HeLa cells with stable expression of RFP-GFP-LC3 were exposed to the extract. As illustrated in **Figure 2c**, a marked accumulation of red-only fluorescent puncta occurred in HeLa cells exposed to rapamycin, an established MTOR inhibitor known to accelerate autophagic flux. In a similar manner, administration of PCRR triggered a substantial rise in red-only LC3-II puncta, indicating the generation of autolysosomes. Conversely, treatment with CQ, a well-recognized inhibitor of late-stage autophagy, prominently induced the development of yellow LC3-II puncta, pointing to an impairment in the fusion between autophagosomes and lysosomes. Additionally, the presence of CQ counteracted the stimulatory impact of PCRR on autolysosome generation. As anticipated, HeLa cells exposed to PCRR demonstrated a marked elevation in GFP-LC3-II puncta, which exhibited extensive colocalization with the lysosomal marker LAMP1, matching the patterns observed under rapamycin administration. Notably, the administration of PCRR considerably enhanced the fluorescent signal intensity of LAMP1 (**Figure 2d**). Equivalent outcomes were noted in LO2 cells expressing either RFP-GFP-LC3 or GFP-tagged LC3. Collectively, these findings imply that PCRR stimulates autophagic flux.

Immunoblotting further revealed that PCRR exposure elicited both a dose-dependent and time-dependent upregulation of lipidated LC3-II expression in LO2 cells. Concurrently, an accumulation of the SQSTM1 (sequestosome-1) protein was detected in the PCRR-treated LO2 cells (**Figure 2e**). Comparable trends were recorded when HepG2 cells were treated with PCRR. Because SQSTM1 serves as an autophagic substrate, it is conventionally broken down when autophagy is activated. However, SQSTM1 expression is also under the transcriptional control of TFEB, which serves as a master regulator of lysosomal biogenesis [28]. Elevated expression of TFEB has been documented to increase the abundance of both LC3B-II and SQSTM1 [29]. To clarify whether PCRR treatment ultimately supports the breakdown of SQSTM1, LO2 cells were treated with PCRR in combination with cycloheximide (CHX), an inhibitor of protein synthesis. In the presence of CHX, PCRR facilitated a further decline in SQSTM1 concentrations, whereas CQ did not, indicating that PCRR exposure promotes the clearance of SQSTM1 in tandem with the stimulation of autophagy (**Figure 2f**). Moreover, the expression levels of LC3B-II were further augmented in PCRR-treated LO2 cells when co-administered with CQ (**Figure 2g**). Beyond LC3 and SQSTM1, the expression of other core autophagy-associated proteins, specifically ATG3, ATG5, ATG7, and BECLIN1, was evaluated. The findings revealed that protein levels for ATG3 and ATG7 were markedly elevated in LO2 cells following PCRR exposure, while the levels of ATG5 and BECLIN1 showed no significant changes. These insights suggest that PCRR potentially drives the generation of autophagosomes through the upregulation of ATG3 and ATG7 in vitro. In summary, these data demonstrate that PCRR enhances autophagic flux, resulting in elevated levels of LC3B-II.

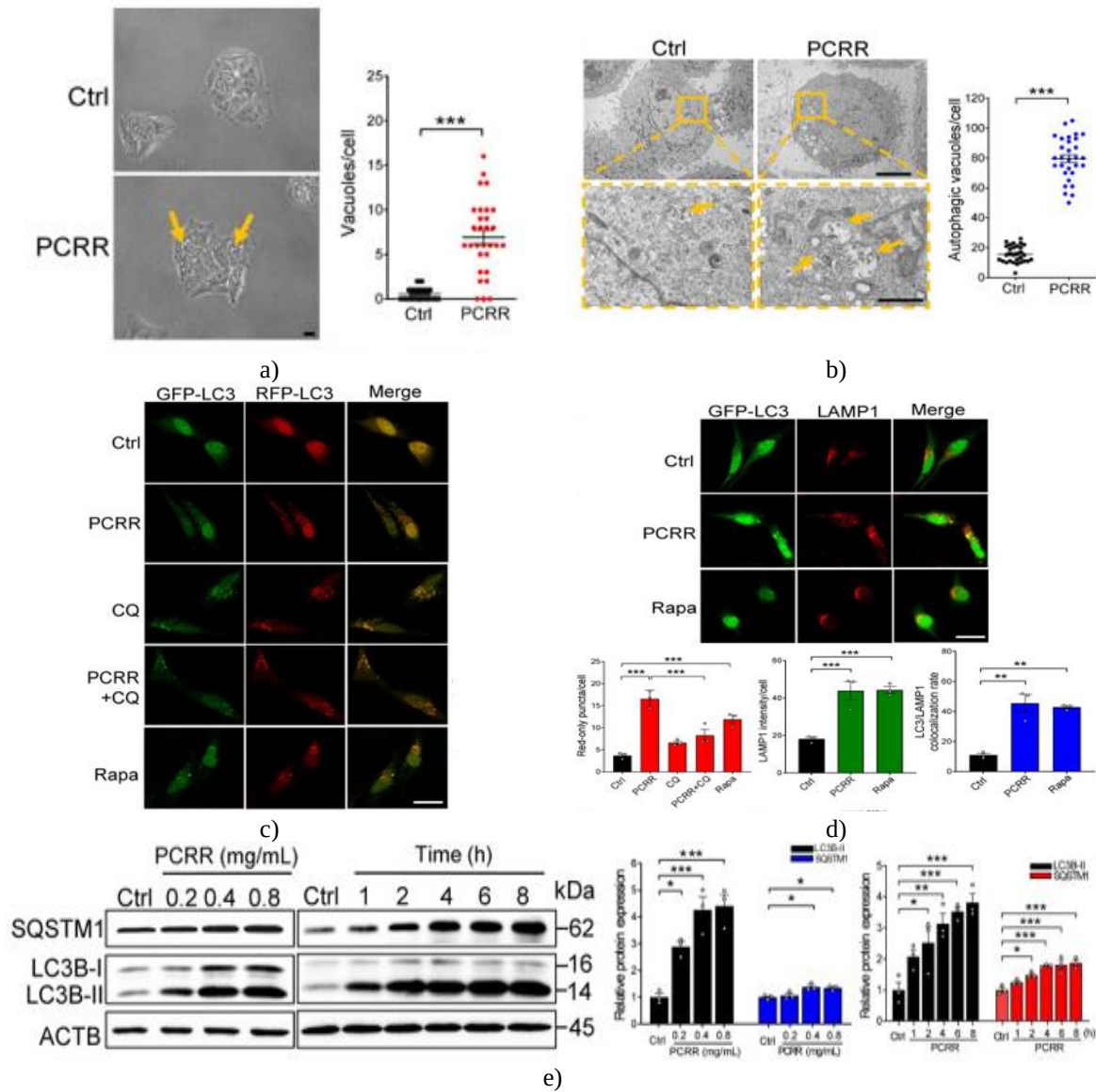
PCRR triggers TFEB nuclear import and enhances lysosome production in vitro

As previously noted, LO2 cells exposed to PCRR exhibited a pronounced rise in LAMP1 fluorescence signal intensity, suggesting potential TFEB activation [28, 29]. To verify that PCRR stimulates TFEB activation, TFEB-GFP was transiently overexpressed in HeLa cells prior to PCRR exposure. This administration prompted a time-dependent migration of TFEB from the cytoplasm into the nucleus (**Figure 3a**). Corresponding behaviors were demonstrated in LO2 cells overexpressing TFEB-GFP. Subcellular fractionation profiling verified that PCRR strongly drove the nuclear import of FLAG-TFEB within HeLa cells (**Figure 3b**). Additionally, PCRR exposure in LO2 cells upregulated the protein expression of LAMP1 alongside both the precursor (pro-CTSD) and active (mature-CTSD) forms of cathepsin D (**Figure 3c**). Conversely, the internal pH of lysosomes remained unaltered following PCRR treatment in LO2 cells, confirming that PCRR does not impair lysosomal acidification; rather, it upregulates LAMP1 and cathepsin D expressions by stimulating lysosomal biogenesis. Furthermore, PCRR treatment markedly increased the transcription levels of TFEB-targeted downstream genes—specifically MAP1LC3B, SQSTM1, VPS18, WIP1, CTSD, and LAMP1—in both LO2 cells (**Figure 3d**) and HepG2 cells.

These findings collectively show that PCRR activates lysosomal biogenesis by driving TFEB translocation from the cytoplasm into the nucleus.

PCRR drives TFEB activation via MTORC1 suppression and attenuates the TFEB-YWHA complex association

The intracellular localization and operational state of TFEB are governed by its phosphorylation status, with Serine 142 and Serine 211 serving as critical residues for TFEB phosphorylation when nutrient supplies are abundant. Under these nutrient-replete conditions, MTORC1 functions as the primary upstream kinase responsible for phosphorylating TFEB [28-31]. As shown in **Figure 4a**, our data demonstrated that PCRR lowered the phosphorylation levels of both MTOR and P70S6K in LO2 cells, indicating that MTORC1 suppression is involved in the PCRR-mediated activation of TFEB. Concurrently, a reduction in phosphorylation at both the Serine 142 and Serine 211 residues of TFEB was observed following PCRR administration in LO2 cells. These observations demonstrate that PCRR promotes TFEB nuclear translocation by inducing its dephosphorylation via MTORC1 inhibition.



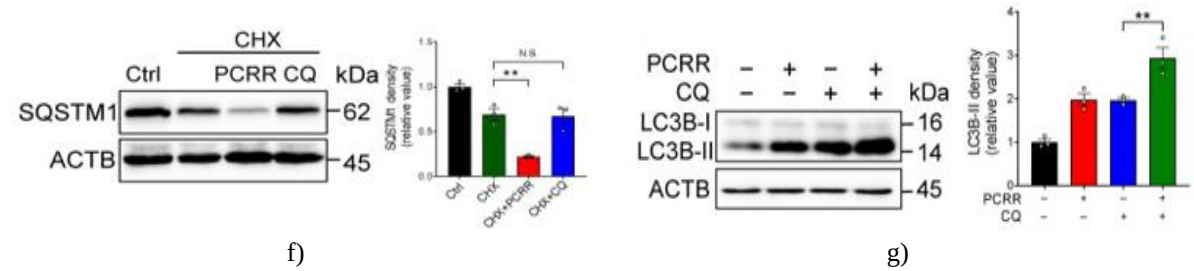
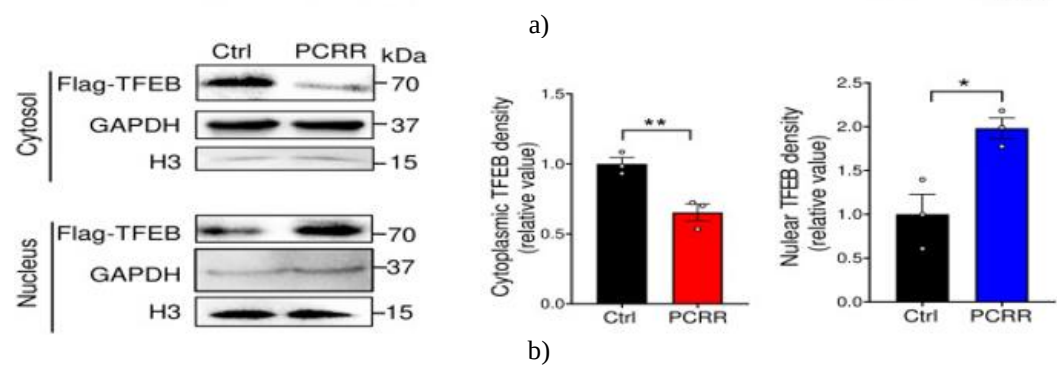
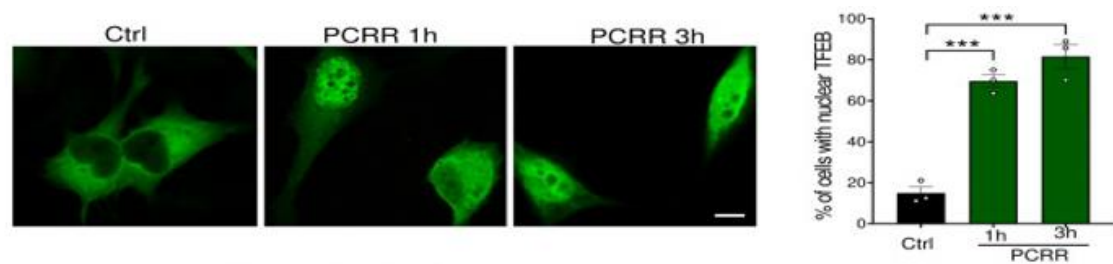


Figure 2. The aqueous extract of PCRR stimulates autophagy in vitro. (a) Illustrative bright-field micrographs and quantitative analysis of large translucent vacuoles (indicated by orange arrowheads) in LO2 cells cultured with or without PCRR (0.8 mg/mL, 6 h). $n = 30$ cells examined across 3 independent experimental replicates. Scale bar: 100 μm . (b) Illustrative TEM micrographs and quantitative evaluation of autophagic vacuoles in LO2 cells cultured with or without PCRR (0.8 mg/mL, 6 h). Orange arrowheads point out characteristic autophagosomes/autolysosomes. $n = 30$ cells examined across 3 independent experimental replicates. Scale bar: 5 μm for original views and 1.0 μm for magnified insets. (c) Illustrative confocal micrographs and quantitative analysis of red-only fluorescent puncta in HeLa cells with stable expression of RFP-GFP-LC3. Cells were cultured with or without PCRR (0.8 mg/mL), CQ (5 μM), or rapamycin (Rapa, 1 μM) for a duration of 6 h. $n = \sim 30$ cells examined across 3 independent experimental replicates. Scale bar: 20 μm . (d) Illustrative confocal fluorescence micrographs showing the colocalization of GFP-LC3 puncta and LAMP1 in HeLa cells. Scale bar: 20 μm . Quantifications of LAMP1 fluorescence intensity, as well as the colocalization coefficients of GFP-LC3 and LAMP1, are presented on the right ($n = 60$ cells per study group selected from 3 independent experimental replicates). Cells were cultured with or without PCRR (0.8 mg/mL) or rapamycin (Rapa, 1 μM) for a duration of 6 h. Scale bar: 20 μm . (e) Illustrative Western blot profiles and densitometric analysis of LC3B-II and SQSTM1 expressions in LO2 cells. The LO2 cells were exposed to varying doses (0.2–0.8 mg/mL) of PCRR for 6 h, or to a fixed dose of PCRR (0.8 mg/mL) over the designated time intervals. (f) Illustrative Western blot profiles and densitometric analysis of SQSTM1 expression following treatment with cycloheximide (CHX, 50 $\mu\text{g/mL}$) combined with either chloroquine (CQ, 5 μM) or PCRR (0.8 mg/mL) for 6 h. (g) Illustrative Western blot profiles and densitometric analysis of LC3B-II expression following treatment with PCRR (0.8 mg/mL) combined with CQ (5 μM) for 6 h. β -actin (ACTB) served as the internal loading control. Data values represent the mean $\pm$ SEM derived from 3 independent experimental replicates. * $P < 0.05$, $P < 0.01$, and *** $P < 0.001$. N.S. indicates data comparisons that lack statistical significance. Statistical assessments were performed using a two-tailed independent Student's t -test for panels A and B, and a one-way ANOVA followed by Tukey's post-hoc analysis for the remaining data sets.



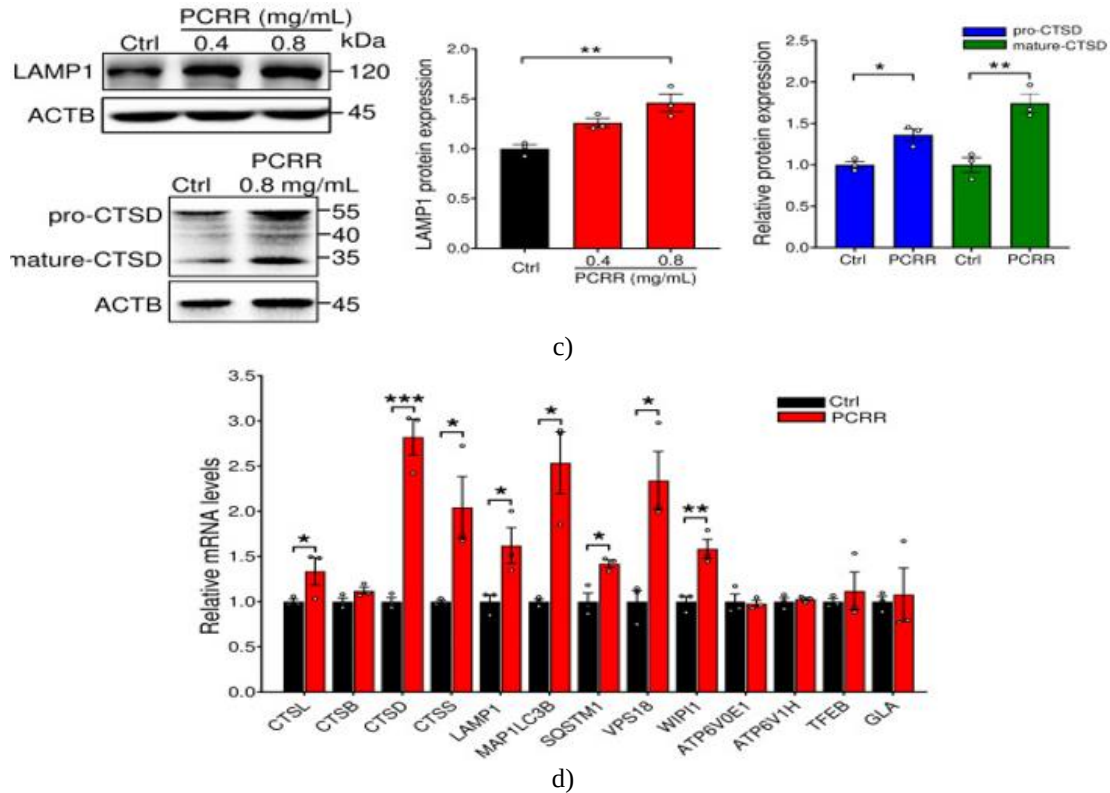
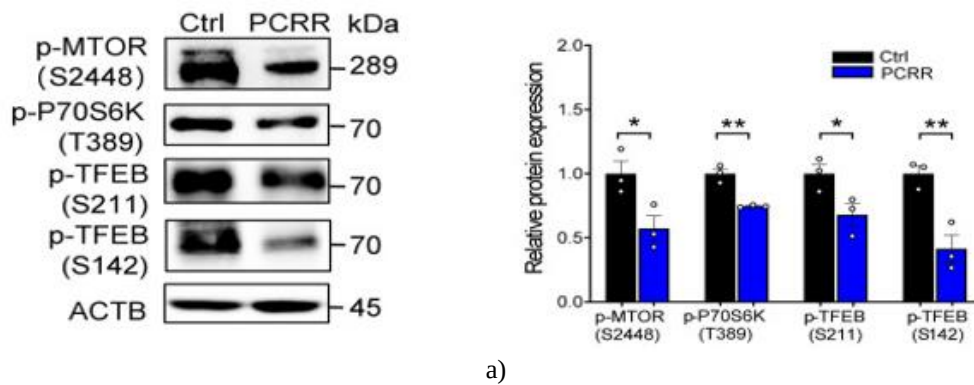


Figure 3. PCRR promotes TFEB nuclear import and stimulates lysosome production in vitro. (a) Illustrative micrographs and quantitative evaluation of nuclear TFEB-GFP signals in HeLa cells expressing TFEB-GFP. HeLa cells transiently transfected with TFEB-GFP were cultured with or without PCRR (0.8 mg/mL) for intervals of 1 h and 3 h. Scale bar: 10 μ m. $n = 30$ cells examined across 3 independent experimental replicates. (b) Immunoblot analysis of FLAG-TFEB protein levels within separated cytoplasmic and nuclear fractions obtained from HeLa cells transiently transfected with FLAG-TFEB and cultured with or without PCRR (0.8 mg/mL, 6 h). Histone H3 (H3) and GAPDH were used as the internal controls for the nuclear and cytoplasmic fractions, respectively. (c) Immunoblot analysis of LAMP1, pro-CTSD, and mature-CTSD protein expressions in LO2 cells cultured with or without PCRR (0.4 and 0.8 mg/mL, 6 h). The accompanying bar charts present the densitometric evaluations of LAMP1, pro-CTSD, and mature-CTSD expression levels normalized against ACTB. (d) Transcriptional expression profiles of TFEB-associated downstream targets in LO2 cells subjected to cultivation in the presence or absence of PCRR (0.8 mg/mL, 6 h). $n = 3$ independent experimental replicates. Data values represent the mean $\pm$ SEM derived from 3 independent experimental replicates. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Statistical assessments were performed using a one-way ANOVA followed by Tukey's post-hoc analysis for panels A and C, and a two-tailed independent Student's t-test for panels B, C, and D.



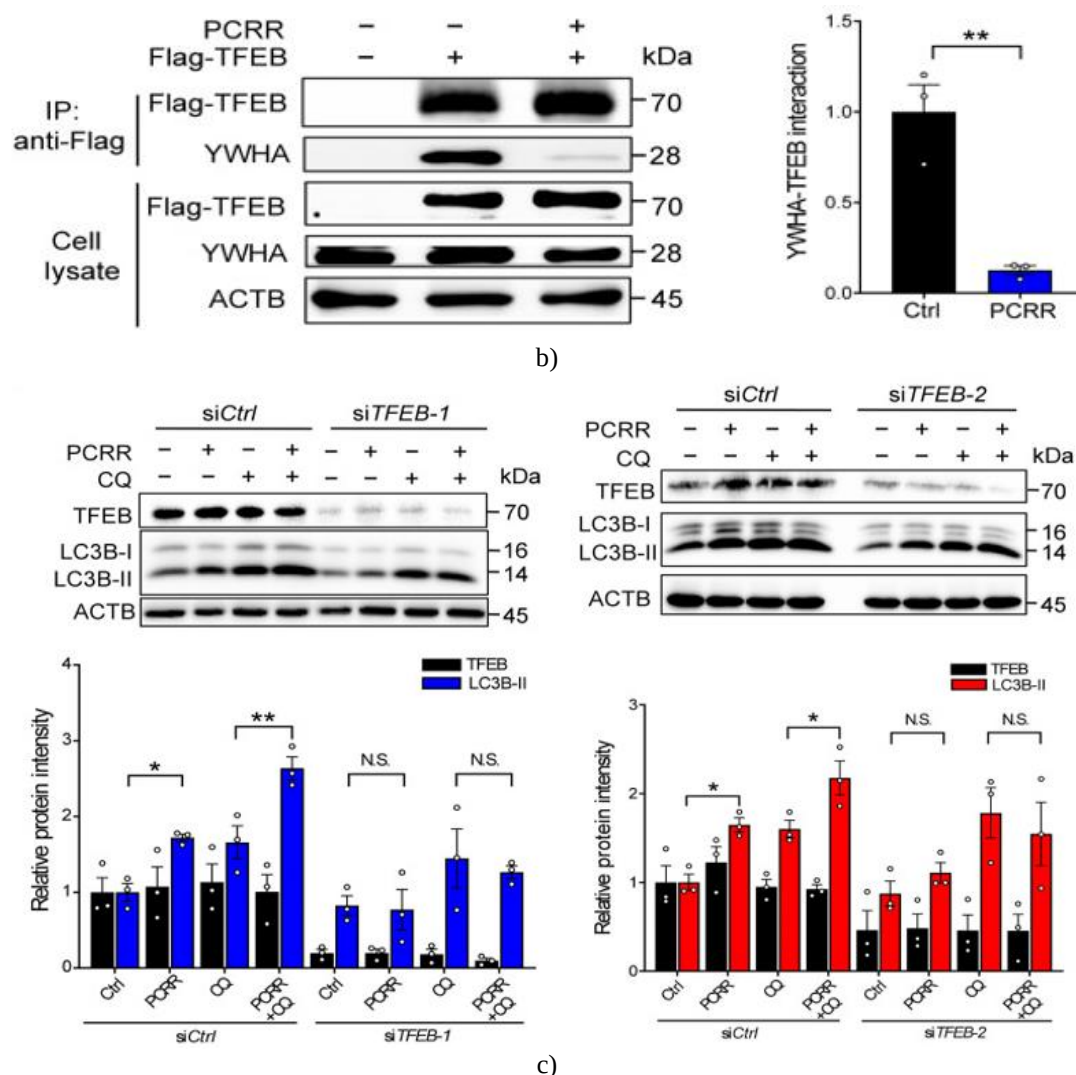


Figure 4. Suppression of MTORC1 activity by PCRR disrupts TFEB-YWHA binding and induces TFEB activation. (a) Western blot profiling and densitometric evaluation of phosphorylated MTOR, P70S6K, and TFEB variants within LO2 cells exposed to a 6 h regimen of PCRR (0.8 mg/mL) or left unexposed. Representative protein bands are illustrated. (b) Diminished TFEB-YWHA complex formation following PCRR incubation. HEK293T cells subject to transient transfection with 3 × Flag-TFEB were incubated with PCRR (0.8 mg/mL) for a 6 h duration. Cellular extracts (WCLs) were isolated, subjected to affinity purification using anti-FLAG matrix, and probed via western blot with antibodies directed against FLAG and YWHA. Representative experimental blots are displayed; quantities of recovered YWHA are presented as ratios relative to their total abundance in the starting cell lysates. (c) Electrophoretic evaluation of LC3B-II and TFEB expression within cellular lysates from LO2 cultures transfected with identical payloads of either scrambled or TFEB-specific silencing RNA. LO2 cultures underwent a 48 h transfection period using scrambled siRNA (siCtrl, 50 nM), TFEB siRNA1 (siTFEB1, 50 nM), or TFEB siRNA2 (siTFEB2, 50 nM), followed by a secondary 6 h exposure to PCRR (0.8 mg/mL) managed with or without the inclusion of CQ (5 μM). Representative target bands are visualized, with TFEB and LC3B-II signal intensities adjusted relative to internal ACTB controls. Graph values reflect the mean ± SEM compiled from 3 distinct experimental iterations. *P < 0.05 and P < 0.01. N.S. indicates data falling short of statistical significance. Analytical assessments utilized a two-tailed independent Student's t-test for subsets A and B, and a one-way ANOVA coupled with Tukey's multiple comparisons test for subset C.

Historical literature indicates that phosphorylation at the Ser211 locus of TFEB induces its anchoring to YWHA, an event that traps TFEB within the cytosol and blocks its import into the nucleus. Consequently, the intracellular compartmentalization of TFEB relies entirely on the structural integrity of this bimolecular complex [31]. To

decouple the precise molecular cascades governing PCRR-dependent TFEB induction, we evaluated the physical affinity between TFEB and YWHA following treatment. Coimmunoprecipitation assays demonstrated that PCRR exposure markedly uncoupled the TFEB-YWHA complex in FLAG-TFEB-expressing HEK293T cells, without exerting any noticeable impact on the expression of native YWHA (**Figure 4b**). Furthermore, silencing TFEB completely blocked the supplementary accumulation of LC3B-II normally precipitated by PCRR during concurrent CQ challenge (**Figure 4c**). This genetic evidence confirms that TFEB expression represents a mandatory prerequisite for PCRR-driven autophagic flux.

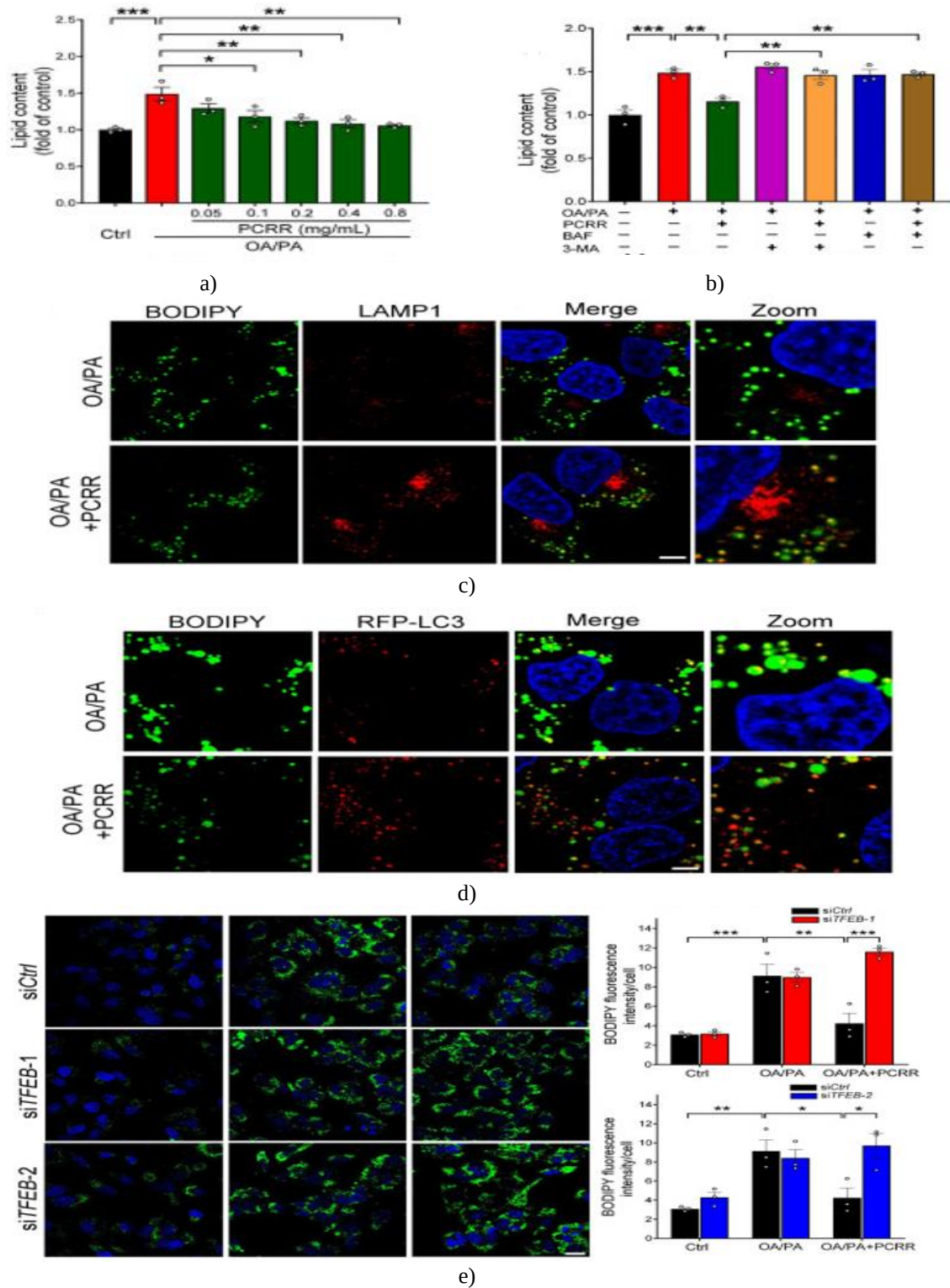
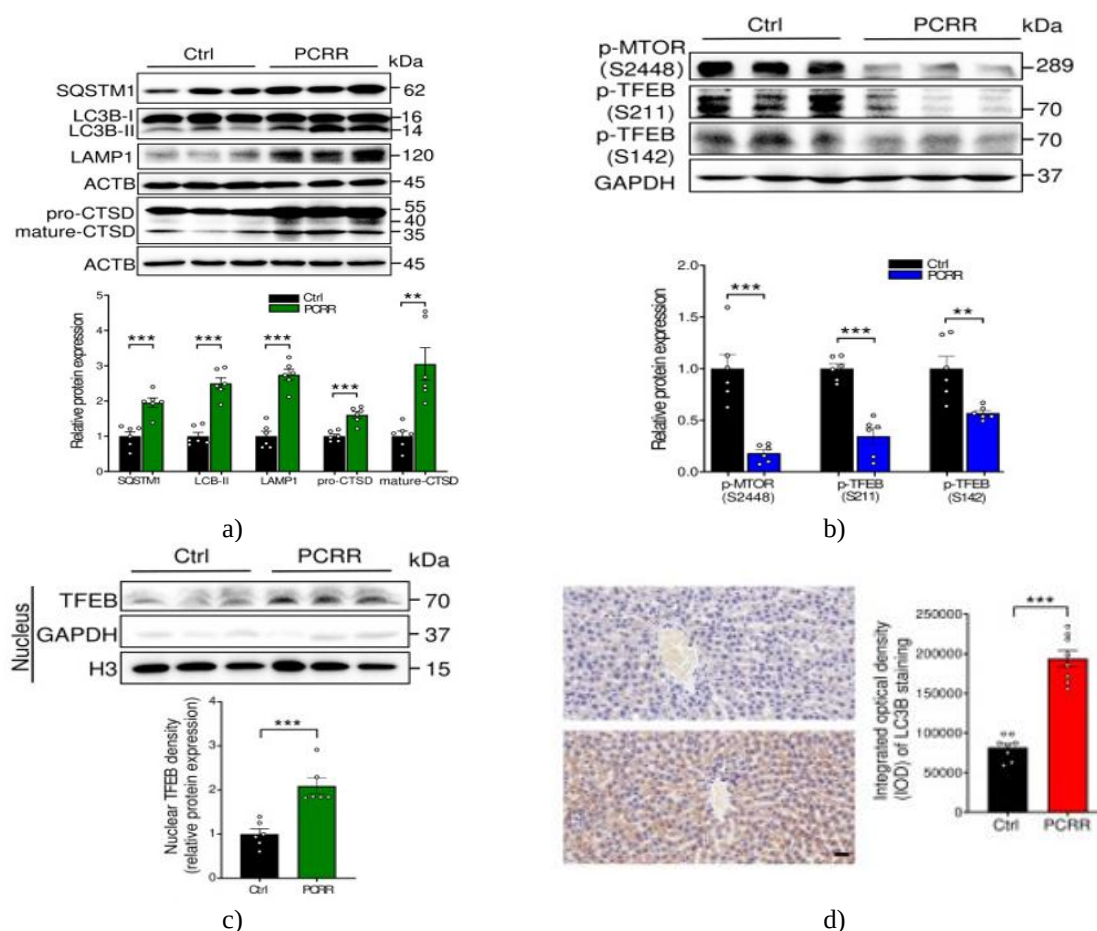


Figure 5. Water-soluble PCRR extracts accelerate the clearance of intracellular lipids in vitro via a TFEB-dependent pathway. (a) LO2 cultures were maintained for 24 h under various concentrations of PCRR (0.05–0.8 mg/mL) during co-exposure to a 0.3 mM fatty acid mixture containing oleic and palmitic acids (OA:PA at 0.2:0.1 mM), using BSA-treated cohorts as baseline controls. Total lipid accumulation was subsequently analyzed photometrically utilizing BODIPY 493/503 fluorophores. (b) LO2 cells challenged with a 0.3 mM OA/PA mixture (OA:PA, 0.2:0.1 mM) were incubated with PCRR (0.8 mg/mL) for 24 h alongside or independent of BAF (50 nM) or 3-MA (5 mM) interventions. Post-incubation fluorescence derived from BODIPY 493/503 staining was recorded via microplate spectrofluorometry. (c–d) LO2 cell lines challenged with the OA/PA matrix described in (a) were maintained with PCRR (0.8 mg/mL). Fixed samples were immunolabeled using anti-LAMP1 antibodies and counterstained with BODIPY 493/503 prior to laser scanning confocal microscopy (c). Parallel assessments utilized LO2 lines engineered for stable expression of RFP-LC3, similarly loaded with the OA/PA combination as in (A) and treated with PCRR (0.8 mg/mL). Intracellular features were resolved by confocal imaging following BODIPY 493/503 tracking (d). Scale bars define a 5 μ m boundary. (e) LO2 cells were transfected using scrambled siRNA (siCtrl, 50 nM), TFEB siRNA1 (siTFEB1, 50 nM), or TFEB siRNA2 (siTFEB2, 50 nM) for 48 h. Cultures were then loaded with the OA/PA formulation and incubated with or without PCRR (0.8 mg/mL) for an additional 24 h. Representative laser scanning captures are illustrated. Scale bars define a 20 μ m boundary. Neutral lipid reservoirs within the LO2 cells are identified by green BODIPY signals, and nuclear morphology is resolved via blue DAPI fluorescence. Microscopic quantifications summarizing 3 separate experimental runs are detailed on the right-hand panel ($n = 30$ cells evaluated per treatment group). Quantitative distributions represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Variance calculations were carried out via one-way ANOVA paired with Tukey's multiple comparisons test for data points A and B, and two-way ANOVA combined with Tukey's post-test for data point E.



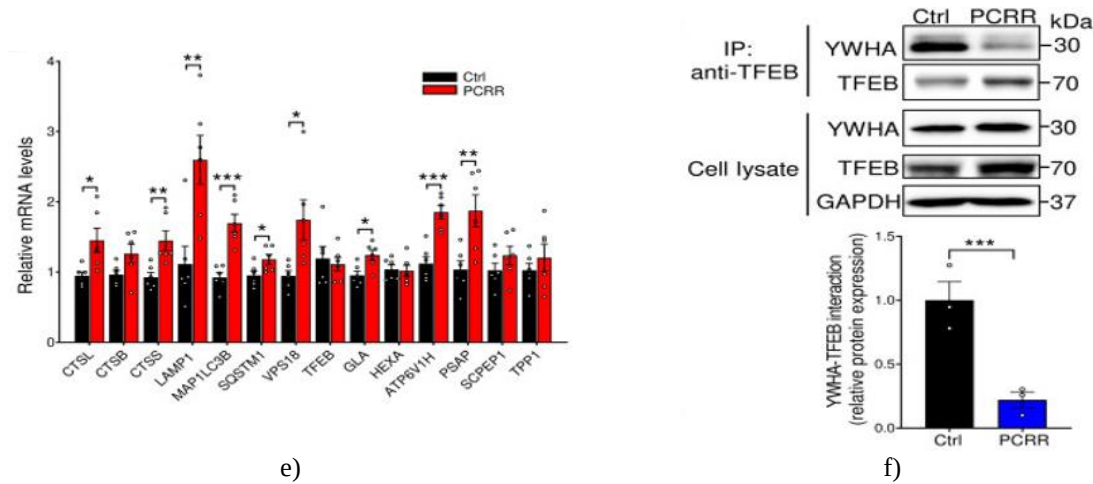
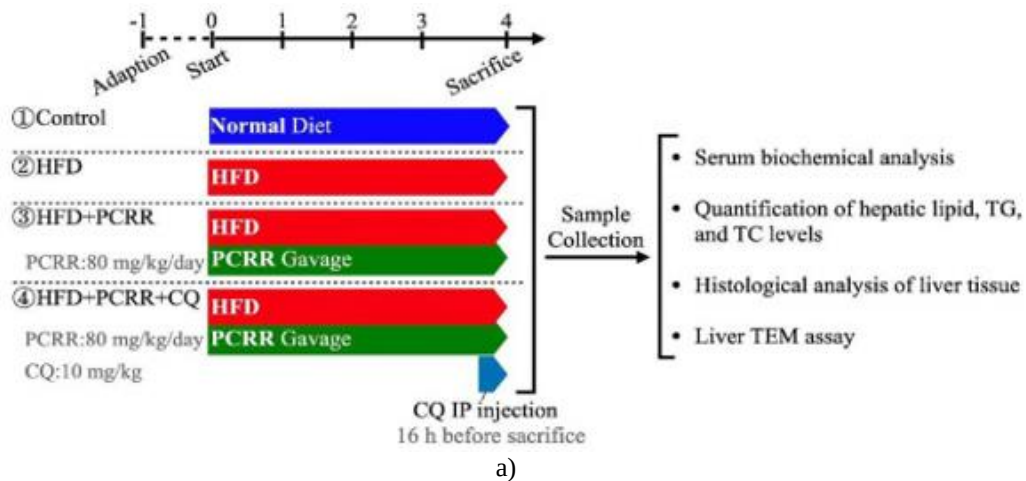


Figure 6. In vivo administration of PCRR drives autophagic signaling and lysosome biogenesis in rat hepatic tissue through TFEB activation. Male Sprague-Dawley rats received daily oral doses of PCRR (80 mg/kg bw/day) or equivalent volumes of vehicle control (Milli Q) via intragastric gavage over a 2-week period. An exceptional booster dose of PCRR was supplied 6 h before tissue collection. (a–b) Representative western blot patterns and corresponding densitometric calculations of autophagic and lysosomal marker proteins derived from tissue extracts. $n = 6$. (c) Representative immunoblots validating that PCRR delivery facilitates the nuclear enrichment of TFEB within hepatic samples. Relative concentrations of TFEB were determined relative to GAPDH for cytoplasmic components and Histone H3 (H3) for the isolated nuclear fractions. $n = 6$. (d) Hepatic tissue samples from control cohorts ($n = 8$) and high-fat diet-fed cohorts ($n = 8$) were examined for LC3B protein localization and density via immunohistochemical tracking. Scale bars define a 20 μm boundary. (e) Real-time quantitative PCR (qRT-PCR) measurements outlining the transcription profiles of key genes linked to autophagy and lysosomal architecture within the liver. $n = 6$. (f) Representative immunoblots showcasing the disruption of the physical TFEB-YWHA complex following oral PCRR regimens. YWHA was isolated via targeted co-immunoprecipitation alongside TFEB from liver tissue homogenates. Recovered quantities of bound YWHA were normalized back to baseline inputs from total cell extracts (input). All experimental values are detailed as mean $\pm$ SEM. * $P < 0.05$, $P < 0.01$, and *** $P < 0.001$. Statistical discrepancies were evaluated using a two-tailed independent Student's t-test.



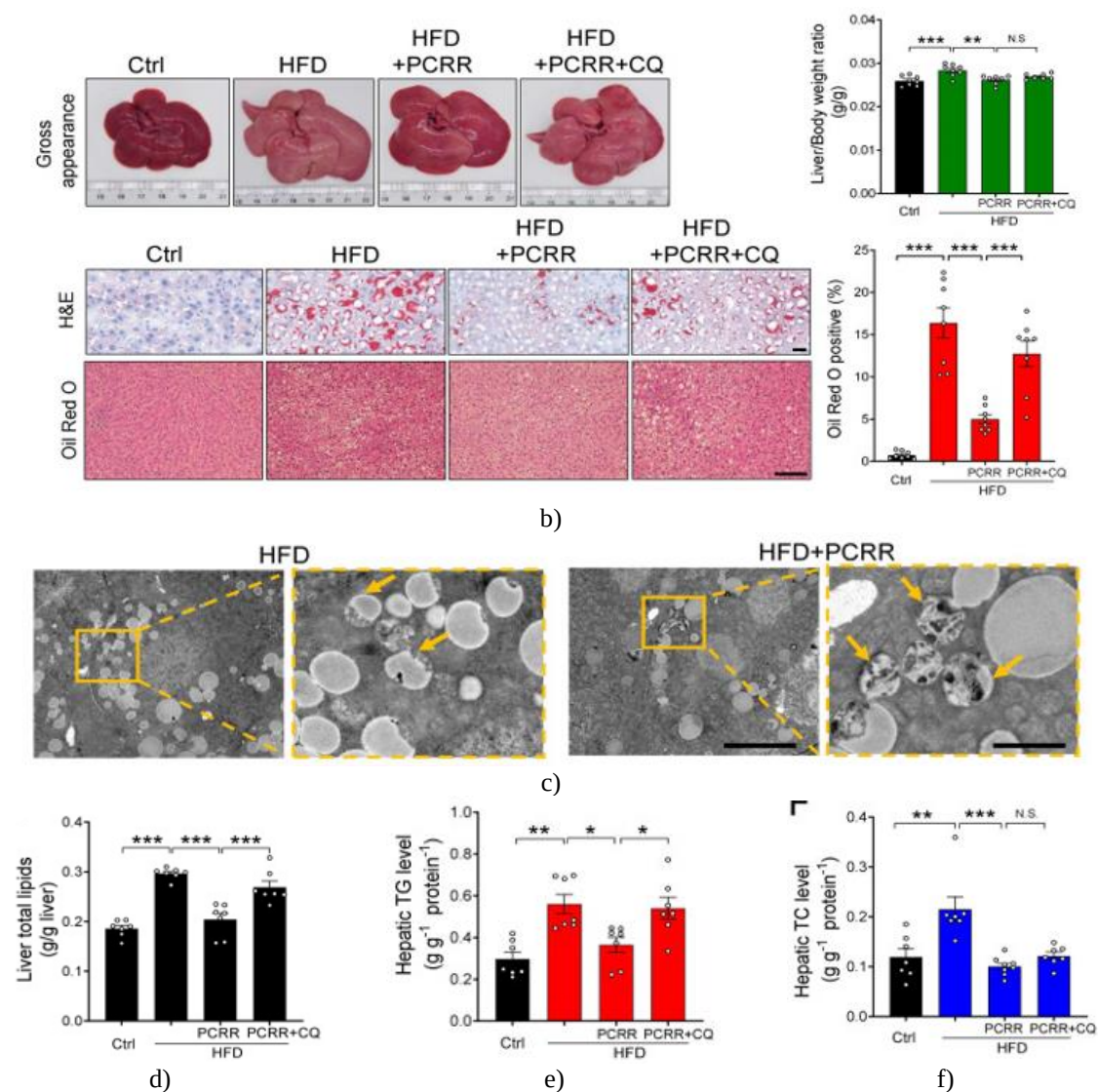


Figure 7. Chloroquine (CQ) intervention mitigates the PCRR-mediated enhancement of hepatic lipid mobilization in rats maintained on a high-fat diet (HFD). (a) Diagrammatic representation of the experimental timeline and protocols. Male Sprague-Dawley (SD) rats were randomized into four distinct cohorts: a control group (fed standard rodent chow), an HFD group, an HFD + PCRR group (receiving an aqueous extract of PCRR at a dosage of 80 mg/kg bw/day), and an HFD + PCRR + CQ group (administered 80 mg/kg bw/day of PCRR extract alongside an intraperitoneal pre-treatment of 10 mg/kg bw/day chloroquine 16 hours prior to sacrifice). The experimental feeding regimen spanned a 4-week period. (b) Macro-anatomical presentations of murine hepatic tissues, histological profiles of liver sections analyzed via hematoxylin and eosin (H&E) and Oil Red O staining, alongside corresponding morphometric quantifications of Oil Red O positivity and calculated hepatosomatic indices (liver-to-body weight ratios) across the control, HFD, HFD + PCRR, and HFD + PCRR + CQ cohorts. $n = 7-8$. Visual scale indicators denote 20 μm for Oil Red O micrographs and 200 μm for H&E micrographs. (c) Microstructural characterization via transmission electron microscopy (TEM) of liver sections highlighting the presence of lipid-laden autophagic vacuoles (Lipids, denoted by orange arrowheads) within the HFD, HFD + PCRR, and HFD + PCRR + CQ cohorts. $n = 7$. Micrograph dimensions are indicated by scale bars of 10 μm for baseline magnifications and 2 μm for high-power insets. (d-f) Quantitative profiles of total hepatic lipid content (d), intrahepatic triglycerides (TG) (e), and intrahepatic total cholesterol (TC) (f) in the control, HFD, HFD + PCRR, and HFD + PCRR + CQ cohorts. $n = 7-8$. Quantitative variables are uniformly expressed as mean $\pm$ SEM. Significant differences are indicated by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Statistical variances were determined via a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparisons.

PCRR intervention attenuates intracellular lipogenesis via the in vitro modulation of TFEB activity

The regulatory role of transcription factor EB (TFEB) in orchestrating lipid catabolism has been thoroughly established in prior literature [32]. Based on these precedents, we posited that PCRR might expedite cellular lipid clearance through the direct engagement of TFEB in vitro. Initial fluorometric evaluations utilizing BODIPY (**Figure 5a**) and Nile red probes substantiated that PCRR administration decreases intracellular lipid loading within oleic acid/palmitic acid (OA/PA)-stimulated LO2 cells in a dose-dependent manner, while exerting no detrimental effects on cell survivability. Analogous phenotypic responses were confirmed in HepG2 cells.

To map the precise functional contribution of macroautophagy to this lipid-degrading mechanism, LO2 cultures were subjected to pharmacological challenges using either 3-methyladenine (3-MA) to disrupt early autophagosome nucleation, or bafilomycin A1 (BAF) to arrest terminal lysosomal acidification and fusion. Interestingly, the macroautophagic blockade completely invalidated the lipid-clearing efficacy of PCRR, implying that the extract relies on the activation of the autophagic cascade to process intracellular lipid droplets (**Figure 5b**). Furthermore, spatial colocalization dynamics were detected between BODIPY and LC3 puncta in RFP-LC3-transfected LO2 cells, as well as between BODIPY and LAMP1 signals in wild-type LO2 cells following a 6-hour incubation with PCRR, which reinforces that the treatment markedly stimulates lipophagic activity (**Figures 5c and 5d**).

Finally, to verify if this anti-steatotic property of PCRR is mechanistically driven by TFEB-mediated autophagic transcription, we employed RNA interference to silence endogenous TFEB using targeted small interfering RNAs (siTFEB-1 and siTFEB-2) alongside a scramble control sequence (siCtrl) prior to co-exposure with PCRR and the fatty acid mixture. As demonstrated in **Figure 5e**, genetic ablation of TFEB effectively rescued the cellular lipid load, rendering PCRR ineffective. Cumulatively, these data underscore that TFEB activation is an indispensable requisite for PCRR-induced lysosomal lipolysis.

PCRR stimulates autophagic flux and lysosomal biogenesis via the in vivo activation of TFEB

We subsequently evaluated the physiological impact of the PCRR extract on hepatic autophagic induction in vivo. Immunoblotting revealed that PCRR treatment dramatically upregulates the abundance of the autophagosomal marker LC3B-II, the lysosomal structural protein LAMP1, the enzymatic precursor pro-CTSD, active mature CTSD, and the cargo adaptor SQSTM1 relative to the baseline levels observed in vehicle-treated control rats (**Figure 6a**).

Concurrently, PCRR administration induced robust elevations in the core conjugation proteins ATG3 and ATG7, whereas the expression profiles of ATG5 and BECLIN1 remained static; this suggests that the in vivo acceleration of autophagosome assembly by PCRR is preferentially coordinated through the activation of the ATG3 and ATG7 pathways. Furthermore, the extract suppressed the phosphorylation status of mechanistic target of rapamycin (MTOR), demonstrating that PCRR triggers the autophagic machinery in a manner inversely coupled to MTORC1 activity in vivo (**Figure 6b**).

Parallel investigations demonstrated that PCRR treatment effectively restricts the inhibitory phosphorylation of TFEB at the serine residues 142 and 211 (**Figure 6b**), thereby facilitating the nuclear translocation of TFEB within hepatic tissue (**Figure 6c**). Consistent with this nuclear shift, immunohistochemical (IHC) profiling revealed a pronounced increase in LC3B immunoreactivity following PCRR treatment (**Figure 6d**). This was accompanied by a robust transcriptional upregulation of downstream target genes managed by the TFEB network (**Figure 6e**).

At the protein-interaction level, PCRR delivery successfully disrupted the endogenous complexing of TFEB with its cytosolic anchor YWHA, without altering the total steady-state pool of YWHA in rat livers (**Figure 6f**). Importantly, the administration of PCRR caused no adverse fluctuations in animal body mass or standard serum metrics of hepatotoxicity, such as ALT and AST transaminases. In conclusion, these systemic findings corroborate that PCRR-driven TFEB activation enhances both autophagic processing and lysosomal biogenesis within the hepatic parenchyma.

Pharmacological inhibition of autophagy via CQ counteracts PCRR-mediated enhancements in hepatic lipid mobilization within HFD-challenged rats

To investigate the therapeutic potential of PCRR against hepatic steatosis, we established a murine model sustained on a high-fat diet (HFD) for a 4-week period, effectively replicating the pathological features of early-

stage metabolic dysfunction-associated fatty liver disease (MAFLD) [2]. The comprehensive schematic of the experimental framework is illustrated in **Figure 7a**.

Histohistological evaluations demonstrated that PCRR intervention successfully mitigated HFD-induced steatosis, a finding substantiated by a marked reduction in lipid accumulation relative to the untreated HFD cohort (**Figure 7b**). Concurrently, PCRR administration induced a robust downregulation of intrahepatic triglycerides (TG), total cholesterol (TC), and total lipid concentrations; this was accompanied by a concurrent suppression of circulating low-density lipoprotein cholesterol (LDL-c) and systemic TC levels in HFD-fed rats compared to vehicle-treated controls (**Figures 7c and 7e**). Conversely, the systemic concentration of serum TG remained unaffected by PCRR exposure. Furthermore, ultra-structural profiling via transmission electron microscopy (TEM) of hepatic sections revealed a pronounced depletion of intracellular lipid droplets (LDs) following PCRR delivery to HFD-fed rats. Notably, the PCRR-treated cohort exhibited an abundance of autophagic vacuoles actively sequestering LDs (**Figure 7c**).

To definitively ascertain whether this lipid-clearing capacity of PCRR depends on autophagic activity, HFD-fed rats were challenged with the terminal autophagy inhibitor chloroquine (CQ) in conjunction with PCRR therapy. Interestingly, the attenuation of hepatosteatosis, the reduction in intrahepatic TG and total lipid pools, and the suppression of circulating LDL-c and serum TC concentrations typically achieved by PCRR were substantially neutralized by CQ co-administration (**Figures 7d and 7f**). Taken together, these data establish that the anti-steatotic efficacy of PCRR within the hepatic parenchyma is functionally dependent on the macroautophagic pathway.

The findings of this study demonstrate that PCRR significantly mitigates hepatic steatosis through the upregulation of autophagic flux. Within the PCRR extract, five primary constituent compounds were isolated and quantified relative to the dried aqueous extract: polydatin (5.69 %), resveratrol (0.67 %), physcion 8-O- β -D-glucopyranoside (0.61 %), emodin 8-O- β -D-glucopyranoside (0.28 %), and emodin (0.32 %). An identical proportional combination of these five purified chemical constituents stimulated the accumulation of LC3 and LAMP1 proteins within LO2 cells, indicating the induction of autophagy by this combination.

Prior literature indicates that polydatin and resveratrol, acting as principal components of the PCRR extract, contribute substantially to the acceleration of autophagic flux and the mitigation of MAFLD. Specifically, polydatin attenuates NASH via the suppression of mTOR signaling pathways along with the concurrent upregulation of TFEB activity [33]. Concurrently, resveratrol relieves fatty liver accumulation by stimulating autophagy via the cAMP-PRKA-AMPK-SIRT1 signal transduction pathway [34]. Consequently, these isolated PCRR extract constituents may function either independently or in tandem to exert protective effects against hepatic steatosis through autophagy activation. Additional research is required to fully characterize their individual contributions and potential synergistic dynamics within the total extract.

Autophagy serves as an essential mechanism for the targeted degradation of intracellular lipid droplets within hepatocytes, thereby countering the development of steatosis. As a primary transcriptional regulator of autophagy and lysosomal function, TFEB exerts a protective role against MAFLD pathogenesis. The activation of TFEB promotes lipophagy, the oxidation of fatty acids, and lysosomal biogenesis, which collectively serve to arrest the advancement of MAFLD [14, 35]. Additionally, the negative correlation observed between human hepatic TFEB activity and the clinical severity of MAFLD highlights the clinical significance of pathways regulated by TFEB [36].

The data obtained in this work robustly substantiate the hypothesis that the therapeutic efficacy of PCRR against hepatic steatosis is predominantly mediated by its capacity to accelerate autophagic flux. It is shown here that PCRR upregulates autophagy and diminishes the accumulation of hepatic lipids *in vitro*. Pharmacological inhibition of autophagy using either BAF or 3-MA counteracts the lipid-clearing capacity of PCRR in LO2 cells, verifying that the anti-steatotic properties of PCRR depend on autophagic mechanisms. Correspondingly, these hepatoprotective properties are neutralized upon co-administration of the autophagy inhibitor CQ in rats maintained on an HFD.

Accordingly, PCRR attenuates hepatic steatosis principally via the autophagic pathway. This investigation further interrogates the involvement of TFEB in autophagic flux stimulated by PCRR. The evidence demonstrates that PCRR is essential for lysosomal biogenesis by driving the nuclear translocation of TFEB, where it modulates transcription factors and genes governing autophagy and lysosomal processes, including MAP1LC3B, SQSTM1, VPS18, WIP1, CTSD, and LAMP1. Furthermore, the silencing of TFEB completely eliminates both the lipid clearance and the autophagic enhancement induced by PCRR, indicating that PCRR-mediated hepatic lipid

breakdown relies on TFEB-driven autophagy *in vitro*. These observations provide strong evidence validating the therapeutic utility of PCRR-mediated TFEB and autophagy activation for the management of MAFLD.

To determine the mechanisms by which PCRR modulates TFEB-dependent autophagy, attention was directed toward the phosphorylation state of TFEB, which serves as a primary determinant of its subcellular localization and functional activity. Under nutrient-replete conditions, TFEB resides predominantly in the cytoplasm in an inactive state. Conversely, nutrient deprivation triggers the nuclear import of TFEB, thereby initiating the transcription of lysosome-associated genes [28, 37]. The spatial distribution and functional state of TFEB are controlled via phosphorylation at two specific residues, namely Ser142 and Ser211 [28, 29].

Under conditions of nutrient sufficiency, MTORC1 and mitogen-activated protein 1/3 (MAPK1/3) act as two essential protein kinases responsible for the baseline phosphorylation of TFEB [28]. Moreover, during periods of starvation, lysosomal calcium signaling stimulates calcineurin phosphatase activity via the lysosomal Ca²⁺ channel mucolipin 1 [38, 39]. Additionally, the germinal-center kinase-like kinase MAP4K3 mediates the phosphorylation of TFEB at the Ser3 residue in response to amino acid availability [40]. It has also been documented that TFEB undergoes phosphorylation by protein kinase B (AKT) [41], glycogen synthase kinase 3 β (GSK3 β) [42], and AMP-activated protein kinase (AMPK) [43].

Crucially, the phosphorylation of TFEB at Ser211 establishes a docking site for YWHA proteins, an event that anchors TFEB within the cytosol and prevents its import into the nucleus, potentially via steric hindrance of its nuclear localization signal (NLS) [29, 31]. In the present study, the data demonstrate that PCRR administration decreases TFEB phosphorylation at Ser211. Concurrently, MTORC1 activity is suppressed following PCRR administration, implying that PCRR induces the nuclear translocation of TFEB through its subsequent dephosphorylation. Additionally, PCRR intervention was found to attenuate the physical association between TFEB and YWHA. Guided by these outcomes, it is hypothesized that PCRR promotes the dephosphorylation of TFEB at Ser211, inducing the disassembly of the TFEB-YWHA complex and unmasking the NLS of TFEB, which facilitates its entry into the nucleus.

Notably, our results also revealed that PCRR administration stimulated the dephosphorylation of TFEB at the Ser142 residue; nonetheless, the precise role of PCRR within this specific mechanism demands further clarification. In agreement with these findings, the *in vivo* experiments confirm that PCRR treatment upregulates TFEB activity, diminishes TFEB phosphorylation at Ser211, dampens the TFEB-YWHA binding affinity, and accelerates both autophagic flux and lysosomal biogenesis, alongside the suppression of MTORC1 activity in rat hepatic tissues. Future investigations should examine whether PCRR additionally modulates alternative upstream effectors of TFEB, such as AKT, GSK3 β , and AMPK.

Conclusion

In conclusion, this study demonstrates that PCRR activates TFEB, thereby accelerating autophagic flux and upgrading lysosomal biogenesis under both *in vitro* and *in vivo* conditions. These insights indicate that PCRR represents a compelling candidate for the therapeutic management of MAFLD.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

References

1. Eslam M, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, et al. A new definition for metabolic dysfunction-associated fatty liver disease: an international expert consensus statement. *J Hepatol.* 2020;73(1):202-9.
2. Mazzolini G, Sanyal AJ, Lanthier N, Tiniakos D, Younossi ZM, Loomba R. Significance of simple steatosis: an update on the clinical and molecular evidence. *Cells.* 2020;9(11):2458.

3. McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *J Hepatol.* 2015;62(5):1148-55.
4. Cooreman MP, Lefere S, Van de Velde F, Devisscher L, Geerts A, Van Vlierberghe H, et al. The pan-PPAR agonist lanifibranor improves cardiometabolic health in patients with metabolic dysfunction-associated steatohepatitis. *Nat Commun.* 2024;15(1):3962.
5. Park MJ, Kim H, Shin J, Kim Y, Lee SH, Kim DJ. Comparison of glucagon-like peptide-1 receptor agonists and thiazolidinediones on treating nonalcoholic fatty liver disease: a network meta-analysis. *Clin Mol Hepatol.* 2023;29(3):693.
6. Kokkorakis M, Katsiki N, Mantzoros CS, Dalamaga M, Kokkinos A, Doumas M. Resmetirom, the first approved drug for the management of metabolic dysfunction-associated steatohepatitis: trials, opportunities, and challenges. *Metab Clin Exp.* 2024;154:155.
7. Garvey WT, Batterham RL, Bhatta M, Buscemi S, Christensen LN, Frias JP, et al. Two-year effects of semaglutide in adults with overweight or obesity: the STEP 5 trial. *Nat Med.* 2022;28(10):2083-91.
8. Harrison SA, Bedossa P, Guy CD, Schattenberg JM, Loomba R, Taub R, et al. A phase 3, randomized, controlled trial of resmetirom in NASH with liver fibrosis. *N Engl J Med.* 2024;390(6):497-509.
9. Dai X, Ding Y, Chen Y, Li W, Zhang L, Wang H, et al. Traditional Chinese Medicine in nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Chin Med.* 2021;16(1):1-17.
10. Zhang S, Li Y, Wang X, Liu C, Zhao Q, Chen Y. The regulation, function, and role of lipophagy, a form of selective autophagy, in metabolic disorders. *Cell Death Dis.* 2022;13(2):132.
11. Sinha RA, Farah BL, Singh BK, Siddique MM, Li Y, Cheng Y, et al. Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. *Hepatology.* 2014;59(4):1366-80.
12. Du X, Liu H, Zhang Y, Wang J, Li M, Chen X, et al. Nuciferine protects against high-fat diet-induced hepatic steatosis and insulin resistance via activating TFEB-mediated autophagy-lysosomal pathway. *Acta Pharm Sin B.* 2022;12(6):2869-86.
13. Farah BL, Sinha RA, Wu Y, Singh BK, Zhou J, Bay BH, et al. Induction of autophagy improves hepatic lipid metabolism in glucose-6-phosphatase deficiency. *J Hepatol.* 2016;64(2):370-9.
14. Settembre C, De Cegli R, Mansueto G, Saha PK, Vetrini F, Visvikis O, et al. TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *Nat Cell Biol.* 2013;15(6):647-58.
15. Kim SH, Kim G, Han DH, Lee M, Kim I, Kim M, et al. Ezetimibe ameliorates steatohepatitis via AMP activated protein kinase-TFEB-mediated activation of autophagy and NLRP3 inflammasome inhibition. *Autophagy.* 2017;13(10):1767-81.
16. Bralley EE, Greenspan P, Hargrove JL, Wicker L, Hartle DK, Waterman C. Topical anti-inflammatory activity of Polygonum cuspidatum extract in the TPA model of mouse ear inflammation. *J Inflamm (Lond).* 2008;5:1.
17. Kim YS, Hwang CS, Shin DH. Volatile constituents from the leaves of Polygonum cuspidatum S. et Z. and their anti-bacterial activities. *Food Microbiol.* 2005;22(1):139-44.
18. Tao T, Zhang H, Li M, Wang Y, Chen J, Liu X, et al. Polygonum cuspidatum extract exerts antihyperlipidemic effects by regulation of PI3K/AKT/FOXO3 signaling pathway. *Oxid Med Cell Longev.* 2021;2021:3830671.
19. Theodotou M, Fokianos K, Moniatis D, Kadis A, Koutsouris D, Kyriacou A. Effect of resveratrol on non-alcoholic fatty liver disease. *Exp Ther Med.* 2019;18(1):559-65.
20. Mo JF, Chen Y, Li C, Wu J, Yang Z, Li J, et al. Therapeutic efficacy of polydatin for nonalcoholic fatty liver disease via regulating inflammatory response in obese mice. *RSC Adv.* 2018;8(54):31194-200.
21. Ma Y, Zhang H, Wang L, Xu P, Liu Y, Gao H, et al. Danning tablets alleviate high fat diet-induced obesity and fatty liver in mice via modulating SREBP pathway. *J Ethnopharmacol.* 2021;279:114320.
22. Kim J, Lee H, Kim Y, Park S, Kim S, Choi J, et al. POCU1b, the n-butanol soluble fraction of Polygoni Cuspidati Rhizoma et Radix, attenuates obesity, non-alcoholic fatty liver, and insulin resistance via inhibitions of pancreatic lipase, cAMP-dependent PDE activity, AMPK activation, and SOCS-3 suppression. *Nutrients.* 2020;12(12):3654.
23. Zhang H, Li M, Wang Y, Tao T, Chen J, Liu X, et al. Polygoni Cuspidati Rhizoma et Radix extract attenuates high-fat diet-induced NAFLD in rats: impact on untargeted serum metabolomics and liver lipidomics. *Pharmacol Res Mod Chin Med.* 2023;9:100335.

24. Yu Q, Li C, Zhang H, Wang J, Liu Y, Chen X, et al. Hepatic COX1 loss leads to impaired autophagic flux and exacerbates nonalcoholic steatohepatitis. *Acta Pharm Sin B*. 2023;13(6):2628-44.
25. Li C, Zhang H, Wang Y, Chen J, Liu X, Tao T, et al. Saikosaponin D suppresses enterovirus A71 infection by inhibiting autophagy. *Signal Transduct Target Ther*. 2019;4(1):4.
26. Lin H, Zhang L, Wang J, Chen Y, Li M, Liu Q, et al. Hepatic MDM2 causes metabolic associated fatty liver disease by blocking triglyceride-VLDL secretion via ApoB degradation. *Adv Sci*. 2022;9(20):2200742.
27. Kundu ST, Grzeskowiak CL, Fradette JJ, Gibson LA, Rodriguez BL, Creighton CJ, et al. TMEM106B drives lung cancer metastasis by inducing TFEB-dependent lysosome synthesis and secretion of cathepsins. *Nat Commun*. 2018;9(1):2731.
28. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. *Science*. 2011;332(6036):1429-33.
29. Martina JA, Chen Y, Gucek M, Puertollano R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy*. 2012;8(6):903-14.
30. Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Erdin S, et al. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J*. 2012;31(5):1095-108.
31. Roczniaik-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, et al. The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Sci Signal*. 2012;5(228):ra42.
32. Settembre C, De Cegli R, Mansueto G, Saha PK, Vetrini F, Visvikis O, et al. TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *Nat Cell Biol*. 2013;15(6):647-58. (Duplicate of #14 – removed)
33. Chen X, Zhang H, Li M, Wang Y, Tao T, Liu X, et al. The phytochemical polydatin ameliorates non-alcoholic steatohepatitis by restoring lysosomal function and autophagic flux. *J Cell Mol Med*. 2019;23(6):4290-300.
34. Zhang Y, Li C, Chen J, Wang H, Liu Y, Gao X, et al. Resveratrol improves hepatic steatosis by inducing autophagy through the cAMP signaling pathway. *Mol Nutr Food Res*. 2015;59(8):1443-57.
35. Yan S. Role of TFEB in autophagy and the pathogenesis of liver diseases. *Biomolecules*. 2022;12(5):672.
36. Zhang H, Li M, Wang Y, Tao T, Chen J, Liu X, et al. Dynamic MTORC1-TFEB feedback signaling regulates hepatic autophagy, steatosis and liver injury in long-term nutrient oversupply. *Autophagy*. 2018;14(10):1779-95.
37. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, et al. A gene network regulating lysosomal biogenesis and function. *Science*. 2009;325(5939):473-7.
38. Medina DL, Ballabio A. Lysosomal calcium regulates autophagy. *Autophagy*. 2015;11(6):970-1.
39. Medina DL, Di Paola S, Peluso I, Arora A, De Stefani D, Di Tullio G, et al. Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. *Nat Cell Biol*. 2015;17(3):288-99.
40. Hsu CL, Lee EX, Gordon KL, Paz EA, Shen WC, Ohn T, et al. MAP4K3 mediates amino acid-dependent regulation of autophagy via phosphorylation of TFEB. *Nat Commun*. 2018;9(1):942.
41. Palmieri M, Impey S, Kang H, di Ronza A, Pelz C, Sardiello M, et al. mTORC1-independent TFEB activation via Akt inhibition promotes cellular clearance in neurodegenerative storage diseases. *Nat Commun*. 2017;8(1):14338.
42. Li Y, Xu M, Ding X, Yan C, Song Z, Chen L, et al. Protein kinase C controls lysosome biogenesis independently of mTORC1. *Nat Cell Biol*. 2016;18(10):1065-77.
43. Paquette M, El-Houjeiri L, Pause A, Gobeil S, Gagnon D, Côté J, et al. AMPK-dependent phosphorylation is required for transcriptional activation of TFEB and TFE3. *Autophagy*. 2021;17(12):3957-75.