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Proteostasis disturbances and endoplasmic reticulum stress contribute to polycystic liver disease: new therapeutic targets

Alvaro Santos-Laso[1], **Laura Izquierdo-Sanchez**[1,2], **Pedro M. Rodrigues**[1], **Bing Q. Huang**[3], **Mikel Azkargorta**[2,4], **Ainhoa Lapitz**[1], **Patricia Munoz-Garrido**[1], **Ander Arbelaiz**[1], **Francisco J. Caballero**[1,5], **Maite G. Fernandez-Barrena**[2,6], **Raul Jimenez-Agüero**[1], **Josepmaria Argemi**[6], **Tomas Aragon**[6], **Felix Elortza**[2,4], **Marco Marzioni**[7], **Joost P.H. Drenth**[8], **Nicholas F. LaRusso**[3], **Luis Bujanda**[1,2], **Maria J. Perugorria**[1,2,9,§], **Jesus M. Banales**[1,2,9,§]

[1]Department of Liver and Gastrointestinal Diseases, Biodonostia Research Institute – Donostia University Hospital –, University of the Basque Country (UPV/EHU), San Sebastian, Spain; [2]National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd, Instituto de Salud Carlos III), Madrid, Spain; [3]Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA; [4]Proteomics Platform, CIC bioGUNE, ProteoRed-ISCIII, Bizkaia Science and Technology Park, Derio, Spain; [5]Department of Organic Chemistry I, Center for Innovation in Advanced Chemistry (ORFEO-CINQA), University of the Basque Country (UPV/EHU), San Sebastian, Spain; [6]Division of Hepatology, CIMA-University of Navarra, Pamplona, Spain; [7]Department of Gastroenterology and Hepatology, Università Politecnica delle Marche, Ancona, Italy; [8]Department of Gastroenterology and Hepatology, Radboud University Medical Center, Nijmegen, The Netherlands; [9]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain.

Abstract

Background & Aims: Polycystic liver diseases (PLDs) are genetic disorders characterized by progressive development of multiple biliary cysts. Recently, novel PLD-causative genes, encoding for endoplasmic reticulum (ER)-resident proteins involved in protein biogenesis and transport, were identified. We hypothesized that aberrant proteostasis contributes to PLD pathogenesis, representing a potential therapeutic target.

Methods: ER stress was analyzed at transcriptional (qPCR), proteomic (mass spectrometry), morphological (transmission electron microscopy, TEM) and functional (proteasome activity) levels in different PLD models. The effect of ER stress inhibitors [4-phenylbutyric acid (4-PBA)] and/or activators [tunicamycin (TM)] was tested in polycystic (PCK) rats and cystic cholangiocytes *in vitro*.

***Correspondence*:** Jesus M. Banales, PhD, Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, Paseo del Dr. Begiristain s/n, E-20014, San Sebastian, Spain; jesus.banales@biodonostia.org / Phone: +34 943006067; Fax: +34 943006250, or, Maria J. Perugorria, PhD, Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute – Donostia University Hospital, Paseo del Dr. Begiristain s/n, E-20014, San Sebastian, Spain; matxus.perugorria@biodonostia.org / Phone: +34 943006125; Fax: +34 943006250.
[§]Both authors share co-seniorship.

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Results: The expression levels of unfolded protein response (UPR) components were upregulated in liver tissue from PLD patients and PCK rats, as well as in primary cultures of human and rat cystic cholangiocytes, compared to normal controls. Cystic cholangiocytes showed altered proteomic profiles, mainly related to proteostasis (i.e., synthesis, folding, trafficking and degradation of proteins), marked enlargement of the ER lumen (by TEM), and hyperactivation of the proteasome. Notably, chronic treatment of PCK rats with 4-PBA decreased liver weight, as well as both liver and cystic volumes, of animals under baseline conditions or after TM administration compared to controls. *In vitro*, 4-PBA downregulated the expression (mRNA) of UPR effectors, normalized proteomic profiles related to protein synthesis, folding, trafficking and degradation, and reduced the proteasome hyperactivity in cystic cholangiocytes, reducing their hyperproliferation and apoptosis.

Conclusions: Restoration of proteostasis in cystic cholangiocytes with 4-PBA halts hepatic cystogenesis, emerging as a novel therapeutic strategy.

Lay summary

Understanding the molecular mechanisms involved in the pathogenesis of PLDs is crucial in order to find new targets for therapy. This study provides evidence that cystic cholangiocytes are characterized by abnormal ER-related proteostasis that prompts adaptive pro-survival mechanisms, favouring PLD progression. Normalization of proteostasis in cystic cholangiocytes with the chemical chaperone 4-PBA halts hepatic cystogenesis in experimental models *in vivo* and *in vitro*, opening a new therapeutic avenue for PLDs.

Keywords

hepatic cystogenesis; cholangiocyte; endoplasmic reticulum stress; unfolded protein response (UPR); proteostasis; pathogenesis

Introduction

Polycystic liver disease (PLD) constitutes a group of genetic cholangiopathies characterized by bile duct dilatation and/or progressive development of multiple fluid-filled biliary cysts (>10), which are the main cause of morbidity.(1–3) Despite most patients with PLD remain clinically asymptomatic through the years, progressive hepatic cystogenesis may cause massive hepatomegaly in a proportion of them, potentially resulting in severe symptoms such as abdominal distention and bloating, dyspnea, back pain, gastroesophageal reflux, hypertension, as well as cyst hemorrhage, infection and rupture, among others.(1–3) To date, surgical and/or pharmacological strategies exert short-term and/or modest benefits, and liver transplantation emerges as the only curative option for patients with advanced PLD.(1–3)

According to the genetic traits, hepatic cystogenesis may solely affect the intrahepatic bile ducts [i.e., autosomal dominant polycystic liver disease (ADPLD)] or arise associated with renal cysts [i.e., autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), Caroli's disease and congenital hepatic fibrosis (CHF) in infants].(2, 3) Noteworthy, the majority of genes known to cause ADPLD (i.e., *PRKCSH*, *SEC63*, *ALG8, GANAB* and *SEC61β*)(2, 4) and ADPKD (i.e., *PKD2* and

GANAB)(2, 4) encode for endoplasmic reticulum (ER)− resident proteins.(1, 2, 4) These proteins play a relevant role in the ER protein biogenesis, governing the synthesis of functional membrane and secreted proteins.(1, 2, 4) In this regard, mutations (i.e., missense, nonsense, splice site and/or frameshift) in the aforementioned genes may result in partial or complete loss-of-function of the protein products, disrupting the folding, maturation and trafficking of nascent proteins and compromising ER proteostasis.(4, 5) Moreover, abnormalities in ER proteostasis may subsequently activate the unfolded protein response (UPR) signaling cascades, composed by sensor (i.e., IRE1α, PERK and ATF6) and effector (i.e., CHOP, GRP78 and XBP1) proteins, in order to promote protein folding, ER-associated protein degradation (ERAD), and the activation of pro-survival mechanisms.(6, 7) Nevertheless, when the UPR is unable to alleviate the burden of un/misfolded proteins, chronic/excessive ER stress might result in proteotoxicity-induced apoptosis.(6, 7)

In this study, we hypothesized that mutations in PLD-related genes could trigger proteostatic abnormalities in cystic cholangiocytes resulting in ER stress, which might contribute to hepatic cystogenesis, thus representing potential druggable targets.

Materials and methods

Human samples

Cystic wall tissue from patients with PLD (n=16), and healthy human liver (n=14) and gallbladder (n=14) biopsies were obtained from Radboud University Medical Center (Nijmegen, The Netherlands) and Donostia University Hospital (San Sebastian, Spain), respectively. Cystic tissues were collected from patients with ADPLD displaying either *PRKCSH* (protein kinase C substrate 80K-H: c.1341–2A>G or c.292+1G>C) or *SEC63* (translocation protein SEC63 homolog: 1702delGAA) mutations, whereas healthy liver and gallbladder tissues were gathered from individuals after surgical resection of colon cancer metastasis or cholecystectomy, respectively. Supporting Table 1 summarizes the main demographic and clinical features of the patients included in the study. Research protocols were approved by the Clinical Research Ethics Committee of the supporting institutions (MSA-MMR-2017–01 and 2012/317), and an informed consent was obtained from all subjects.

PLD animal model

The polycystic (PCK) rat [PCK/CrljCrl-*Pkhd1*^{pck}/Crl] constitutes a well-characterized animal model of ARPKD, presenting a mutation in the *PKHD1* (polycystic kidney and hepatic disease 1) orthologous gene and developing hepato-renal cysts that recapitulate the course of the human disease.(8, 9) Hepatic cystogenesis, serum biochemical parameters and molecular markers were analyzed in wild-type (WT; n=12) and control PCK (n=12) rats, as well as in PCK rats chronically treated with 4-phenylbutyric acid sodium salt (4-PBA: 100 mM in drinking water; Scandinavian formulas, PA, USA; n=12) and/or Tunicamycin (TM: 0.02 mg/kg intraperitoneal injection; Sigma, MO, USA; n=12) for 5 months. All details are described in Supporting Information.

Human and rat cholangiocyte primary cultures

Normal human cholangiocytes (NHC) and polycystic human cholangiocytes were isolated and characterized by our group as previously described.(10) Two primary cultures of PLD cholangiocytes (i.e., ADPKD and ADPLD) were used. ADPKD cells have a missense mutation (c.2515C>T) in exon 22 of *GANAB* (glucosidase II alpha subunit) gene,(11) whereas ADPLD cells present a splice site mutation (c.292+1G>C) in intron 4 of the *PRKCSH* gene. In addition, PCK rat cholangiocytes, which hold a splicing mutation (IVS35–2A→T) in *Pkhd1* gene,(8) as well as the corresponding normal rat cholangiocytes (NRC), were used.(12) All primary cultures were cultured in fully-supplemented DMEM/F-12 medium as previously described.(10)

RNA isolation, retrotranscription and quantitative polymerase chain reaction (qPCR)

RNA purification, retrotranscription and quantification of UPR factors (i.e., sensors and effectors), as well as, pro-fibrotic and pro-inflammatory genes were performed as detailed in Supporting Information. Primer sequences are included in Supporting Table 2.

Cell proliferation and death

The rates of cell proliferation and death were measured as described in Supporting Information.

Proteasome activity

Ubiquitinated protein degradation was determined measuring the proteasomal chymotrypsin-like activity with the *Proteasome Activity Assay Kit* (Abnova, Taiwan), according to the manufactureŕs instructions. Free 7-amino-4-methylcoumarin (AMC) fluorescence intensity was detected using the Infinite® 200 Pro plate reader (Tecan, Switzerland).

Transmission electron microscopy (TEM)

Ultrastructural examination of the ER morphology and size in human (i.e., NHC, ADPKD and ADPLD) and rat (i.e., NRC and PCK) cholangiocyte cultures were performed as previously described.(9) The total surface area occupied by the ER in each TEM image was determined with the ImageJ software version 1.50 (NIH, Bethesda, MA, USA) by calculating the sum of the area (μm^2)/length (μm) ratio of each ER portion present in the captured images.

Mass spectrometry and proteomic analysis

Comparative proteomic analysis of whole cell extracts (WCEs) from normal and cystic cholangiocytes (both human and rat incubated with 4-PBA or vehicle) were performed by mass spectrometry as described in Supporting Information.

Statistical Analysis

Data are shown as mean ± standard error of mean (SEM). Once normality was assessed with *Shapiro–Wilk* test, parametric *Student t* test or non-parametric *Mann-Whitney U* test were used for statistical comparisons between two groups. For comparisons between more than two groups, the parametric one-way analysis of variance (ANOVA) test followed by Tukey's

post hoc test or the non-parametric *Kruskal-Wallis* test followed by *a posteriori Dunn's* test were used. In the proteomics analysis, protein abundance differences and p-values were calculated and quantified using logarithmic values (log2 transformation and imputation). As samples did follow a Gaussian distribution, parametric *Student t* test was applied. Differences were considered significant when $p<0.05$ (*, ** and *** are p values <0.05, <0.01 and <0.001, respectively). Results were statistically analyzed using the *GraphPad Prism* version 6.01 software (La Jolla, CA, USA).

Results

UPR sensors and effectors are upregulated in human and rat PLD tissues and cystic cholangiocyte cultures

The levels (mRNA) of the main UPR components were measured in cystic wall tissue obtained from patients with PLD (i.e., ADPLD), as well as in healthy human gallbladder (mainly composed by cholangiocytes) and liver tissue. NHC were also included in this analysis, as they constitute a pure cellular population of healthy human cholangiocytes. Interestingly, human cysts exhibited an upregulation of the UPR components compared to normal controls (Fig. 1A). When compared with gallbladder tissue samples or NHCs, the expression levels of the three UPR sensors (i.e., *ATF6*, *IRE1α* and *PERK*) and the effectors *GRP78* and *CHOP* were found upregulated in PLD tissue (Fig. 1A); likewise, increased levels of the effector *XBP1* were observed in cystic tissue compared to NHC, while no differences were evident when compared to gallbladder tissue samples (Fig. 1A). In addition, *PERK* and *CHOP* were also found upregulated in cysts compared with healthy livers, whereas no significant differences in the expression of the other UPR components were noticed (Fig. 1A). Primary cultures of cystic human cholangiocytes (i.e., ADPKD and ADPLD) also exhibited upregulated levels of all the UPR components (i.e., sensors and effectors) when compared to NHC (Fig. 1B). Of note, ADPKD (*GANAB*-mutated) cholangiocytes displayed higher levels of the UPR effectors *XBP1* and *CHOP* than ADPLD (*PRKCSH*-mutated) cholangiocytes (Fig. 1B).

The expression levels of the UPR components were also determined in both liver tissue samples and cultured cholangiocytes derived from WT and PCK rats. The UPR sensors *Atf6* and *Perk*, as well as the effectors *Grp78* and *Chop*, were all found upregulated in PCK rat livers compared to WT livers (Fig. 1C). Although *Ire1α* and *Xbp1* expression levels displayed a tendency to increase in liver tissue from PCK rats, no significant differences were observed compared to matched liver tissue from healthy rats (Fig. 1C). Additionally, ER stress was also evidenced in PCK cholangiocytes *in vitro*, as shown by marked upregulation of most UPR components when compared to NRC (Fig. 1D).

Cystic cholangiocytes exhibit marked ER dilatation

Considering the overexpression of UPR components in human and rat cysts (Fig. 1A–D), which suggest aberrant ER proteostasis and potential accumulation of un/misfolded proteins within the ER lumen, the morphological and ultrastructural features of this organelle were evaluated. For this purpose, TEM images from normal and cystic cholangiocytes in culture were captured and analyzed. As expected, NHC and NRC exhibited a typical narrow ER

lumen (Fig. 1E). However, pronounced dilatation of the ER lumen was found (Fig. 1E) in all primary cultures of cystic cholangiocytes (i.e., ADPKD, ADPLD, PCK) compared to normal controls, being this phenotype a well-documented feature linked to ER stress.(6, 7, 13) In particular, the ER area underwent a 1.79, 1.47 and 1.62-fold increase in ADPKD, ADPLD and PCK cholangiocytes respectively, compared to the respective normal controls (Fig. 1F). Of note, ADPKD cholangiocytes presented increased ER dilatation compared to ADPLD cholangiocytes.

The proteomic profile of cystic cholangiocytes is characteristic of aberrant proteostasis

The proteomic profiles of normal and cystic cholangiocytes were determined under baseline conditions using mass spectrometry. A total of 2878, 2775 and 2715 proteins were identified in NHC, ADPKD and ADPLD cholangiocytes, respectively (Fig. 2A). Among the significantly dysregulated proteins, 87 were found upregulated and 328 downregulated similarly in ADPKD and ADPLD (PLD) compared to normal cholangiocytes (Figs. 2B,C). Gene ontology (GO) analysis revealed that most of these dysregulated proteins are involved in ER proteostasis-related mechanisms (i.e., synthesis, folding, trafficking and degradation pathways) (Fig. 2D), while the STRING analysis revealed functional interactions between several protein clusters (Fig. 2E). Among these proteins, overexpression of ubiquitin-protein ligases (i.e., HERC3), flavoproteins that catalyze the formation of disulfide bonds (i.e., QSOX1) and enzymes that mediate lysosomal proteolysis (i.e., LGMN) stand out, whereas the levels of other proteins involved in the late stages of protein maturation [such as HSP90 family chaperones (i.e., HSP90AA1) and co-chaperones (i.e., UN45A)], ER to Golgi transport mediators (i.e., GDS1), and oxidoreductases (i.e., QOR) were found downregulated in cystic *vs* normal human cholangiocytes (Fig. 2F).

Similar to human cholangiocytes, the proteomic profiles of NRC and PCK rat cholangiocytes was determined. A total of 2575 and 2531 proteins were identified in NRC and PCK cholangiocytes, respectively (Fig. 3A). Among the significantly dysregulated proteins, 980 were found upregulated and 476 downregulated in PCK *vs* NRC cholangiocytes (Figs. 3B,C). Further functional analysis revealed a substantial enrichment of proteins involved in protein synthesis, folding, transport and degradation, as well as in other pivotal processes related to PLD pathogenesis such as ciliogenesis (Fig. 3D). The protein interaction network evidenced their roles along the different steps of protein maturation, from protein biogenesis to degradation (Fig. 3E). Among these proteins, overexpression of components related to protein synthesis (i.e., Eif3h) and folding (i.e., Calr, Pdia4), as well as of the 20S proteasome (i.e., Psmb3), Hsp70 family chaperones (i.e., Hspa4) and co-chaperones (i.e., Hspbp1) was evident, whereas the expression of other families of chaperones and mediators of nascent protein trafficking (i.e., Hspb1 and Bloc1s6, respectively) were downregulated in PCK *vs* NRC cholangiocytes (Fig. 3F).

ER proteostasis disturbance and stress contributes to hepatic cystogenesis *in vivo*

The contribution of ER proteostasis disturbance and proteotoxic stress to hepatic cystogenesis, as well as the potential therapeutic value of its modulation, was evaluated in PCK rats. 4-PBA is a chemical chaperone that inhibits ER stress,(14) while tunicamycin (TM) prevents the glycosylation and proper folding of proteins within the ER, being used as

a “bona fide” ER stressor.(15) Therefore, 4-PBA and/or TM were administered to 8-weeks-old PCK rats for 5 months. In comparison to WT animals, control PCK rats developed hepatomegaly as shown by a significant increase in liver weight and volume (Fig. 4A), without displaying any differences in total body weight, thus resulting in an increased liver/body weight ratio (Supporting Table 3). Remarkably, chronic administration of 4-PBA to PCK rats reduced their total liver weight, as well as the liver and cystic volumes, when compared with control PCK rats (Fig. 4A,B). Administration of TM to PCK rats did not further increase their liver weight, volume and cystogenesis (Figs. 4A,B), but it induced a significant increase of the total liver/body weight ratio and in the levels of serum markers of cholestasis (ALP) and liver injury (AST), when compared to control PCK rats (Supporting Table 3). Noteworthy, 4-PBA was also able to decrease the liver weight and volume, as well as the hepatic cystic volume, of TM-induced PCK rats, when compared with rats exposed to TM alone (Fig. 4A and 4B, respectively).

Modulation of ER stress impacts on cholangiocyte proliferation and cell death *in vitro*

The effect of ER stress regulation was also evaluated in normal and cystic (ADPKD and ADPLD) cholangiocytes *in vitro*. The mRNA levels of the UPR sensors *ATF6* and *PERK* remained mostly constant in normal and cystic cholangiocytes after 4-PBA and/or TM incubation, whereas *IRE1α* was upregulated in both cell types under the presence of 4-PBA and/or TM (Supporting Fig. 1). Of note, 4-PBA reduced the expression of *CHOP*, *GRP78* and *XBP1* in ADPKD, but not in ADPLD cholangiocytes and also reduced the levels of *GRP78* and *XBP1* in NHC (Fig. 5A). On the other hand, TM induced the expression of the UPR effectors *CHOP*, *GRP78* and *XBP1* in both normal and cystic human cholangiocytes compared to corresponding control conditions, with 4-PBA almost reverting these alterations to basal levels (Fig. 5A).

Cystic cholangiocytes exhibit higher proliferative rates than normal cholangiocytes.(16, 17) Noteworthy, the proliferation of both ADPKD and ADPLD cholangiocytes was significantly reduced upon 4-PBA treatment, an effect that was also evident in NHC (Fig. 5B). On the other hand, no significant differences in the baseline apoptotic rate were observed between normal and cystic cholangiocytes *in vitro* (Fig. 5C). 4-PBA also decreased ADPKD and ADPLD cholangiocytes baseline cell death, while no differences were evident in NHC (Fig. 5C). Moreover, 4-PBA protected against TM-induced apoptosis in both normal and cystic human cholangiocytes (Fig. 5C). In line with this, baseline caspase 3/7 activity was significantly diminished after 4-PBA exposure in normal and cystic cholangiocytes (Fig. 5D), further substantiating the anti-apoptotic effect of 4-PBA.

The ER stress and ubiquitin-proteasome system are interconnected in cystic cholangiocytes

An integrated part of the ERAD pathway is the ubiquitin-proteasome system (UPS), which ultimately leads to the degradation of the terminally un/misfolded proteins retrotranslocated from the ER,(18) in order to alleviate the burden of accumulated structurally aberrant proteins, in an attempt to decrease the ER stress.(19) Data showed that the 20S proteasome activity is increased in cystic human cholangiocytes (i.e., ADPKD and ADPLD) under baseline conditions, compared to the respective controls (Fig. 5E). In addition, ADPLD

cholangiocytes exhibited increased 20S proteasome activity compared to ADPKD cholangiocytes (Fig. 5E). Interestingly, a decline of 30–60% of the 20S proteasome activity was observed in NHC, ADPKD and ADPLD cholangiocytes under the presence of 4-PBA (Fig. 5F).

4-PBA improves ER proteostasis in cystic cholangiocytes

The impact of 4-PBA administration on the proteomic profile and protein biogenesis pathways of cystic cholangiocytes were further analyzed. A total of 2250 proteins were commonly expressed in cystic cholangiocytes (i.e., ADPKD and ADPLD) under baseline and 4-PBA conditions (Fig. 6A). Importantly, the expression levels of 266 dysregulated proteins in ADPKD and ADPLD cholangiocytes became normalized after 4-PBA administration (Fig. 6B). Among them, the expression of 8 proteins commonly dysregulated in ADPKD and ADPLD cholangiocytes and involved in protein folding, trafficking and degradation were reverted to baseline levels after 4-PBA administration (Fig. 6C). As shown in Fig. 6D, the levels of most of the aforementioned key proteins found dysregulated under baseline conditions in cystic cholangiocytes (Fig. 2F) were reverted by 4-PBA. Similarly, the levels of 225 from a total of 2092 proteins differentially expressed between PCK and NRC cholangiocytes were subsequently normalized, at least in part, by 4-PBA (Figs. 7A–C).

Discussion

The findings reported in this study indicate that: **(I)** the UPR signaling pathways are induced in PLD, which is revealed by increased expression of ER stress sensors and effectors in cystic tissue from patients with PLD and in cholangiocytes in culture; **(II)** morphologically, the ER lumen of cystic cholangiocytes undergoes a marked dilatation under baseline conditions, which is a characteristic of ER stress; **(III)** the proteomic profile of cystic cholangiocytes is consistent with alterations in protein synthesis, folding, trafficking and degradation; **(IV)** chronic administration of the 4-PBA chaperone reduces liver weight and volume, as well as the hepatic cystic volume in PCK rats, both in the presence or absence of the ER stress inducer TM, thus delaying disease progression; **(V)** 4-PBA exerts anti-proliferative and anti-apoptotic effects, and re-establishes ER proteostatic mechanisms in cystic cholangiocytes *in vitro*; **(VI)** TM increases the expression of the UPR effectors and promotes cell death, two events that were efficiently attenuated by 4-PBA; **(VII)** cystic cholangiocytes exhibits 20S proteasome hyperactivity, which is reduced after 4-PBA incubation *in vitro*. All these data are consistent with our hypothesis that abnormal proteostasis and ER stress play a pivotal role in the pathogenesis of PLD, while its attenuation may halt hepatic cystogenesis, ameliorating the disease pathogenesis and thus representing a novel and promising therapeutic strategy.

Different molecular mechanisms are behind the pathogenesis of PLD, including primary cilium and centrosome dysfunctions, matrix metalloproteinase hyperactivity, aberrant fluid secretion, epigenetic alterations, and dysregulated intracellular levels of cAMP and Ca^{2+}, leading to the characteristic hyperproliferative phenotype of cystic cholangiocytes that govern the onset and progression of hepatic cystogenesis.(3, 17) However, the fact that the majority of PLD-causative genes encode for ER-resident proteins involved in the biogenesis

and trafficking of nascent proteins,(2) suggests that these processes may be primarily or secondary altered in PLD, contributing to cyst growth and disease severity. In this regard, it was proposed that impaired biogenesis and/or transport of polycystin-1 to the primary cilia reduce its functional dosage, which is the rate-limiting determinant of cystogenesis and consequently of PLD severity.(4, 5) Taking these observations into consideration, we aimed to study the morphological and molecular features of the ER in cystic cholangiocytes, ascertain their role in the development and progression of PLD, and evaluate the therapeutic value of its regulation. Our data indicate that mutations in the PLD-related genes *PRKCSH*, *SEC63*, *GANAB and Pkhd1* are associated with upregulation of the UPR signaling pathways (i.e., sensors and effectors). Further proteomic studies confirmed an abnormal ER-related proteostasis in cystic cholangiocytes associated with overexpression of the Hsp70 family, as a potential compensatory mechanism, but without changes in the expression other chaperone families involved in late stages of protein folding (such as Hsp90, among others). In line with this, pronounced enlargement of the ER lumen, 20S proteasome hyperactivity and overexpression of several ubiquitin ligases were evident in cystic cholangiocytes. *PRKCSH*, *SEC63* and *GANAB* encode for the ER-resident proteins glucosidase II beta subunit, SEC63, and glucosidase II alpha subunit respectively, which play key roles in co- and post-translational modifications of nascent proteins.(4) Hence, partial or total abolishment of the function of these proteins may impair the folding, maturation and/or trafficking of nascent ER proteins, promoting their accumulation inside the ER and triggering ER stress. Of note, ADPKD (*GANAB* mutant) cholangiocytes exhibited increased levels of the UPR effectors *XBP1* and *CHOP*, as well as enlarged ER lumen, compared to ADPLD (*PRKCSH* mutant) cholangiocytes at baseline conditions. These effects were in line with the 20S proteasome activity in both types of diseased cholangiocytes, supporting the notion that the 20S proteasome activity aims to relieve the levels of un/misfolded proteins and the subsequent ER stress in cystic cholangiocytes. Based on all these data, the levels of ER stress and the interplay between the ER proteostasis and the proteasome activity seem to be dependent on the type of gene found mutated in PLD. On the other hand, *Pkhd1* encodes for fibrocystin/polyductin, a protein localized in the primary cilium that seems to participate in tubulogenesis and the maintenance of ductal lumen epithelium architecture.(3, 17) The splicing mutation of the *Pkhd1* orthologous gene in PCK rats compromise the correct maturation of fibrocystin/polyductin, which is retained within the ER and rapidly degraded, reducing the amount of protein located in the primary cilia, where it usually interacts with polycystin-2 (PC2) and modulate the activity of this calcium channel.(20) Furthermore, fibrocystin/polyductin also interacts with the calcium modulating cyclophilin ligand, a primary cilia and ER-associated protein involved in the regulation of cytosolic Ca^{2+} levels.(21) Interestingly, ER stress is also induced when the ER-calcium storage is depleted, as some ER chaperones require an optimal Ca^{2+} concentration for the correct folding and maturation of nascent proteins,(14, 22) supporting our data. Under all of these pathological circumstances, and in order to restore the protein homeostasis within the ER lumen and resolve ER stress, UPR signaling cascades become activated, leading to the upregulation of genes involved in both protein folding and ERAD.(6, 7) It is well documented that the transcription factors XBP1 and ATF6 (i.e., N-terminal cytosolic domain) translocate to the nucleus,(23) where they induce the expression of GRP78 (a member of the Hsp70 family of chaperones), XBP1 and components of the ERAD machinery to promote the folding and

UPS-mediated degradation of structurally aberrant proteins, respectively.(23) Interestingly, both mechanisms counteract the mild pro-apoptotic signals motivated by the transcriptional activation of CHOP via ATF4.(23) However, when the UPR is unable to relieve the burden of un/misfolded proteins, excessive/chronic ER stress might result in proteotoxicity-induced cell death.(6, 7) In this scenario, it has been reported that ATF4 and ATF6 markedly upregulate the expression of CHOP,(7, 24) reaching levels that are unmanageable by the adaptive responses inducing apoptosis. Of note, sustained UPR activation was previously identified as a pivotal pathogenic factor in several human diseases, such as diabetes mellitus, neurodegenerative disorders, viral infections, cancer, heart failure and a wide range of liver diseases.(25, 26)

Several reports have demonstrated the chaperone-like activity of the small molecule 4-PBA, which promotes protein folding and trafficking, thus preventing the aggregation of un/misfolded proteins within the ER and alleviating the ER stress.(14, 27) Indeed, the therapeutic effects of 4-PBA were assessed in a variety of experimental models of genetic,(28) inflammatory,(29) metabolic,(30) and liver(31) diseases, exerting promising benefits. The results obtained in our *in vitro* and *in vivo* experimental models of PLD are consistent with these findings, as 4-PBA halted the disease progression in PCK rats. On the other hand, the ER stressor TM did not exacerbate the hepatomegaly and liver cystogenesis of PCK rats, probably as a consequence of proteotoxic-induced cell death processes that could be linked to the elevation in AST and ALP levels observed in TM-administered PCK animals. Notably, 4-PBA exerted similar therapeutic benefits when it was administered in combination with TM. Furthermore, 4-PBA markedly attenuated the UPS adaptive response (i.e., proteasome activity and expression of ubiquitin-ligases) in cystic cholangiocytes due to its capacity to assist both folding and trafficking of newly synthesized proteins, contributing to restore ER proteostasis and resolve ER stress. Accordingly, 4-PBA inhibited the hyperproliferation, as well as the baseline and TM-induced apoptosis in cystic cholangiocytes, maintaining a suitable balance that delays the progression of PLD.(32)

Our data are also in line with a previous report indicating that cystic cholangiocytes are characterized by increased autophagy,(12) as overexpression of lysosomal proteolysis components was also evident here in cystic cholangiocytes. In this regard, emerging evidences indicate that activation of UPR signaling can also promote the clearance of un/misfolded proteins via autophagy, specially, of those proteins that are not efficiently degraded by the ERAD-proteasome system.(33–35)

In summary (Fig. 8), this study provides strong evidence that cystic cholangiocytes are characterized by abnormal ER proteostasis, leading to the accumulation of a burden of un/misfolded proteins within the ER lumen that causes ER stress and the subsequent activation of pro-survival mechanisms that promote hepatic cystogenesis, thus representing a novel and promising therapeutic target for patients with PLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

PLDs	polycystic liver diseases
ER	endoplasmic reticulum
qPCR	quantitative polymerase chain reaction
TEM	transmission electron microscopy
4-PBA	4-phenylbutyric acid sodium salt
TM	tunicamycin
PCK	polycystic
UPR	unfolded protein response
mRNA	messenger ribonucleic acid
ADPLD	autosomal dominant polycystic liver disease
ADPKD	autosomal dominant polycystic kidney disease
ARPKD	autosomal recessive polycystic kidney disease
CHF	congenital hepatic fibrosis
PRKCSH	protein kinase C substrate 80K-H
SEC63	translocation protein SEC63 homolog
ALG8	asparagine-linked glycosylation 8
GANAB	glucosidase II alpha subunit
SEC61β	SEC61 translocon beta subunit
PKD2	polycystic kidney disease 2

IRE1α	inositol-requiring enzyme 1 alpha
PERK	PRKR-like endoplasmic reticulum kinase
ATF6	activating transcription factor 6
CHOP	C/EBP-homologous protein
GRP78	glucose-regulated protein 78kDa
XBP1	x-box binding protein 1
ERAD	endoplasmic reticulum-associated protein degradation
WT	wild-type
NHC	normal human cholangiocytes
NRC	normal rat cholangiocytes
DMEM/F-12	dulbecco's modified eagle medium/nutrient mixture F-12
PKHD1	polycystic kidney and hepatic disease 1
AMC	7-amino-4-methylcoumarin
NIH	national institutes of health
WCEs	whole cell extracts
SEM	standard error of the mean
ANOVA	analysis of variance
GO	gene ontology
ALP	alkaline phosphatase
AST	aspartate aminotransferase
UPS	ubiquitin proteasome system
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium
PC2	polycystin-2
ATF4	activating transcription factor 4

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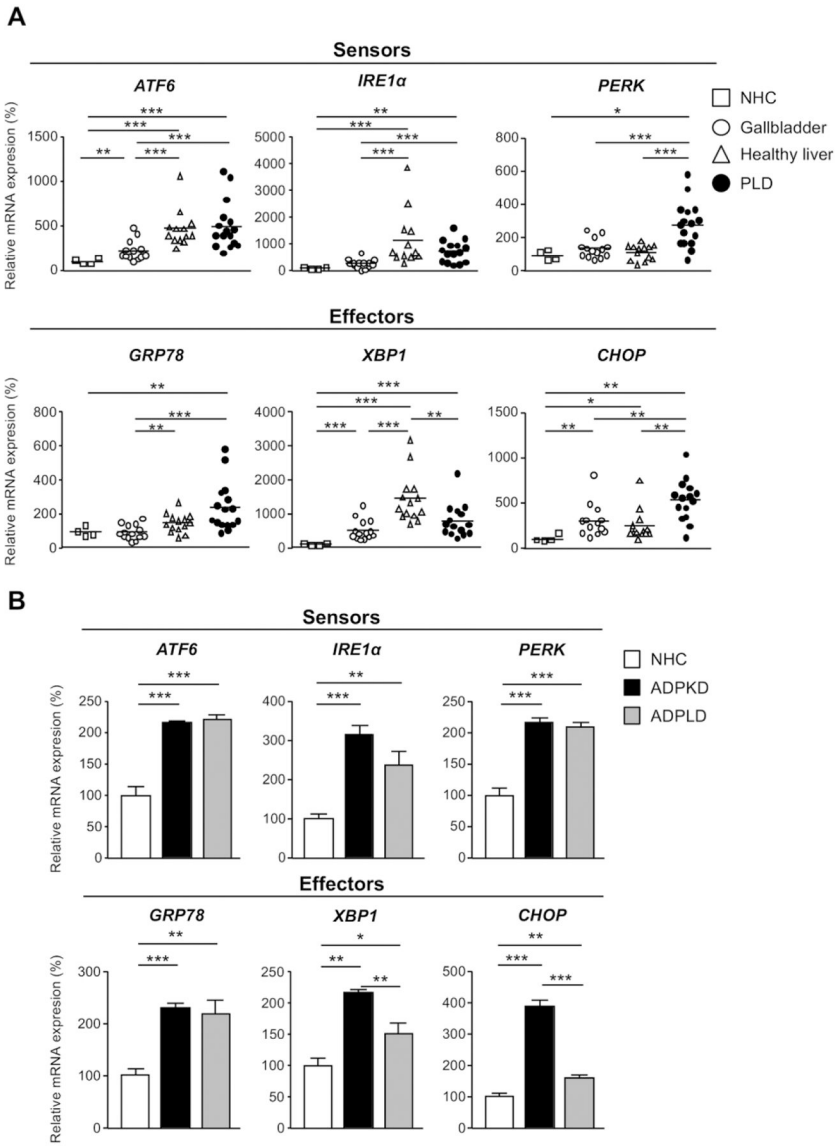
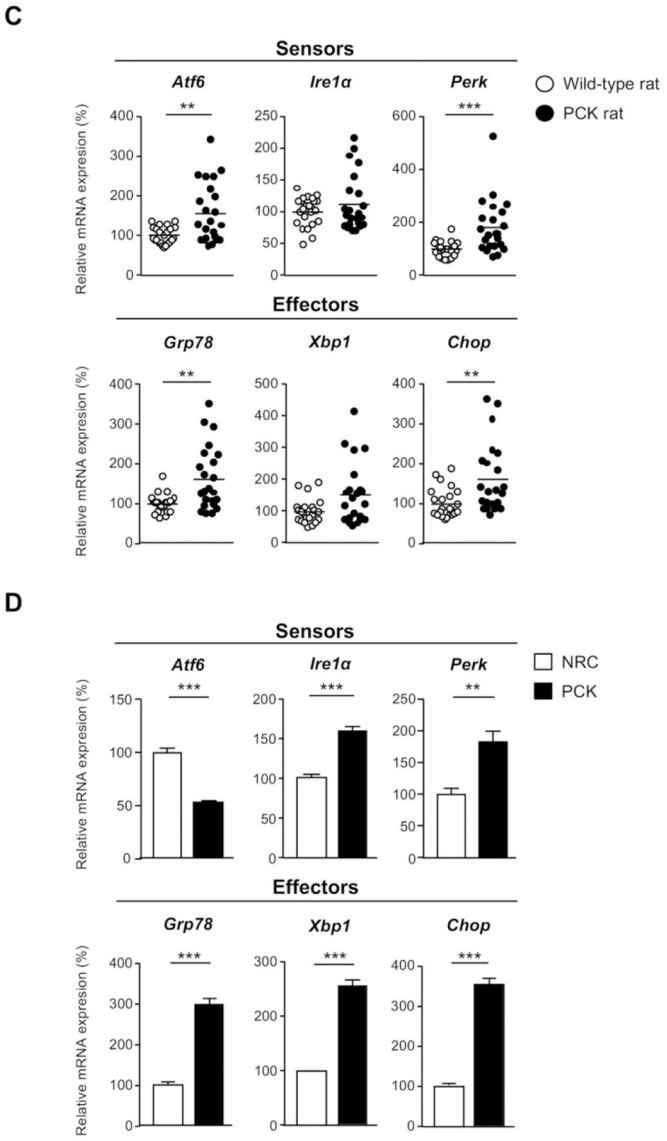




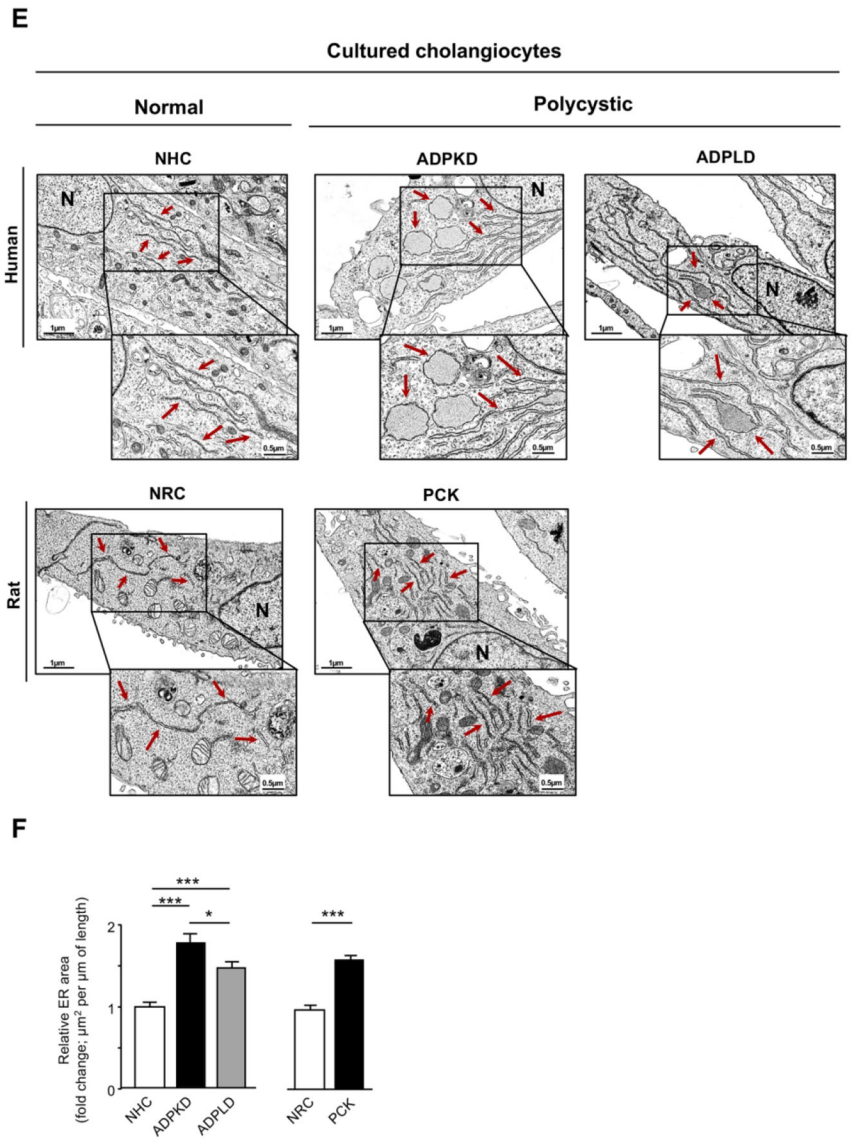
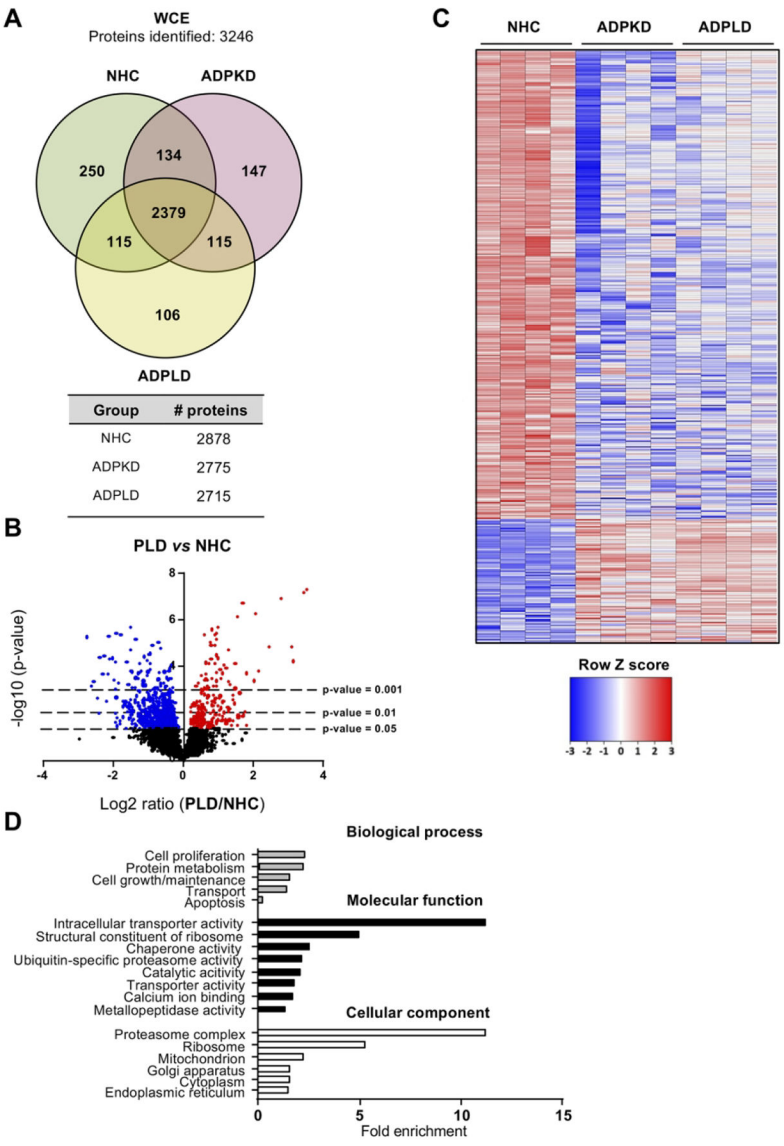


Figure 1. Molecular and morphological ER features of human and rat cystic cholangiocytes *in vivo* and/or *in vitro*.

mRNA levels of UPR sensors and effectors in: (**A**) ADPLD cystic wall tissue (n=16) and healthy controls [liver (n=14), gallbladder (n=14), and NHC (n=4)], (**B**) normal (NHC; n=6) and cystic (ADPKD and ADPLD; n=6) human cholangiocytes in culture, (**C**) wild-type (n=20) and PCK rat (n=22) livers, and (**D**) normal (NRC; n=6) and cystic [PCK; n=6) rat cholangiocytes in culture. (**E**) Representative TEM images showing the ER ultrastructural morphology (red arrows) of normal and cystic cholangiocytes isolated from humans and rats. Scale bar= 0.5 µm. (**F**) Quantification of the ER lumen area in human [NHC (n=47), ADPKD (n=28), and ADPLD (n=28)] and rat [NRC (n=49) and PCK (n=52)] cholangiocytes.



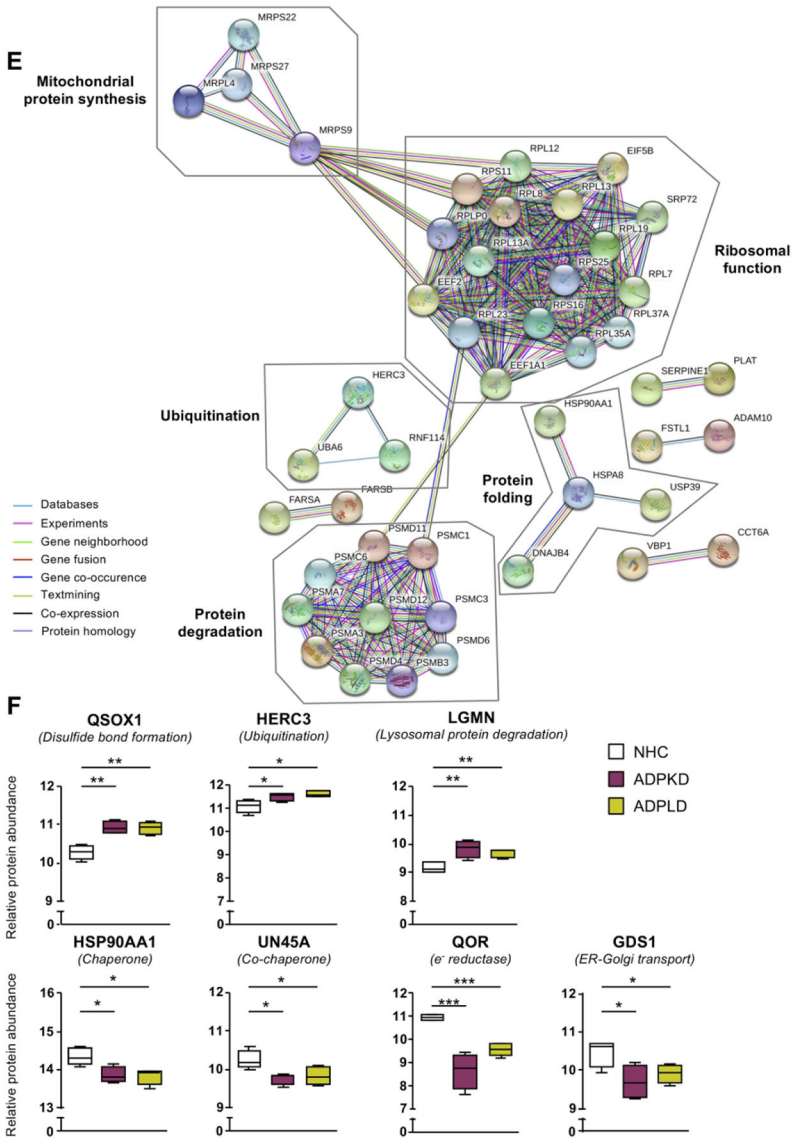
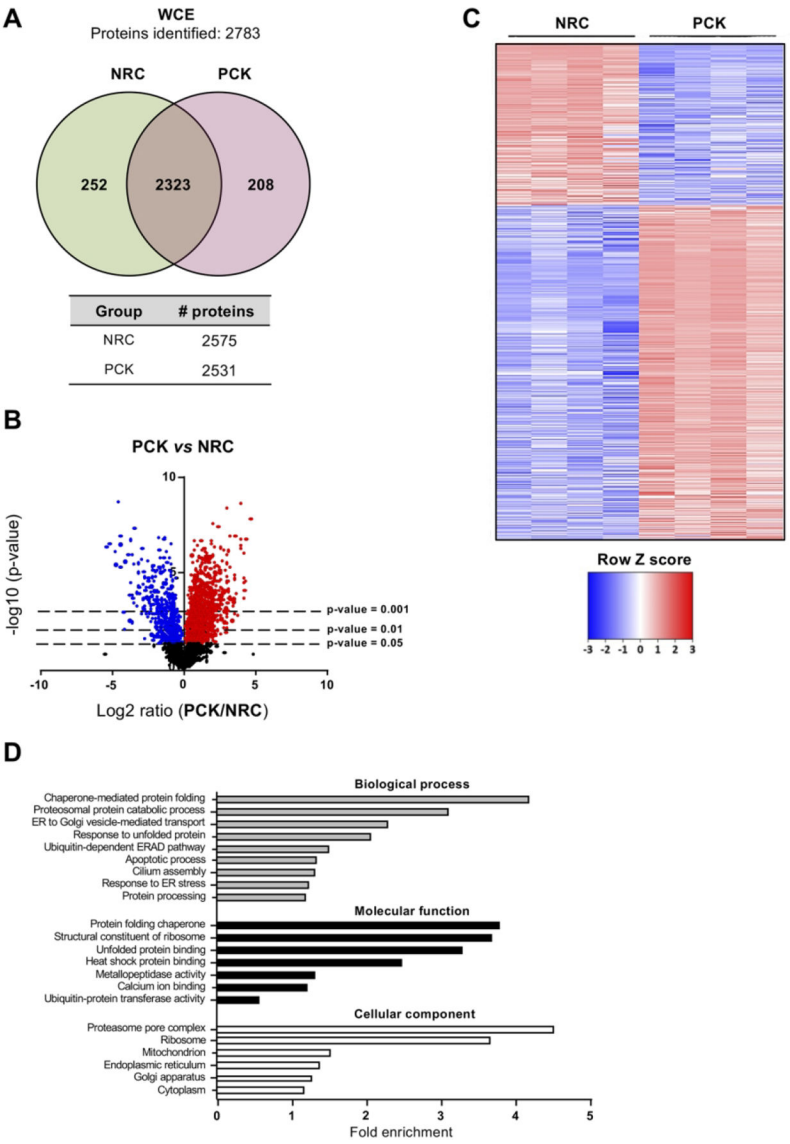


Figure 2. Proteomic profiles of normal and cystic human cholangiocyte primary cultures.

(**A**) Venn diagram with the proteins identified in each human cell type. (**B**) Volcano plot displaying the expression pattern and differences of all proteins identified in PLD *vs* NHC cholangiocytes. (**C**) Heatmap representing only the proteins differentially expressed in PLD (ADPKD and ADPLD) *vs* NHC. (**D**) GO classification of the proteins differentially expressed in PLD *vs* NHC. (**E**) STRING interaction analysis of the proteins differentially expressed in PLD *vs* NHC. (**F**) Box plot diagrams of the relative abundance of each selected protein in the three human cell types.



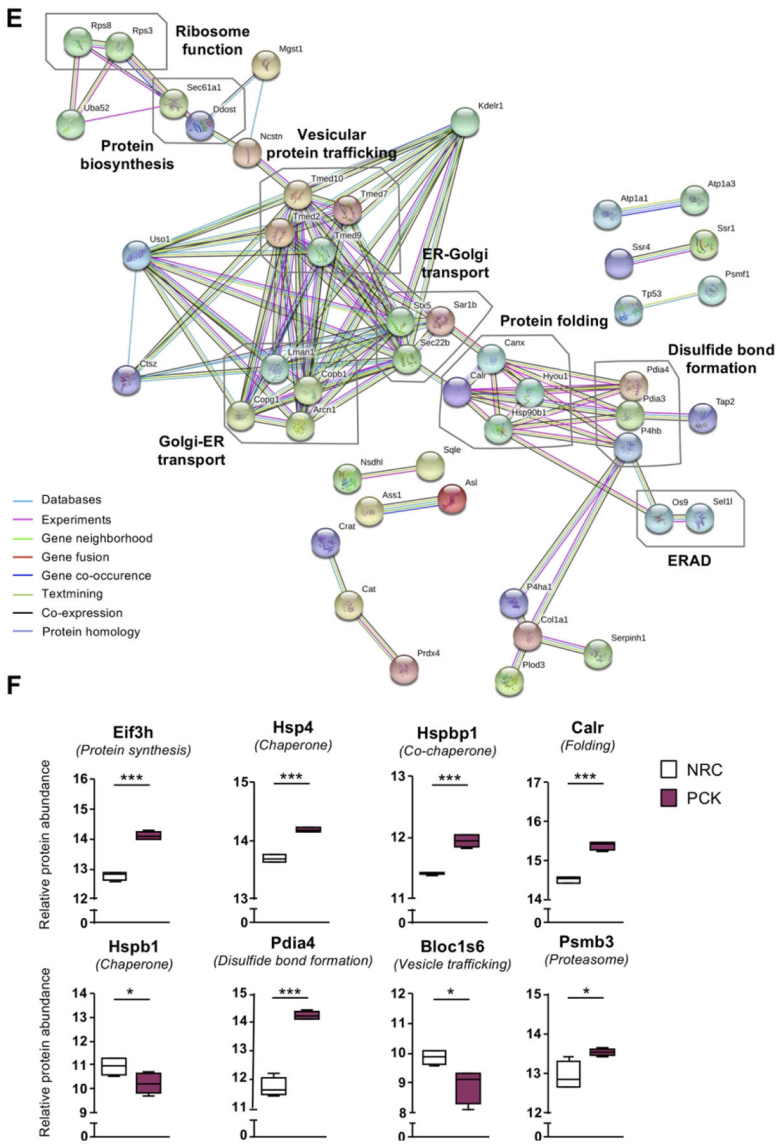


Figure 3. Proteomic profiles of normal and cystic rat cholangiocyte primary cultures.

(**A**) Venn diagram with the proteins identified in normal and cystic rat cholangiocytes. (**B**) Volcano plot displaying the expression pattern and differences of all the identified proteins in PCK *vs* NRC. (**C**) Heatmap representing only the proteins differentially expressed in PCK *vs* NRC. (**D**) GO classification of the proteins differentially expressed in PCK *vs* NHC. (**E**) STRING interaction analysis of the proteins differentially expressed in PCK *vs* NRC. (**F**) Box plot diagrams of the relative abundance of each selected protein in normal and cystic rat cholangiocytes.

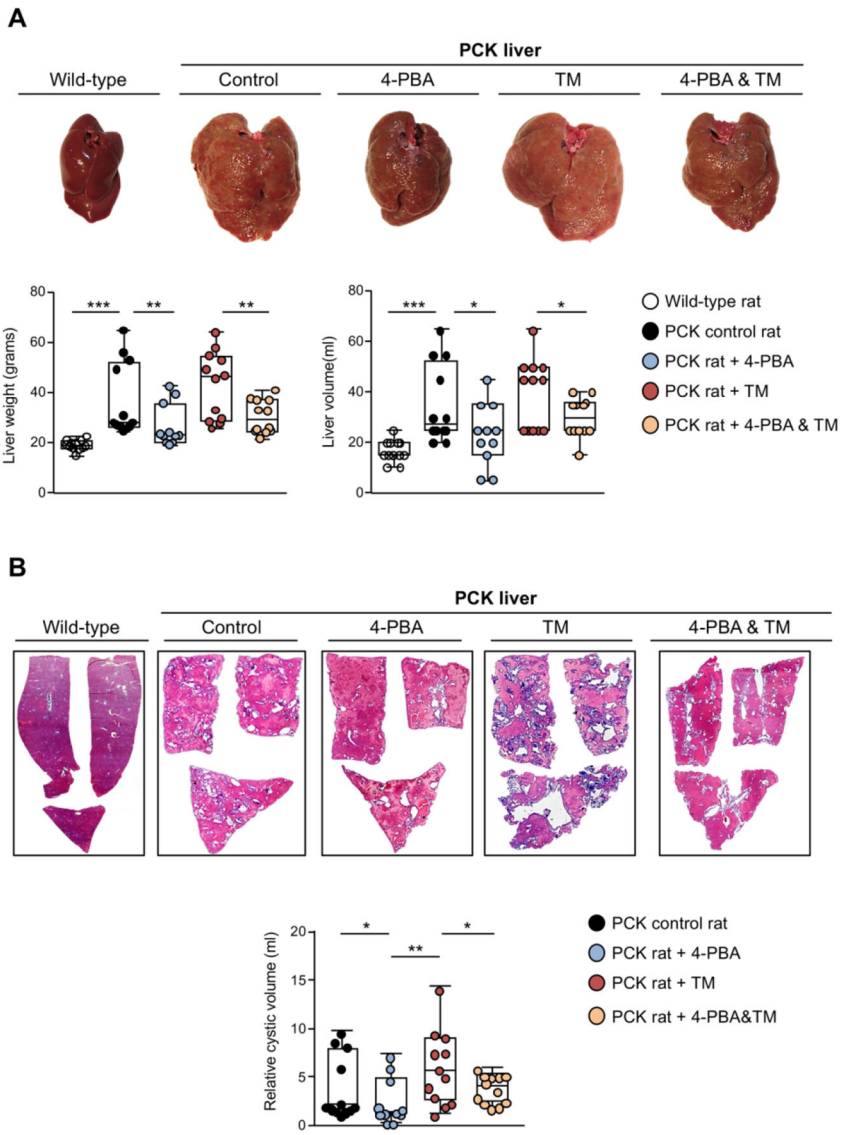
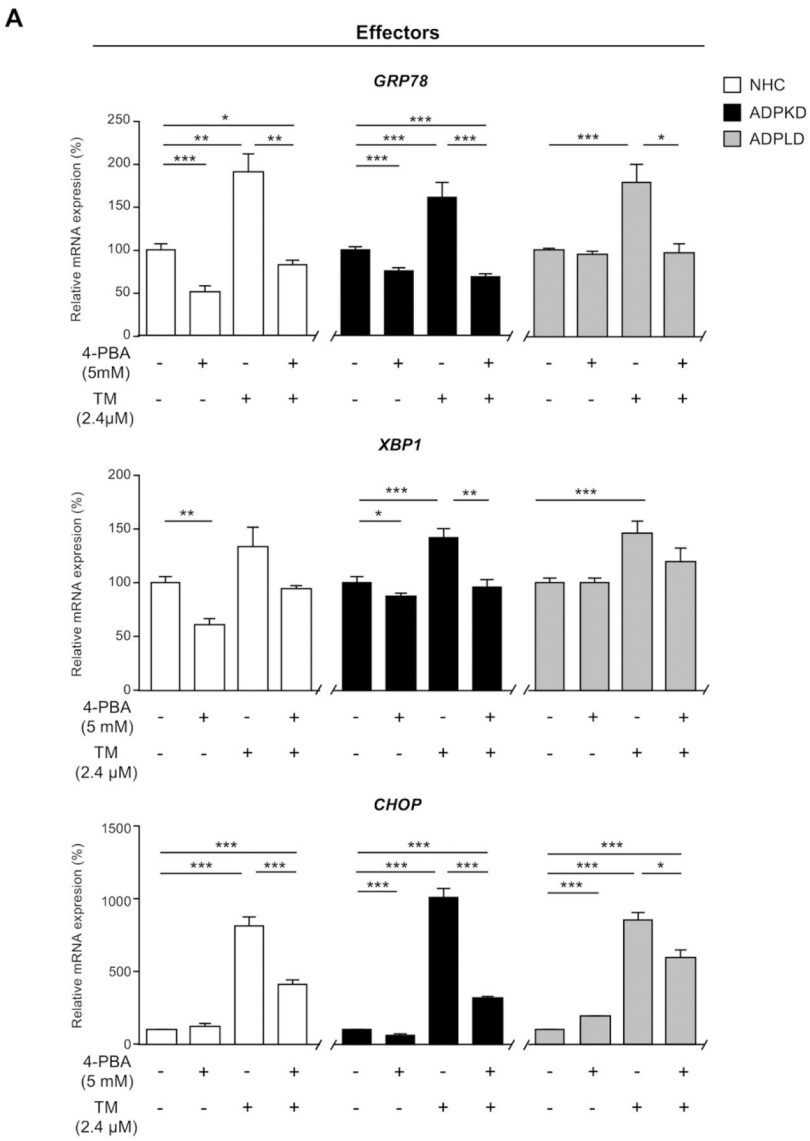
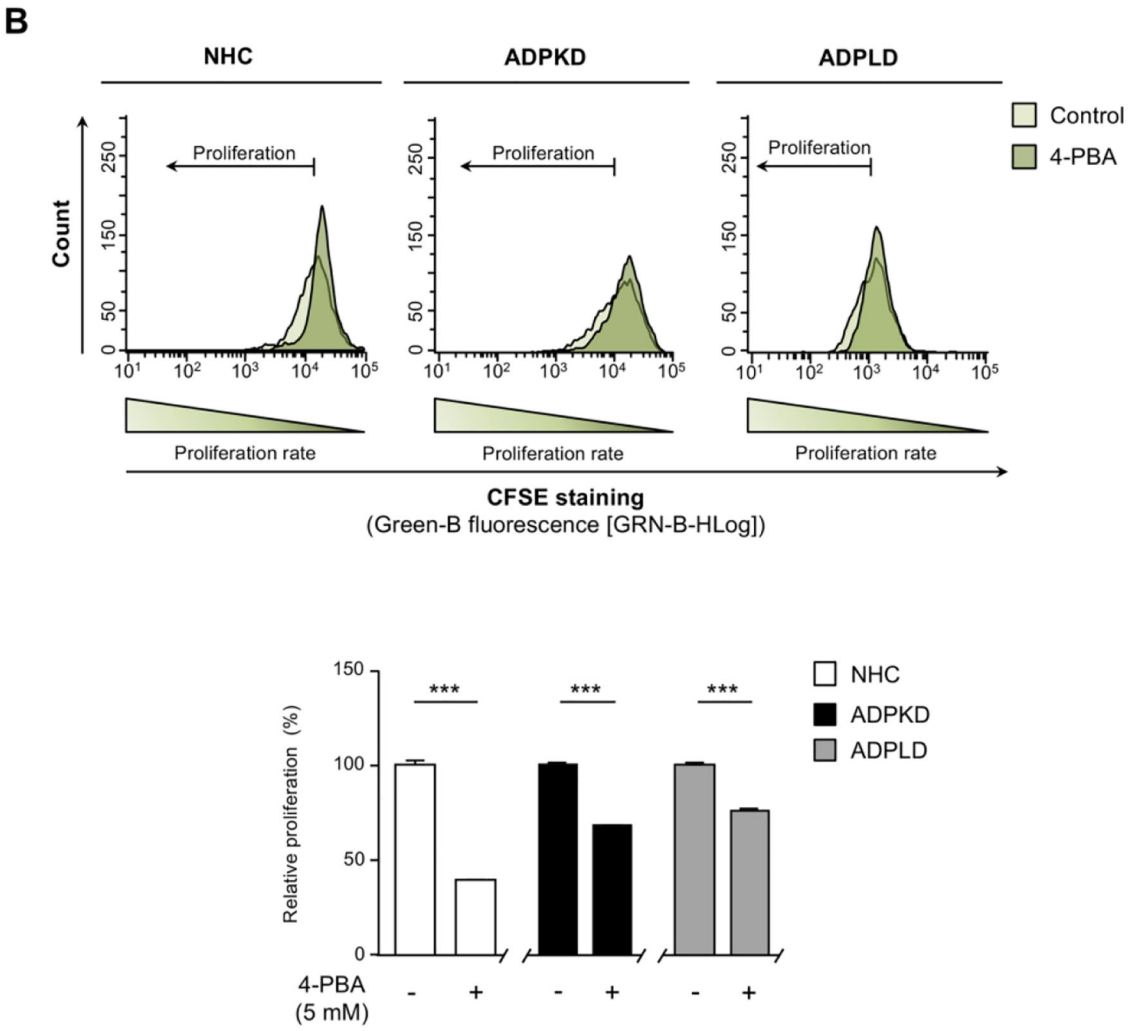
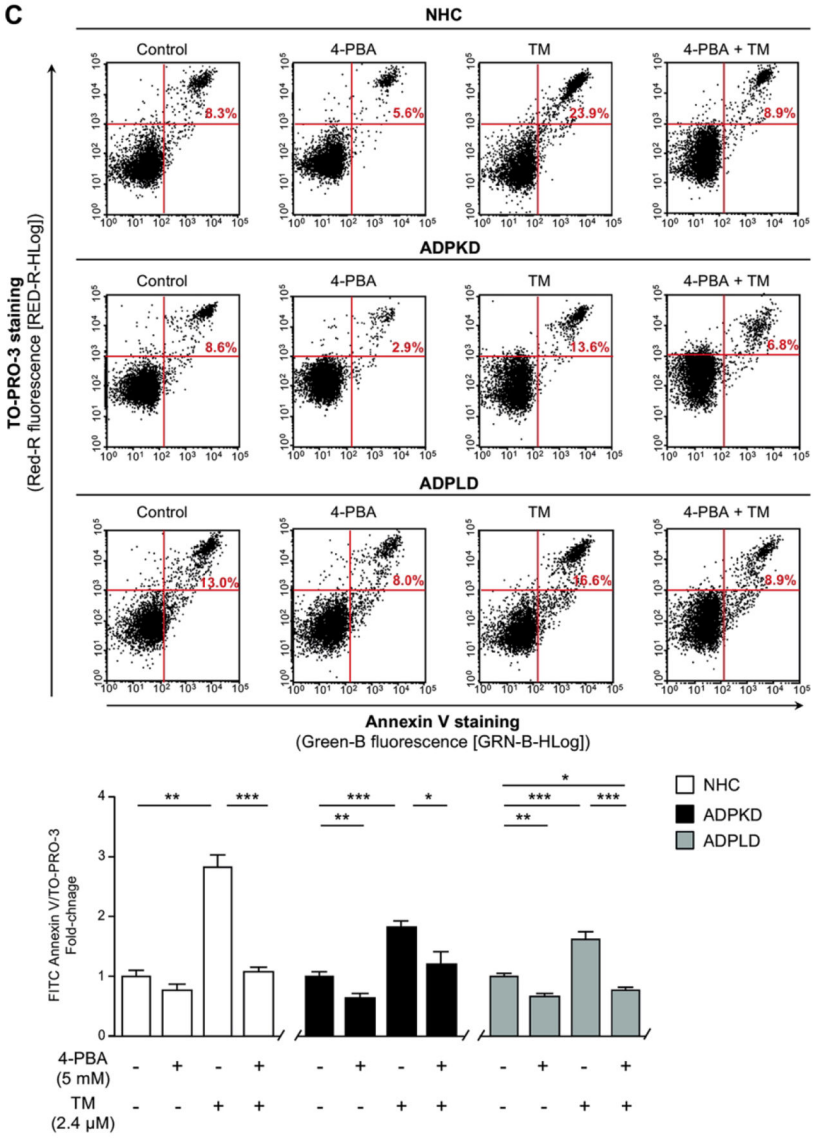


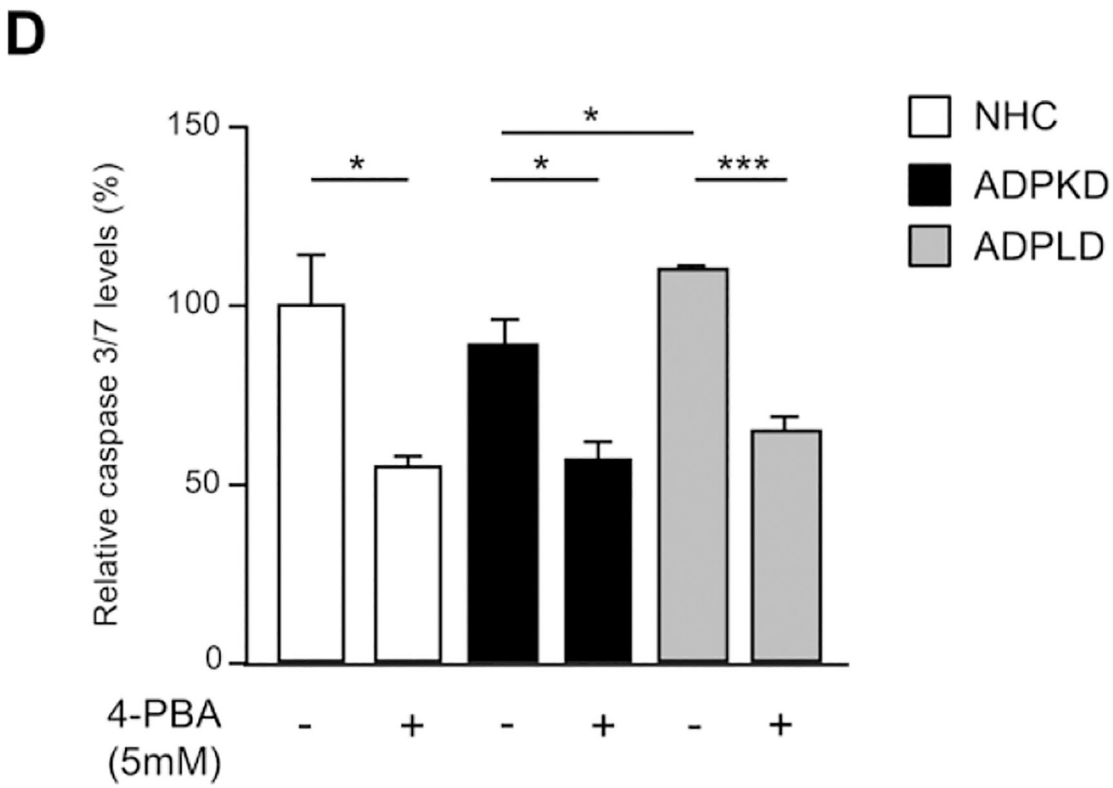
Figure 4. Effect of 4-PBA and/or TM on the liver of PCK rats.

(**A**) Representative macroscopic images of liver tissue and corresponding quantification of its total weight and volume in wild-type and PCK rats [control, 4-PBA (100 mM), TM (0.02 mg/kg) and 4-PBA&TM; n=12 per group]. (**B**) Representative histological images of liver sections stained with hematoxylin/eosin and corresponding measurement of the total cystic volume in the aforementioned *in vivo* experimental groups (n=12 per group).









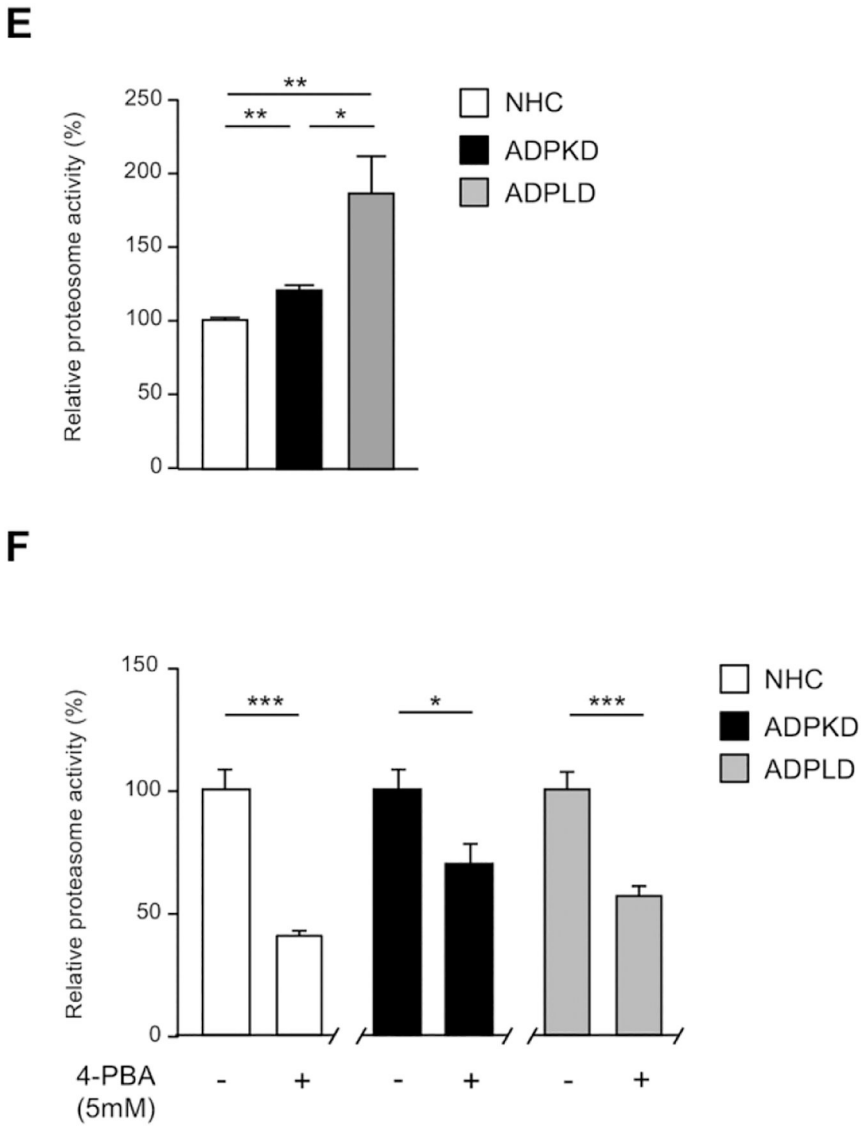
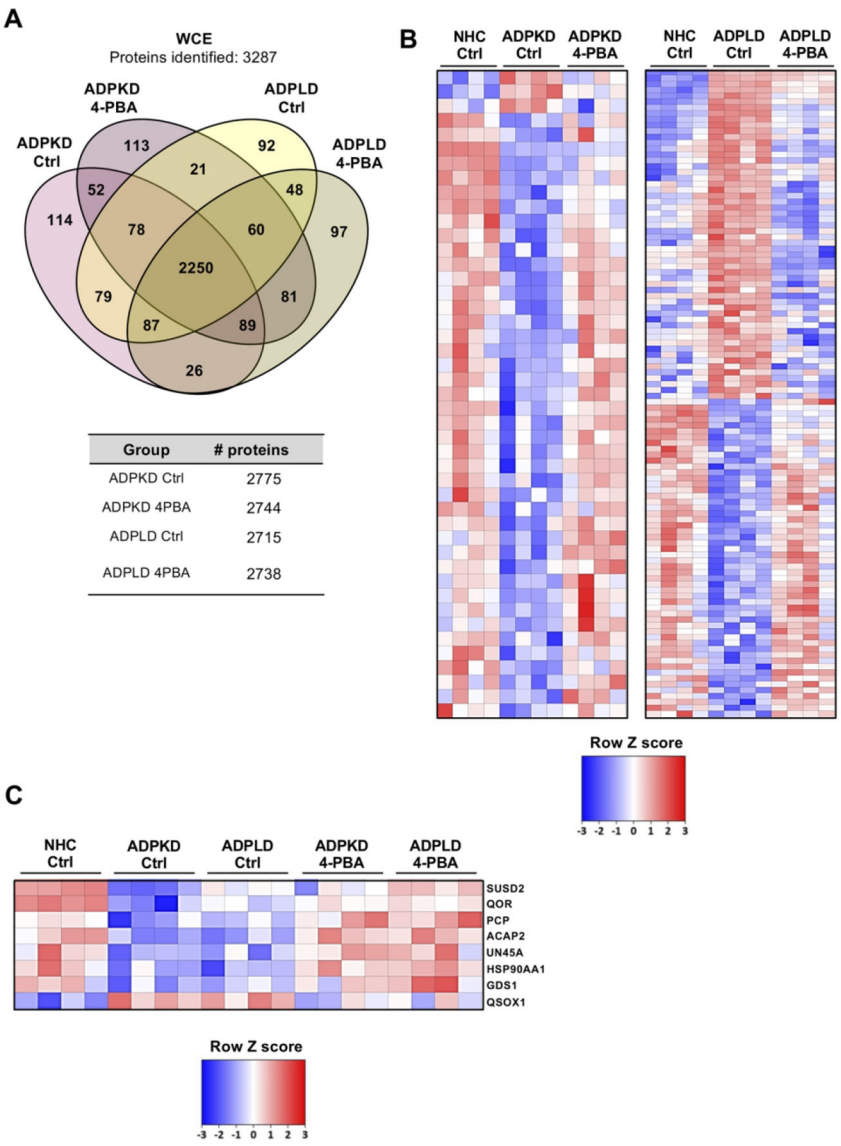


Figure 5. Effect of 4-PBA and/or TM on normal and cystic human cholangiocytes in culture.

(**A**) Transcriptional analysis of the UPR effectors in normal (NHC; n=5) and cystic [ADPKD and ADPLD; n=4–5] cholangiocytes after incubation with 4-PBA and/or TM. (**B**) Representative proliferation histograms and corresponding quantification in NHC, ADPKD and ADPLD (n=4) cholangiocytes incubated with 4-PBA or vehicle. (**C**) Representative scatterplots indicating the apoptotic rate of normal and cystic cholangiocytes (n=6) incubated with 4-PBA and/or TM. (**D**) Caspase 3/7 activity in NHC, ADPKD and ADPLD (n=4) cholangiocytes incubated with 4-PBA or vehicle. Relative 20S proteasome activity of NHC, ADPKD and ADPLD cholangiocytes (n=4–5) under (**E**) baseline and (**F**) 4-PBA incubation (n=6).



Group	# proteins
ADPKD Ctrl	2775
ADPKD 4PBA	2744
ADPLD Ctrl	2715
ADPLD 4PBA	2738

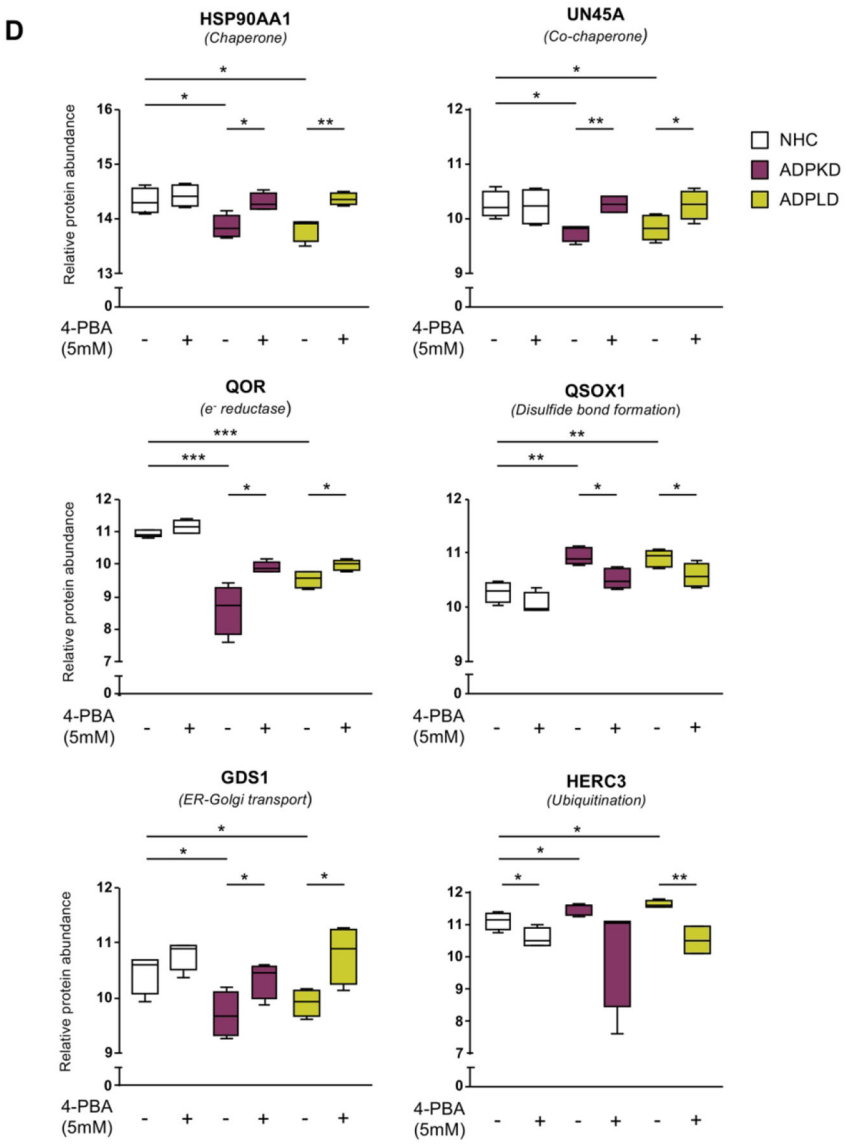
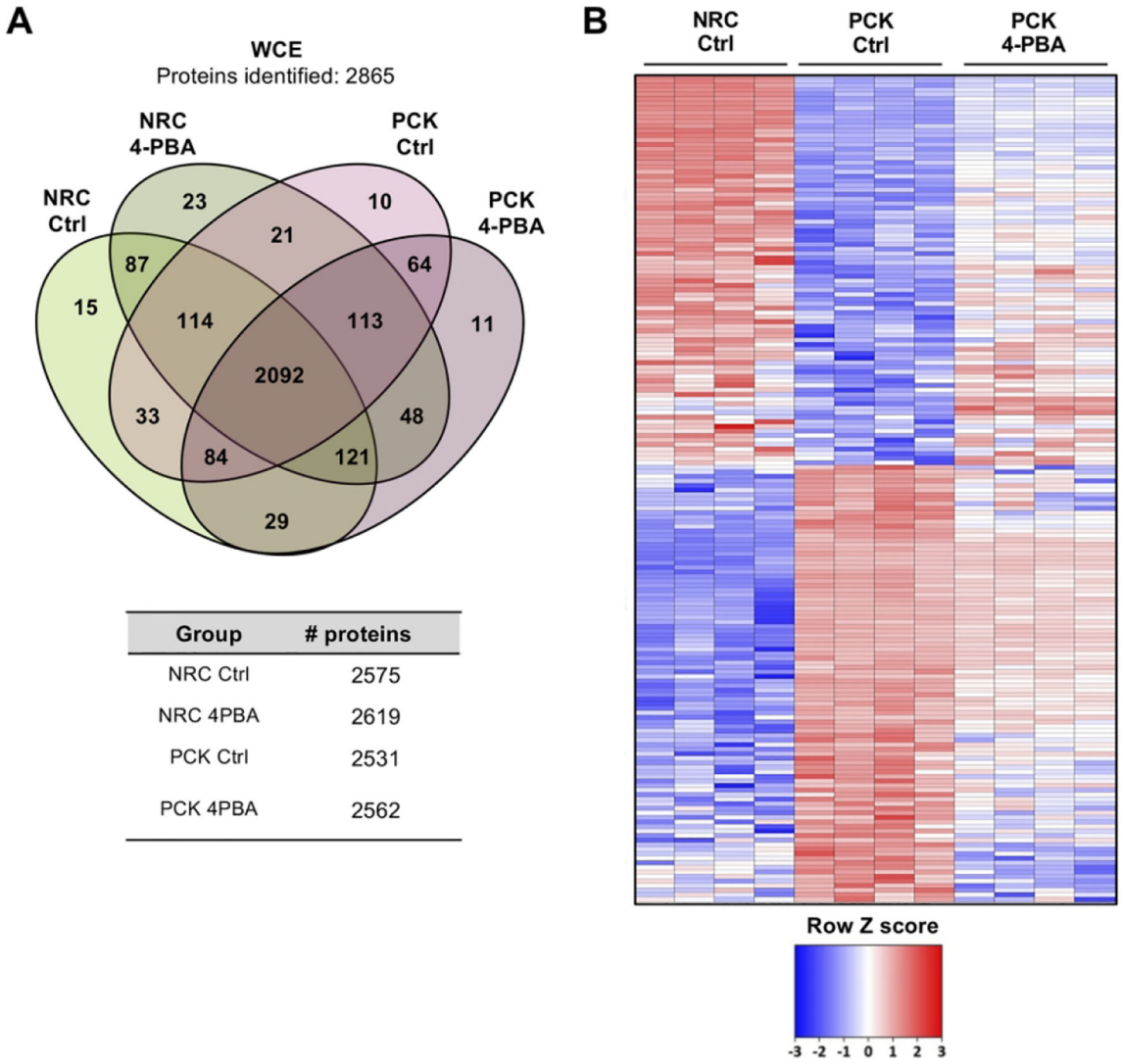


Figure 6. Effect of 4-PBA on the proteomic profiles of normal and cystic human cholangiocyte.

(**A**) Venn diagram depicting the number of proteins identified in cystic human cholangiocytes (ADPKD and ADPLD) under baseline and 4-PBA incubated conditions. (**B**) Heatmaps showing the effect of 4-PBA on the expression pattern of proteins dysregulated in ADPKD and ADPLD cholangiocytes compared to NHC. (**C**) Heatmap showing those proteins that were similarly dysregulated in ADPKD and ADPLD cholangiocytes and equally modulated by 4-PBA. (**D**) Box plot diagrams showing the modulatory effects of 4-PBA on the relative abundance of each selected protein in normal and cystic human cholangiocytes.



Group	# proteins
NRC Ctrl	2575
NRC 4PBA	2619
PCK Ctrl	2531
PCK 4PBA	2562

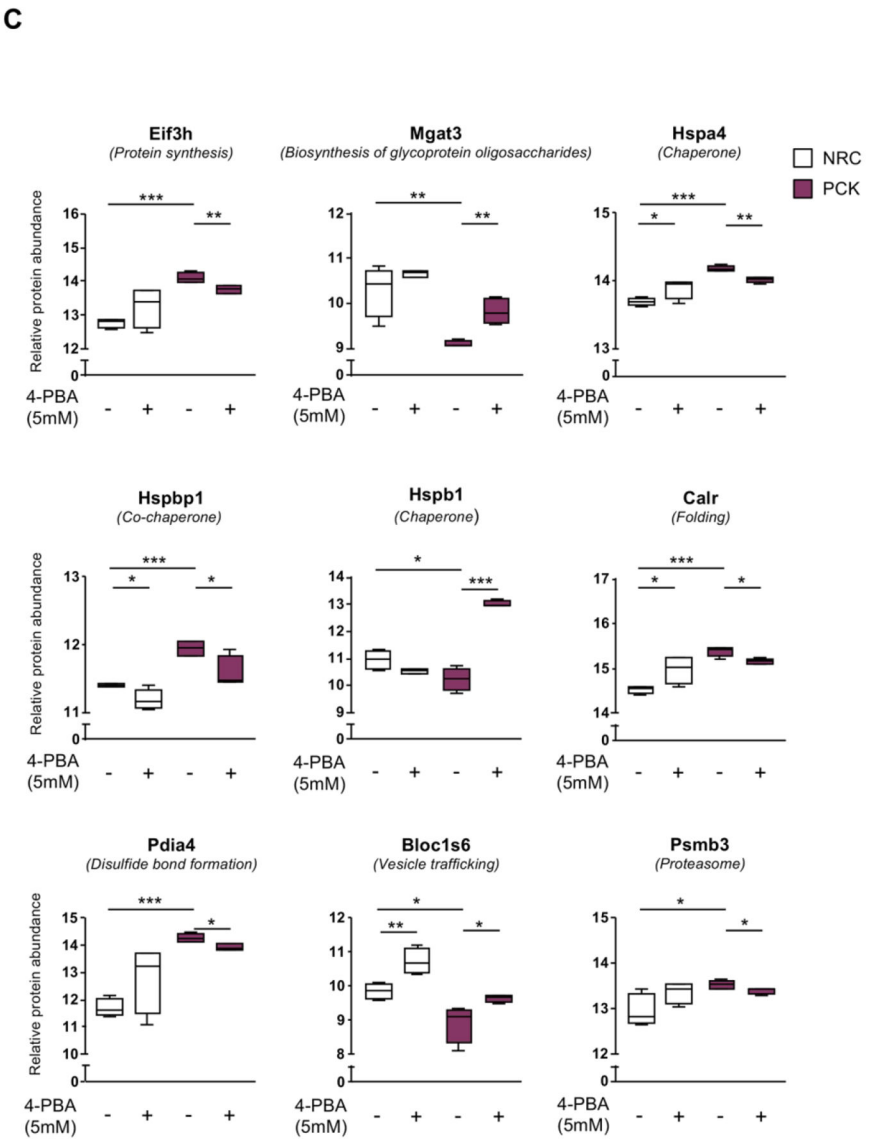


Figure 7. Effect of 4-PBA on the proteomic profiles of normal and cystic rat cholangiocyte.

(**A**) Venn diagram depicting the number of proteins identified in both normal and cystic rat cholangiocytes under baseline and 4-PBA incubated conditions. (**B**) Heatmap showing the effect of 4-PBA on the expression pattern of proteins significantly dysregulated in PCK compared to NRC cholangiocytes. (**C**) Box plot diagrams showing the modulatory effects of 4-PBA on the relative abundance of each selected protein in normal and cystic rat cholangiocytes.

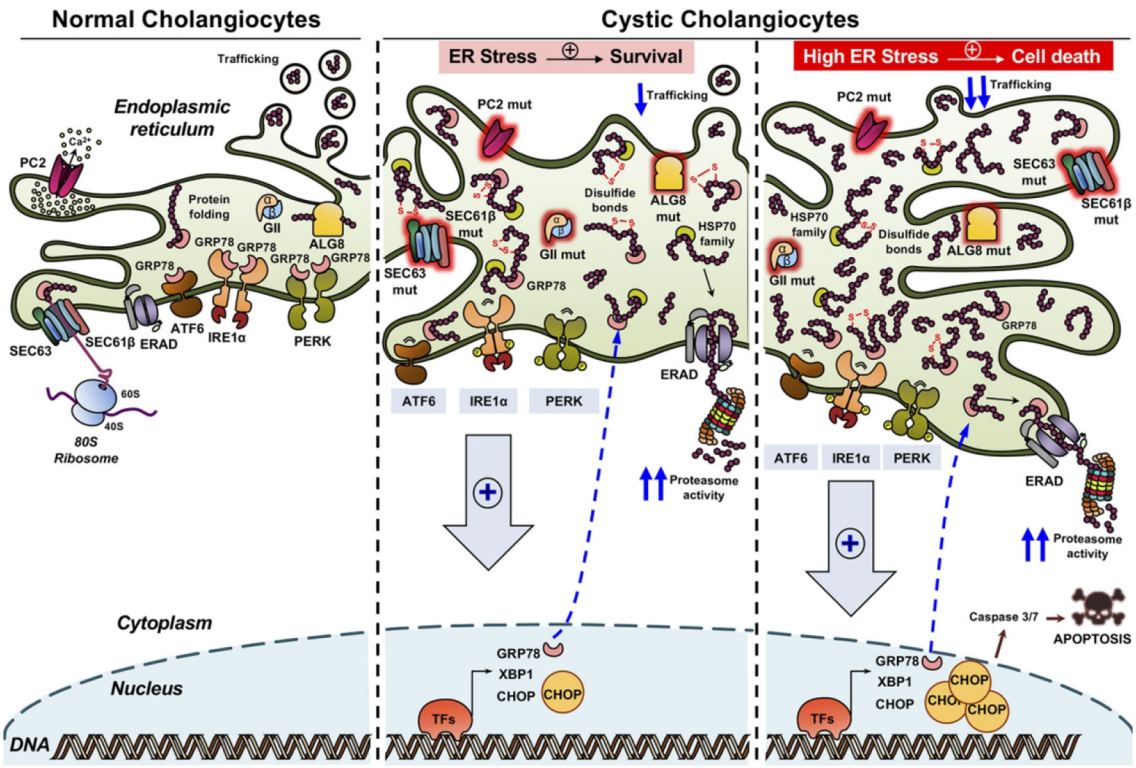


Figure 8. Working model.

Mutations in PLD-related genes that encode for ER-resident proteins (i.e., PC2, ALG8, SEC63, SEC61B, GIIa and GIIb) compromise the ER proteostasis and promote the accumulation of aberrant structurally nascent proteins within the ER lumen, leading to the induction of the UPR signaling cascades, enlargement of the ER lumen and hyperactivation of the cellular degradation mechanisms mediated by the UPS. When ER stress levels are low the three adaptive mechanisms are able to restore the proteostasis and resolve the ER stress reinforcing the survival and proliferation of cystic cholangiocytes. Nevertheless, if the ER stress becomes chronic and excessive the adaptive mechanism fails to restore ER proteostasis, inducing cholangiocyte apoptosis by CHOP and caspase 3/7 activation.