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Histone Deacetylase 4 promotes cholestatic liver injury in the absence of Prohibitin-1

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Abstract

Prohibitin 1 (PHB1) is an evolutionary conserved pleiotropic protein that participates in diverse processes depending on its subcellular localization and interactome. Recent data have indicated a diverse role for PHB1 in the pathogenesis of obesity, cancer and inflammatory bowel disease, among others. Data presented here suggest that PHB1 is also linked to cholestatic liver disease.

PHB1 expression is markedly reduced in patients with primary billiary cirrhosis and biliary atresia and Alagille syndrome, two major pediatric cholestatic conditions. In the experimental model of bile duct ligation, silencing of PHB1 induced liver fibrosis, reduced animal survival and induced bile duct proliferation. Importantly, the modulatory effect of PHB1 is not dependent on its known mitochondrial function. Importantly, d PHB1 interacts with Histone Deacetylase 4 (HDAC4) in the presence of bile acids. Hence, PHB1 depletion leads to increased nuclear HDAC4 content and its associated epigenetic changes. Remarkably, HDAC4 silencing and the administration of the HDAC inhibitor parthenolide during obstructive cholestasis *in vivo* promote genomic reprogramming leading to the regression of the fibrotic phenotype in the liver-specific *Phb1* KO mice.

Conclusion—Our data identify PHB1 as an important mediator of cholestatic liver injury regulating the activity of HDAC4, which controls specific epigenetic marks. These results identify potential novel strategies to treat liver injury and fibrosis, particularly as a consequence of chronic cholestasis.

Keywords

Prohibitin1; HDAC4; cholestasis; fibrosis

INTRODUCTION

Prohibitins (PHBs) comprise an evolutionary conserved and ubiquitously expressed family of proteins with a variety of suggested activities in different cellular compartments[1]. Two homologous PHB proteins, PHB1 and PHB2, form a large multimeric complex predominantly found in the inner mitochondrial membrane where it exerts a chaperone-like function to stabilize newly synthesized mitochondrial proteins[2] and maintains the organization and stability of mitochondrial nucleoids[3]. PHBs are essential for mitochondrial biogenesis in yeast[4] and mammals[5], in part by regulating the stability of optic atrophy 1 (OPA1), a mitochondrial fusion regulating protein[6]. Although PHBs are best known for their role in mitochondria, recent reports indicate that they are involved in other multiple cellular pathways due to their subcellular localization. PHB1 is found in the nucleus, where it interacts with Rb and p53 among other proteins to induce a change in the transcriptional activity of E2F[7] and p53[8,9]. Moreover, its transcriptional repression requires histone-deacetylase (HDAC) activity. Indeed, it has been previously described that PHB1 inhibits E2F activity through a mechanism that involves HDAC1 and co-repressors such as NCoR1[7].

These nuclear effects have been associated with the inhibition of cell cycle progression[9] and the induction of apoptosis[7].

Given the implication of PHB1 in many vital functions, we generated liver-specific *Phb1* knockout (*Phb1* KO) mice[10], which developed liver fibrosis spontaneously. In humans, reduced levels of PHB1 have been observed in obese people at risk of developing non-alcoholic steatohepatitis[11]. Here we describe that PHB1 expression is markedly reduced in common human cholestatic liver diseases and provide the molecular basis underlying the PHB1's protective role in the liver.

Our present studies reveal that a mitochondrial-independent mechanism contributes to the fibrotic phenotype associated with the absence of PHB1. The apoptotic response and the alteration of the gene profile associated to bile acid metabolism in the absence of PHB1 were dependent on an over-stimulation/representation of HDAC4. Notably, we provide evidence that dynamic regulation of acetylation/deacetylation by HDAC4 plays a key role in determining the PHB1-dependent liver injury. Parthenolide treatment leads to a switch in the balance of ubiquitin-dependent protein homeostasis, which consistently reduces the levels of HDAC4 resulting in a regression of the fibrotic phenotype in *Phb1* KO mice. Importantly, similar effects are detected when silencing HDAC4 during obstructive cholestasis in bile duct ligated *Phb1* KO mice.

These results determine that in PHB1 absent cholestatic liver disease, HDAC4 emerges as a new druggable target for liver injury.

MATERIALS AND METHODS

Human samples

PBC liver samples were obtained from the Biodonostia Health Research Institute-Donostia University Hospital and from the Hospital Clinic in Barcelona. Each patient signed an informed consent document. PBC liver samples were obtained by percutaneous biopsy in 7 patients. The diagnosis of PBC was established by liver biopsy with characteristic features of the disease and presence of anti-mitochondrial antibodies. Liver samples from children with biliary atresia (n=9), Alagille syndrome (n=9), and liver disease associated with total parenteral nutrition (n=9) were obtained at the time of transplantation. The diagnosis of biliary atresia was made on clinical, laboratory, radiological, and histopathological findings. Heterozygous mutations in *JAG1* gene were demonstrated in all children with Alagille syndrome. The age of the patients ranged from 22 months to 5 years at the time of transplantation. For the PHB1 mRNA expression analysis in alcoholic and viral cirrhosis we used samples provided by Dr. Erica Villa, from 47 patients with liver cirrhosis detected during surveillance They had preserved liver function and corresponded to BCLC stage A (n=34) and B (n=13)[12]. Healthy human liver was used as control for immunostaining and qPCR analyses. Patients or patient parents gave informed consent to all clinical investigations, in according to the principles embodied in the Declaration of Helsinki.

Experimental procedures in animals

Phb1 KO animals were generated as described[10]. Males from 8–12 weeks of age were treated in accordance with the Spanish Guide for the Care and Use of Laboratory Animals. Parthenolide was intraperitoneally injected at a dose of 3mg/kg 24h and 1h before bile duct ligation (BDL) or twice a week during two weeks. Liver specimens were snap-frozen for subsequent analysis.

Isolation of biliary trees

C57BL6 WT and *Phb1* KO mice were subjected to bile duct ligation and after 7 days biliary trees were isolated via collagenase perfusion of the liver[13].

In vivo silencing

For PHB1 and OPA1 silencing BDL was performed in 12-week-old WT mice and 3 days later they received via tail vein injection, either 200 μl of a 0.75 μg/μl solution of PHB1, OPA1 specific shRNA or control shRNA (pSM2c Open Biosystems). Animals were sacrificed 14 days after BDL. For the HDAC4 silencing, 12-week old *Phb1* KO mice received via tail vein injection, 1 day before and 2 days after the BDL, 100 μl of a 25 μM solution of HDAC4 or control siRNA.

RESULTS

Alteration of PHB1 levels is associated with cholestatic liver disease

We have previously reported that PHB1 protein is frequently down-regulated in obese people at risk of developing non-alcoholic steatohepatitis[11]. Indeed, liver-specific PHB1 knockout (*Phb1* KO) mice develop fibrosis spontaneously, with abnormal ductular proliferation[10]. However, it is unknown if PHB1 levels confer sensitivity to liver cholestatic injury. We assessed the levels of PHB1 in livers from patients with primary biliary cirrhosis (PBC), one of the most common cholestatic liver diseases in the adult population and in two representative severe pediatric cholestatic disorders as biliary atresia and Alagille syndrome[14]. Remarkably, the levels of PHB1 were significantly reduced in these pathologies in comparison to normal healthy livers (Fig. 1A, B). We also measured the levels of PHB1 in samples from children with end-stage liver disease associated with total parenteral nutrition and from a prospective study of patients with liver cirrhosis at mRNA level. In all these conditions, PHB1 gene expression was comparable to that of healthy livers (Fig. 1B, C). Interestingly, liver biopsy specimens from patients with alcohol and viral cirrhosis displayed higher PHB1 protein levels than normal liver (Fig. 1D). Finally, we observed that PHB1 expression decreased at protein and mRNA levels in wild type (WT) mice subjected to bile duct ligation (BDL), a well-established experimental model of obstructive cholestasis leading to fibrosis (Fig. 1E, F). Overall, these data strongly support a role of PHB1 during cholestatic liver injury.

PHB1 deficiency predisposes to liver injury

Cholestasis is the read out of a bile duct damage caused by the accumulation of toxic hydrophobic bile acids. In response to repeated injury, both biliary epithelial cells (BECs)

and hepatocytes loose their balance to evoke inflammation and collagen deposition, which further damage the liver and lead to fibrosis. To examine the potential role of PHB1 in a cholestatic liver we adopted the BDL animal model, an experimental model of human obstructive cholestatic liver disease[14]. We have previously reported that *Phb1* KO mice develop spontaneously liver injury[10]. As expected, the response of *Phb1* KO mice to BDL was more deleterious than WT animals. After 3 days, the survival of *Phb1* KO mice was reduced to 45% (Suppl Fig. 1A). Moreover, features such as F4/80 immunostaining, necrosis and collagen-1 liver expression 3 and 7 days post-BDL and the activation of JNK in *Phb1* KO mice revealed more severe liver damage (Suppl Fig. 1B, C). Indeed, *Phb1* KO mice 3 and 7 days post-BDL had a strong regulation in the gene profile associated to an inflammatory response as showed by TNFα, TNFR2 and IL-6 levels, while markers related to proliferation like HGF decreased, reflecting an impairment also in the regenerative response (Suppl. Fig. 1D). Furthermore, the liver injury associated to the absence of PHB1 implied a dysregulation in bile acid metabolism with the down-regulation of FXR, as well as increased expression of CYP7A1, the rate-limiting enzyme of bile acids synthesis and in proapototic markers such as Bax (Suppl. Fig. 1D).

In order to sort out whether the liver damage observed in *Phb1* KO mice was due to the chronic absence of the gene, we transiently down-regulated PHB1 by i.v injection of shRNAs in WT mice during BDL (Suppl Fig 1E). Lowering PHB1 expression resulted in decreased survival and higher levels of caspase 3 and JNK activity (Fig. 2A–C). We also observed more necrotic areas and collagen deposition, higher CK19, αSMA, and F4/80 immunostaining compared to control animals (Fig. 2D) and increased ALT and AST activities (Fig. 2E). Therefore, the decrease of PHB1 levels after BDL induced a more aggressive fibrotic phenotype linked to an inflammatory and ductular response. PHB1 is important for mitochondrial function predominantly through the regulation of OPA1 stability[6]. We therefore assessed whether the deleterious effect observed in *Phb1* KO mice during BDL was due to the regulation of OPA1. Specific reduction of OPA1 by i.v injection of shRNAs in WT mice did not alter mortality rates and did not show worsening of BDL-induced liver injury (Suppl Fig 1E, Fig. 1A). Indeed, caspase 3 activity (Fig. 2B), JNK activation (Fig. 2C), the presence of necrotic areas, collagen, the staining for αSMA, CK19 and F4/80 (Fig. 2D) and the activities of ALT and AST (Fig. 2E) were equal or even lower in OPA1-silenced animals as compared to controls.

Consistent with the *in vivo* results, increased JNK activity (Suppl. Fig. 1F) correlated with a major apoptotic response in *Phb1* KO and silenced PHB1 hepatocytes after deoxycholic acid (DCA) treatment (Suppl Fig 2A left panel), while OPA1 silencing had the opposite effect (Suppl Fig 2A right panel).

Thus, the effect of PHB1 knockdown in the liver was not mediated through the down-regulation of OPA1 suggesting an independent role of its well-known chaperone activity. Although other functions of PHB1 related to mitochondrial dysfunction cannot be excluded.

PHB1 deficiency modulates cholangiocytes activation after BDL

The reduction of Phb1 levels in cholestatic liver injury and the increased sensitivity of *Phb1* KO to BDL, prompted us to evaluate the behavior of their biliary epithelial cells (BECs).

For that purposed we harvested livers in *Phb1* KO mice and in WT animals at day 7 post-BDL, which corresponds to the peak of ductular proliferation[15]. The RNA was extracted from the 'biliary tree' fraction, in which the bile ducts are separated from the hepatocytes by perfusion digestion[13]. First, we found no contamination of the purified biliary trees with hepatocytes through the evaluation of albumin levels. Importantly, PHB1 levels in BECS from AlbCre *Phb1* KO and WT mice remained unaltered. We detected the enrichment of markers related to a fibrotic phenotype and activation of cholangiocytes as represented by higher levels of CK19, Col1A1 and αSMA in those BECs derived from AlbCre *Phb1* KO mice in comparison to the control mice. Indeed, biliary trees derived from *Phb1* KO showed significantly higher levels of TNFα, an indicator of inflammation and genes related to regenerative and proliferative activities such as cyclin D1, as well as Epcam and VEGF (Fig 2F). These data underscore that the absence of Phb1 in the hepatocytes result in a ductular proliferation leading to a fibrotic phenotype.

PHB1 regulates the expression of HDAC4

It has been shown that regulatory changes in histone acetylation rates occur in the absence of PHB1[16]. Moreover, a strong implication has been identified between HDAC activity and liver fibrosis[17]. Therefore, we analyzed the regulatory pattern of HDACs associated to PHB1 levels. As we mentioned before, the levels of PHB1 decrease significantly after 7 days of BDL in WT mice and in human cholestatic patients (Fig 1A and E). We evaluated the levels of HDAC 1 to 6 and importantly (data not shown), we found that only HDAC4 protein is overexpressed basally in *Phb1* KO livers and hepatocytes, and in livers after 3 and 7 days of BDL (Fig. 3A). This upregulation was also detected at mRNA levels after BDL (Fig. 3B). Moreover, we observed a significant increase of HDAC4 at mRNA level in the i.v PHB1 silenced WT mice 14 days after BDL (Suppl. Fig. 2B).

Interestingly, we observe that the downregulation of PHB1 after 7 days of BDL correlates with higher nuclear HDAC4 localization in WT mice. Also, specifically a nuclear HDAC4 localization was increased in *Phb1* KO (Fig. 3A, 3C, Suppl. Fig. 2C). Altogether, our data suggest that PHB1 could play a role in HDAC4 subcellular localization. No changes in global H3 acetylation levels were detected in *Phb1* KO livers compared to WT animals (Suppl. Fig. 2D). To examine if this shift in HDAC4 compartmentalization and therefore its activity could be mediated by PHB1, we performed coimmunoprecipitation studies. We found a slightly interaction between PHB1 and HDAC4 in WT hepatocytes (Fig. 3D). Importantly, this interaction was enhanced by DCA. These data support the hypothesis that in the livers where PHB1 is absent, an aberrant transcriptional regulation could be mediated through the hyperactivation of HDAC4. Interestingly, immunohistochemical analysis revealed a significant increase of HDAC4 expression in patients with initial stages of PBC where PHB1 is also found down-regulated (Fig. 3E).

HDAC inhibition modulates the apoptotic response in *Phb1* KO hepatocytes

To evaluate if the overactivation of HDACs is part of the mechanism that mediates liver injury in the absence of PHB1 the pan inhibitor of HDAC activity, trichostatin A (TSA) was tested in the presence of bile acids. Interestingly, TSA reduced *Phb1* KO primary hepatocytes sensibility to DCA apoptotic stimuli analyzed by caspase 3 activity (Suppl Fig.

3A). These data support that a hyper-activation of HDAC could participate in the pathological phenotype associated to PHB1 ablation.

In order to discriminate the impact that HDAC class I and II could play in the *Phb1* KO hepatocytes injury, we employed different inhibitors (rocilinostat, PCI34051, mocetinostat, parthenolide and apicidin) and measured the apoptotic response in the presence of DCA. Only parthenolide, the HDAC inhibitor with anti-inflammatory features[18], displayed a potent anti-apoptotic effect in *Phb1* KO hepatocytes (Suppl Fig. 3A). We reasoned that the effect of TSA and parthenolide in *Phb1* KO hepatocytes might result, among other effects, in a proper regulation of the gene expression profile associated with bile acid metabolism and inflammatory response that is essential to preserve liver homeostasis. Indeed, TSA and parthenolide-treated hepatocytes showed increased levels of FXR, and reduced levels of CYP7A1, HDAC4, TNFα, TRAIL and Bax suggesting a less toxic effect of bile acids as a results of specific HDAC inhibition, resulting in the attenuation of the *Phb1* KO hepatocytes apoptotic response (Suppl Fig. 3B).

Parthenolide reduces liver damage after BDL in *Phb1 KO* mice

Importantly, parthenolide exerted a protective effect from the liver injury after BDL in *Phb1* KO mice. Indeed, parthenolide treatment resulted in a reduction of the mortality rate of this mice after BDL (Fig. 4A) associated with a lower apoptotic response as revealed by a reduction of necrotic areas, Tunel-staining (Fig. 4B), as well as decreased ALT (8431±957 vs. 4225±210 U/L) and AST (4805±300 vs. 2242±438 U/L) activities compared to control *Phb1* KO mice. Additionally, markers of fibrosis like αSMA (Fig. 4B) and the proinflammatory cytokines TNFα, and IL-6 as well as TNFR2 (Fig. 4C) were reduced in the presence of the drug in *Phb1* KO mice treated with parthenolide. Importantly, low levels of HDAC4 mRNA were identified under the drug treatment (Fig 4C). Indeed, the protein levels of TNFα in the livers were reduced in the presence of the HDAC inhibitor (Fig. 4D). Interestingly, parthenolide restored FXR and CYP7A1 levels. (Fig. 4C).

Parthenolide has been reported as an inductor of HDAC1 degradation by proteasome activity through the action of Mdm2[19]. While no changes were detected in HDAC1 (data not shown), parthenolide treatment resulted in the decrease of HDAC4 protein levels (Fig. 4E and Suppl. Fig. 3C), which we speculated could be due to proteasomal degradation. Indeed, we found that 26S proteasome activity decreased after BDL in *Phb1* KO mice, and that this activity was restored with parthenolide treatment (Suppl Fig. 3D). Moreover, the amount of ubiquitinated proteins was significantly higher after BDL in *Phb1* KO mice, and, in contrast, parthenolide prevented this accumulation (Suppl Fig. 3E). Certainly, the use of tandem ubiquitin-binding entities (TUBEs)[20] showed lower levels of ubiquitinated hepatic HDAC4 protein after BDL in the presence of parthenolide (Suppl. Fig. 3F). Overall, these data suggest that parthenolide restored protein homeostasis of HDAC4, which significantly attenuated liver injury in *Phb1* KO mice. Importantly, keeping the levels of HDAC4 elevated through its overexpression in *Phb1* KO hepatocytes under DCA treatment counteracts the reduction in the apoptotic response detected in the presence of parthenolide (Fig. 4F). Therefore, this result highlighted the importance of HDAC4 levels in the effect mediated by parthenolide treatment.

Of particular importance was the successful attenuation of the spontaneously developed fibrosis, characteristic of *Phb1* KO mice, after 15 days of parthenolide treatment. We detected lower AST activity (268±68 vs. 63±16 U/L) and marked reduction in Smad2/3 and F4/80 protein levels in parthenolide-treated *Phb1* KO livers (Suppl. Fig. 4A). Moreover, mRNA levels of profibrogenic markers like TGFβ and αSMA were decreased in the presence of this drug. Additionally, the levels of FXR increased while those of CYP7A1 diminished (Fig. 5A). Importantly, the protein levels of the inflammatory cytokine TNFα were also reduced (Fig. 5B).

Alternatively, parthenolide induced the hepatic 26S proteasome activity (Suppl. Fig. 4B). Indeed, this trigger of the proteasome degradation was correlated with a remarkable reduction of HDAC4 protein levels by IHC and Western blot analysis in the liver animals under treatment, with no changes at mRNA level (Fig 5A, C). Additionally, global levels of ubiquitin were also lower (Suppl. Fig. 4C). The use of ubiquitin-traps revealed the formation of ubiquitin chains on HDAC4 in the *Phb1* KO mice that demonstrated the defective proteasome-mediated degradation in these animals, which was corrected upon parthenolide treatment (Suppl Fig 4D). Our results propose a direct effect of parthenolide on protein ubiquitination and therefore protein homeostasis in liver fibrosis. Importantly HDAC4 was identified as a specific target of this drug.

HDAC4 silencing attenuates liver damage after BDL in *Phb1* KO mice

Finally, to confirm that the increase of HDAC4 expression contributed to the liver fibrosis observed in the liver-specific *Phb1* KO, we silenced HDAC4 with siRNAs in *Phb1* KO hepatocytes and evaluated the response to bile acids exposure. Notably, HDAC4 silencing reduced significantly the apoptotic response induced by DCA analyzed by caspase 3 activity (Suppl. Fig. 5A). We further validated this *in vivo* by i.v injection of siHDAC4 in *Phb1* KO mice after BDL, which revealed a decreased in the liver damage as shown by H&E, Sirius red, CK19 and F4/80 staining (Fig. 6A) and by the significant reduction of bilirubin levels from 24±2 to 12±5 mg/dl. Importantly, we observed a decrease in JNK activation both *in vitro* and *in vivo* (Fig. 6B and Suppl. Fig. 5B), together with a deeply down-regulation of genes related to the inflammatory response like TNFα, TNFR2, CXCL1 and CCL2, apoptotic genes like Bax and those linked to bile acids metabolism, with the exception of FXR, which was markedly induced (Fig. 6C and Suppl. Fig. 5C). These data support the implication of HDAC4 during cholestatic liver damage in the context of low levels of PHB1.

DISCUSSION

Recent data have indicated a diverse role for PHB1 in the pathogenesis of diseases such as obesity, cancer and inflammatory bowel disease, among others[11]. Data presented here suggest that PHB1 is also linked to cholestatic liver disease. Thus, reduced PHB1 expression is observed in representative cholestatic disorders that manifest in adulthood or infancy, such as PBC, biliary atresia and Alagille syndrome. This effect can hardly be attributed to the cirrhotic state of the liver, as no changes in PHB1 mRNA levels were detected in patients with alcoholic or viral cirrhosis. Reinforcing these data, we have identified a down-regulation of PHB1 at mRNA and protein levels after the model of obstructive cholestasis

such as BDL. Importantly, the transient silencing of PHB1 in mice has also a harmful effect in the liver after BDL, as well as in hepatocytes treated with bile acids. Altogether, these data identify a new link between PHB1 and cholestatic liver injury paving the way for new therapeutic approaches.

Cholestatic liver injury is characterized by mitochondrial dysfunction and oxidative stress. Although disturbances in the bile acid balance induces apoptosis in hepatocytes through the generation of reactive oxygen species and mitochondrial impairment with the release of cytochrome C, we propose that this is not the main function exerted by PHB1 during cholestatic liver injury. Among other findings, we show that OPA1 silencing upon BDL has the opposite outcome than the profibrotic effect exerted in the absence of PHB1. The dynamin-like GTPase OPA1 is a major organizer of the mitochondrial inner membrane and a repressor of cellular apoptosis through the sequestration of cytochrome C and the regulation of the cell cycle[9]. These data suggest that liver injury linked to the absence of PHB1 is not totally dependent of its well-known chaperone role of OPA1 in the mitochondria and other functions of PHB1 related to mitochondrial dysfunction cannot be excluded.

Exogenous expression of PHB1 in intestinal epithelial cells reduced the inflammatory responses and regulated the transcription of multiple cytokines[21]. Consistently, we have shown that the absence of PHB1 in the liver after BDL resulted in the up-regulation of inflammatory markers, while genes related with bile acid metabolism were deeply regulated in the *Phb1* KO versus WT animals as shown by the down-regulation of FXR a central regulator of bile acid synthesis and CYP7A1. It has been previously reported that FXR-mediated gene expression is suppressed during hepatic inflammation[22]. TNFα is a key cytokine that plays a central role during liver fibrosis orchestrating an inflammatory crosstalk between hepatocytes and hepatic immune cells[23]. Indeed, TNFα levels were also increased not only in the liver but also in *Phb1* KO biliary tree after BDL versus control mice. Proliferating cholangiocytes, typical of obstructive cholestasis, are more sensitive to TNFα-mediated cell injury[24]. Likewise, we have identified an increase in the expression of pro-fibrogenic markers in the biliary tree derived from *Phb1* KO mice and a profuse ductular reaction in those animals compared to control mice.

The different roles proposed for prohibitin proteins are attributed to their subcellular localization and interactome[1]. At the subcellular level, PHB1 has been reported in mitochondrial membranes as well as the nucleus and cytoplasm. PHB1 can interact with Nrf2 and behave as a co-activator of ARE[25]. Additionally, PHB1 could inhibit E2F activity through a mechanism that involves HDAC1 and co-repressors such as NCoR1. HDAC4 expression is regulated by multiple mechanisms including transcriptional regulation. TNFα has been identified as one of the cytokines that regulate HDAC4 levels and interestingly, the levels of TNFα and HDAC4 are elevated in *Phb1* KO in comparison to WT hepatocytes. The treatment with HDAC inhibitors counteracts this effect decreasing the amount of HDAC4 available in the liver. Importantly, we have also revealed that TNFα levels were reduced upon HDAC inhibitors treatment, so we cannot exclude a negative feedback mechanism to control HDAC4 expression mediated by this cytokine.

Regarding HDAC4 activity, this is modulated by multiple mechanisms encompass its interaction with HDAC3 that can associate in a complex with the nuclear receptor corepressors NCoR and SMRT to mediate transcriptional repression by nuclear receptors[26] and postranslational modifications that determine its nucleocytoplasmic shuttling. These include modification by multiple kinases and phosphatases and its interaction with different proteins such as 14-3-3[27]. However, the complete interactome of HDAC4 is still not defined. We now demonstrate that lower levels of PHB1 are linked to a more nuclear localization of HDAC4 as shown in control mice after BDL, in *Phb1* KO mice and very importantly, in PBC patients. These data suggest that a shift in the cellular compartimentalization of HDAC4-PHB1 could be associated with cholestatic liver damage. Indeed, we have found that PHB1 interacts with HDAC4 mainly throughout the response to bile acids avoiding its shuttling to the nucleus. These findings support a role of PHB1 as a negative regulator of HDAC4 activity, sequestering it in the cytoplasm. Although, the nuclear interactome of HDAC4 in the absence of PHB1 needs to be further explored, we can suggest that the absence of PHB1 leads to epigenetic changes sensitizing the liver to the apoptotic response in a HDAC4 dependent manner.

HDAC activity is generally linked to transcriptional repression[28]. The misbalance in HDAC activity is associated with cancer and several disorders including fibrosis, with aberrations in inflammatory and chemokine related genes[29]. Specifically, HDAC4 has been related to fibrogenesis *in vivo*[30] and as we mentioned before, in PBC patients with low expression of PHB1.

Parthenolide is a sesquiterpene lactone derived from the plant feverfew that is actively investigated as a potential therapeutic drug for several human cancers[31]. Notably, parthenolide induces ubiquitination and proteasomal degradation of HDAC1 in treated cells[19]. It has been previously described that PHB1 deficiency induces a down-regulation of proteasome activity in human hepatoma cells[32]. Accordingly, we have found that parthenolide increases hepatic 26S proteasome activity in *Phb1* KO mice resulting in the degradation of HDAC4 and the regression of the liver injury. Indeed, HDAC4 has been reported before as a target of ubiquitination[33]. No changes in HDAC1 were detected under these conditions. This loss of HDAC4 resulted in epigenetic activation of key genes related to bile acid metabolism like FXR and down-regulation of profibrogenic and inflammatory markers.

Finally, stressing the importance of HDAC4 in cholestatic liver injury, its specific knockdown both *in vitro* and *in vivo* was sufficient to diminish the damage detected in the absence of PHB1, restoring the expression of important genes related to inflammation, bile acid metabolism and apoptosis.

Overall, our data identify PHB1 as a critical modulator in cholestatic liver injury regulating HDAC4 activity and specific epigenetic marks. These results also confirm that HDAC4 activity is central during hepatic damage caused by PHB1 deficiency. This mechanism might be particularly important for the identification of novel therapeutic targets, including HDAC4, to treat liver injury and chronic cholestatic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BDL	bile duct ligation
DCA	deoxycholic acid
HDAC	histone deacetylase
OPA1	Optic Atrophy 1
PBC	primary biliary cirrhosis
PHB1	Prohibitin 1
TSA	trichostatin A
TUBE	tandem ubiquitin-binding entity

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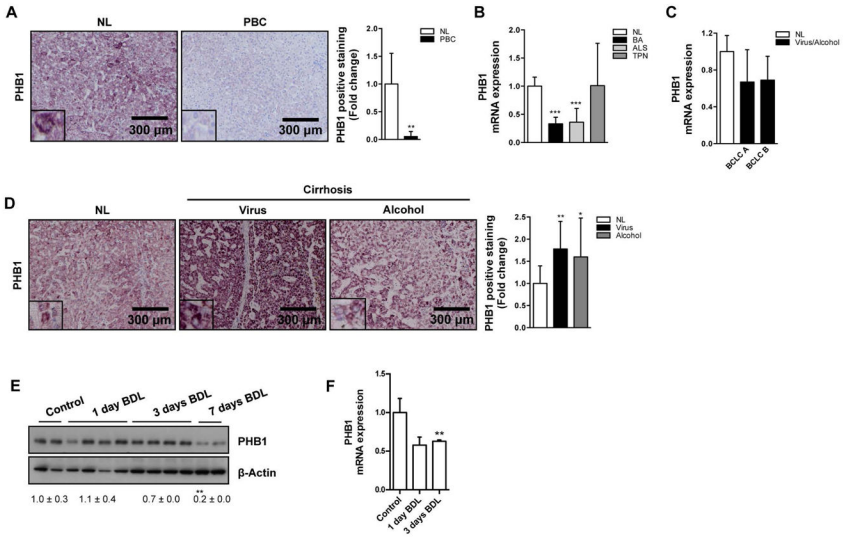

Figure 1. Reduced PHB1 levels are associated with cholestatic liver disease

(A) PHB1 levels in normal healthy liver (NL) and PBC human samples. PHB1 mRNA levels in human (B) NL, biliary atresia (BA), Alagille syndrome (ALS) and parenteral nutrition-associated liver disease (TPN) and (C) NL and BCLC A and B stage viral and alcoholic liver cirrhosis. (D) PHB1 levels in human NL, viral and alcoholic cirrhosis. PHB1 (E) protein and (F) gene expression in WT mice at different time points after BDL. (Values are mean ± Stdev. *p<0.05, **p<0.01, ***p<0.001 [PBC, BA, ALS, Virus or Alcohol *vs* NL, BDL *vs* Control).

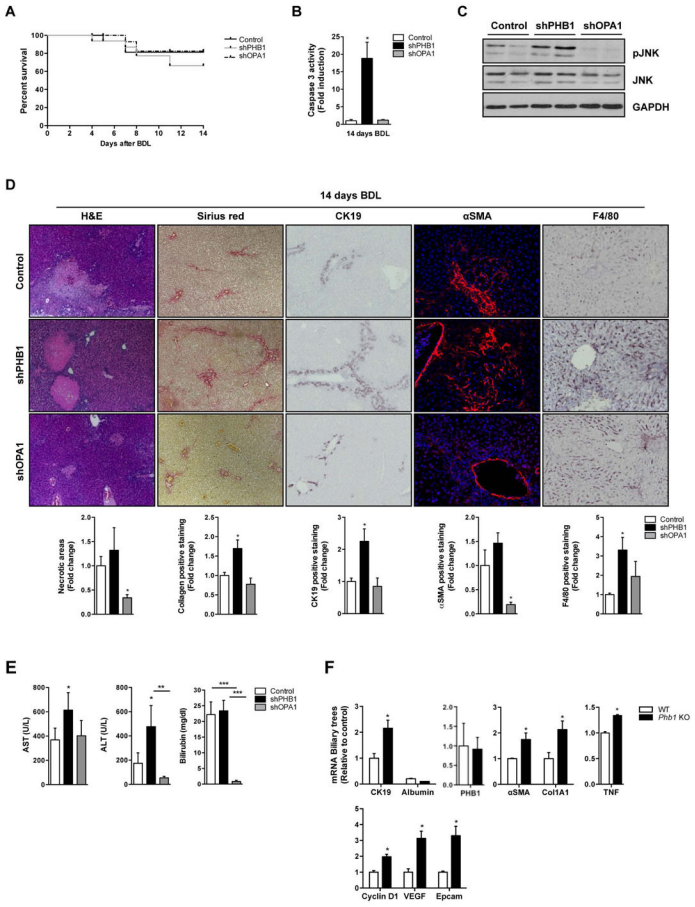

Figure 2. The absence of PHB1 predisposes to liver injury

(A) Kaplan Meier curve, (B) Caspase 3 activity, (C) WB analysis with the indicated antibodies, (D) H&E, CK19, αSMA, Sirius red and F4/80 staining in livers and (E) AST, ALT and bilirubin levels in serum from WT, shPHB1 and shOPA1 livers 14 days after BDL. (F) qPCR analysis of the indicated genes in the biliary trees (n=2) isolated from WT and *Phb1* KO mice 7 days after BDL. (Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 [shPHB1 and shOPA1 *vs* Control, *Phb1* KO *vs* WT).

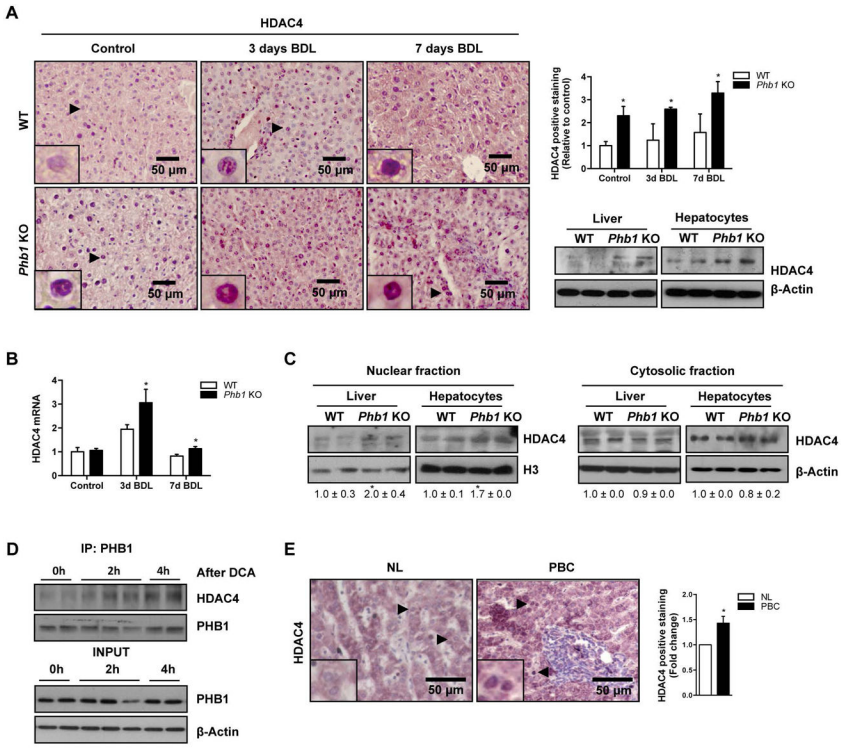

Figure 3. HDAC4 expression in *Phb1* KO mice

HDAC4 expression in (A) liver sections from WT and *Phb1* KO mice at basal conditions and after BDL (left) and WT and *Phb1* KO livers and hepatocytes by WB (right), (B) WT and *Phb1* KO livers at basal conditions and after BDL at mRNA level and (C) nuclear and cytosolic fractions from WT and *Phb1* KO livers and hepatocytes. (D) IP of PHB1 and WB against HDAC4 in WT hepatocytes stimulated with DCA. (E) HDAC4 in human samples from NL and PBC. (Values are mean ± SEM. *p<0.05 [*Phb1* KO *vs* WT; PBC *vs* NL).

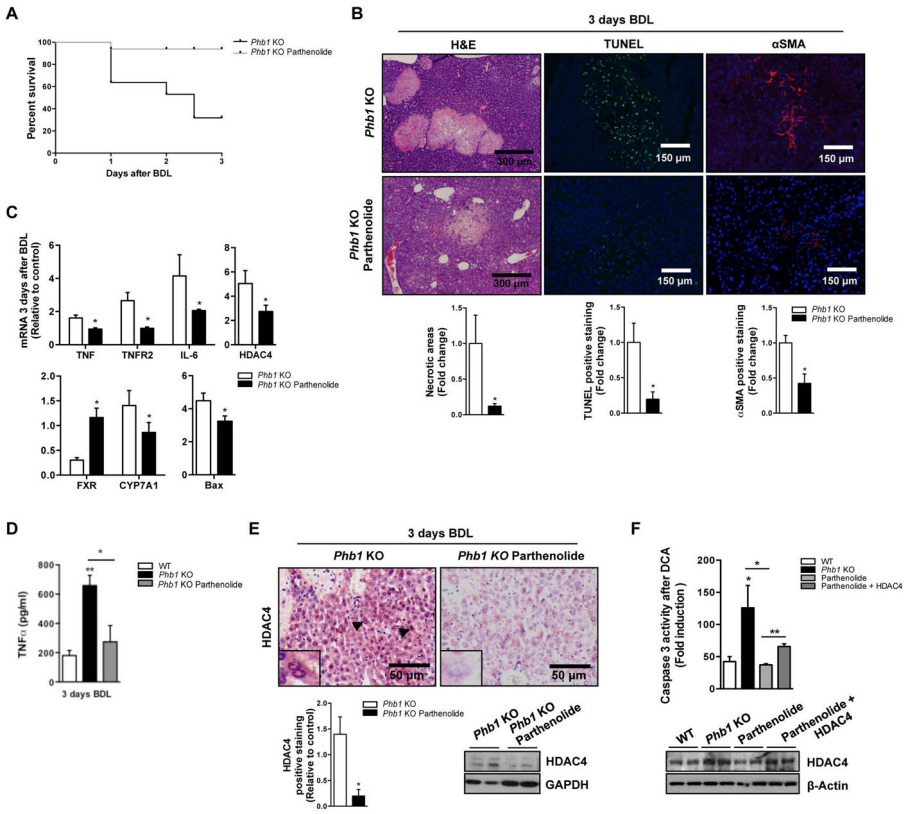

Figure 4. Parthenolide reduces liver damage after BDL in *Phb1* KO mice

(A) Kaplan Meier curve, (B) H&E, αSMA and TUNEL staining, (C) indicated genes expression, (D) TNFα levels measured by ELISA and (E) HDAC4 levels in control and parthenolide treated *Phb1* KO animals after BDL. (F) Caspase 3 activity after DCA (100µM) (upper panel) and WB against HDAC4 (lower panel) in WT, *Phb1* KO, *Phb1* KO treated with parthenolide and *Phb1* KO overexpressing HDAC4 treated with parthenolide primary hepatocytes. (Values are mean ± SEM. *p>0.05, **p>0.01 [Parthenolide *vs Phb1* KO, Phb1 KO *vs* WT and Parthenolide + HDAC4 *vs* Parthenolide]).

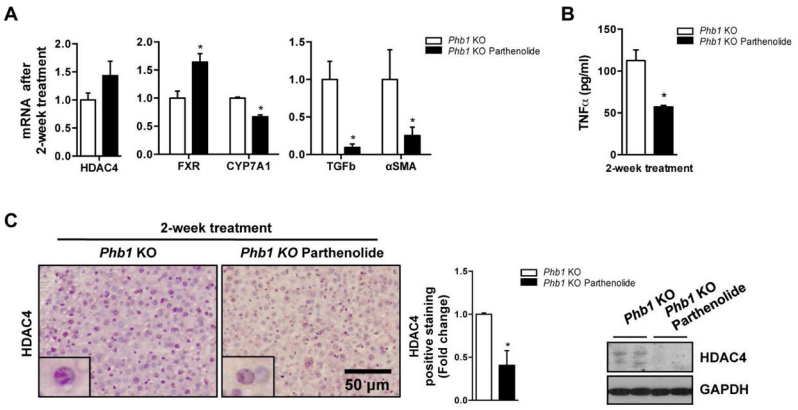

Figure 5. Parthenolide delays liver fibrosis in *Phb1* KO mice

(A) Indicated genes expression, (B) TNFα levels measured by ELISA and (C) HDAC4 levels in control and parthenolide treated *Phb1* KO animals. Values are mean ± SEM. *p > 0.05, **p<0.01 [Parthenolide *vs Phb1* KO]).

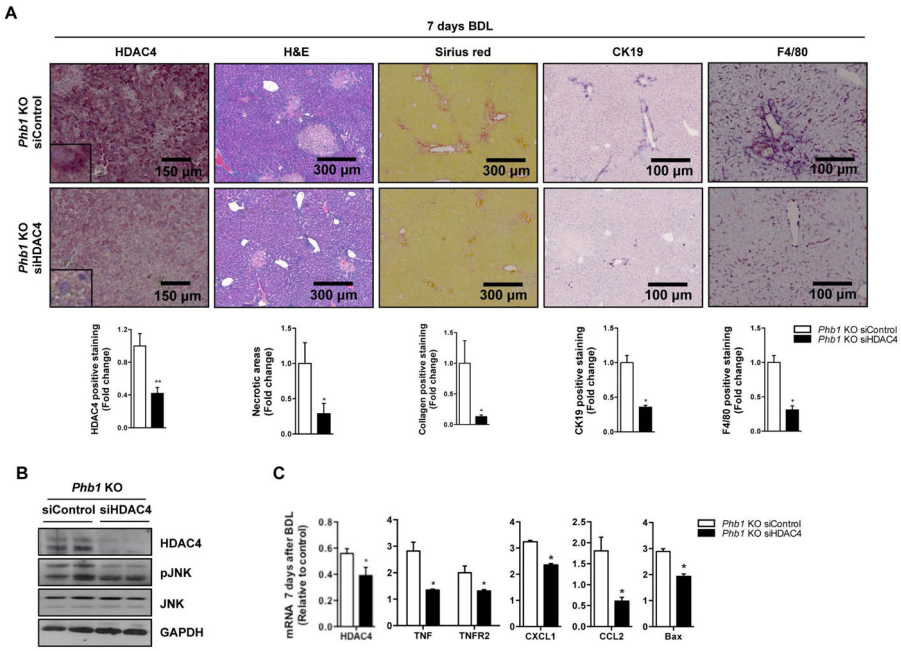

Figure 6. HDAC4 silencing protects *Phb1* KO mice from bile acid injury

(A) HDAC4, H&E, Sirius red, CK19 and F4/80 staining, (B) WB with the indicated antiboides and (C) expression of the indicated genes on livers from *Phb1* KO siControl and siHDAC4 animals 7 days after BDL. (Values are mean ± SEM. *p> 0.05, **p<0.01 [siHDAC4 *vs* siControl]).