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Authors

Fan, Wei
Yang, Heping
Liu, Ting
et al.

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Prohibitin 1 Suppresses Liver Cancers Tumorigenesis in Mice and Human Hepatocellular and Cholangiocarcinoma cells

Wei Fan[1,7,8], **Heping Yang**[1], **Ting Liu**[2], **Jiaohong Wang**[1], **Tony W. H. Li**[1], **Nirmala Mavila**[1], **Yuanyuan Tang**[1,3], **JinWon Yang**[1], **Hui Peng**[1], **Jian Tu**[1,9], **Alagappan Annamalai**[4], **Mazen Noureddin**[1,4], **Anuradha Krishnan**[5], **Gregory J. Gores**[5], **ML Martínez-Chantar**[6], **José M. Mato**[6], and **Shelly C. Lu**[1]

[1]Division of Digestive and Liver Diseases, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

[2]Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China

[3]Department of Oncology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

[4]Comprehensive Transplant Center, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

[5]Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN

[6]CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), Technology, Park of Bizkaia, 48160 Derio, Bizkaia, Spain

[7]Department of Geriatrics, Guangzhou First People's Hospital, Guangzhou 510180, China

[8]State Key Laboratory of Respiratory Diseases, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, 510120, China

[9]Institute of Pharmacy & Pharmacology, University of South China, Hengyang 421001, China

Abstract

Prohibitin 1 (PHB1) is best known as a mitochondrial chaperone and its role in cancer is conflicting. Mice lacking methionine adenosyltransferase α 1 (MATα1) have lower PHB1 expression and we reported c-MYC interacts directly with both proteins. Furthermore, c-MYC and MATα1 expert opposing effects on liver cancer growth, prompting us to examine the interplay between PHB1, MATα1 and c-MYC and PHB1's role in liver tumorigenesis. We found PHB1 is highly expressed in normal hepatocytes and bile duct epithelial cells and down-regulated in most human hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). In HCC and CCA cells, PHB1's expression correlate inversely with growth. PHB1 and MAT1A positively regulate each other's expression, whereas PHB1 negatively regulates the expression of c-MYC, MAFG and c-MAF. Both PHB1 and MATα1 heterodimerize with MAX, bind to the E-box element and repress E-box promoter activity. PHB1 promoter contains a repressive E-box element, is occupied mainly

Contact information: Shelly C. Lu, M.D., Division of Digestive and Liver Diseases, Department of Medicine, Cedars-Sinai Medical Center, Davis Building, Room #2097, 8700 Beverly Blvd., Los Angeles, CA, 90048. Tel: (310) 423-5692, Fax: (310) 423-0653, shelly.lu@cshs.org

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by MAX, MNT and MATα1 in nonmalignant cholangiocytes and noncancerous tissues that switched to c-MYC, c-MAF and MAFG in cancer cells and human HCC/CCA. All 8-month old liver-specific *Phb1* knockout mice developed HCC and one developed CCA. 5-month old *Phb1* heterozygotes but not *Phb1* flox mice developed aberrant bile duct proliferation and one developed CCA 3.5 months after left and median bile duct ligation (LMBDL). *Phb1* heterozygotes had a more profound fall in the expression of GSH synthetic enzymes and higher hepatic oxidative stress following LMBDL.

Conclusion—We have identified PHB1, down-regulated in most human HCC and CCA, heterodimerizes with MAX to repress the E-box. PHB1 positively regulates *MAT1A* while suppressing *c-MYC*, *MAFG* and *c-MAF* expression. In mice, reduced PHB1 expression predisposes to the development of cholestasis-induced CCA.

Keywords

Hepatocellular carcinoma; c-MYC; methionine adenosyltransferase α1; MAFG; c-MAF

Prohibitin 1 (PHB1) belongs to an evolutionary conserved and ubiquitously expressed family of proteins with a myriad of functions in different cellular compartments (1, 2). PHB1 is best known as a mitochondrial chaperone that is essential for mitochondrial function and biogenesis (2). PHB1 is also present in the nucleus where it serves as a transcription co-factor, interacts with many proteins including Rb and p53 to repress or activate the transcriptional activities of E2F (3, 4) or p53 (5), respectively. Recently we reported hepatic PHB1 expression is down-regulated at the mRNA and protein levels in chronic cholestatic injury in experimental murine models and in humans (6, 7). We found PHB1 interacts directly with NRF2 and serves as a co-activator of the anti-oxidant response element (ARE) to positively regulate ARE-dependent genes such as glutamate-cysteine ligase (6). In contrast, c-MYC serves as a co-repressor and inhibits NRF2-ARE activity (6). We also found a cross-talk between c-MYC and methionine adenosyltransferase α1 (MATα1, encoded by *MAT1A*) in cholestatic liver injury and cholangiocarcinoma (CCA) where the two exerts reciprocal regulation against each other at the transcriptional level (8). MATα1 expression falls in chronic cholestatic liver injury while c-MYC expression is induced (8) and we showed the induction of c-MYC is a key driver in cholestasis-associated CCA (9). Consistently overexpressing MAT1A inhibited CCA growth in vivo as effectively as silencing c-MYC, while silencing MAT1A promoted CCA growth comparable to overexpressing c-MYC (8). Interestingly mice lacking MATα1 have reduced PHB1 expression (10) and both *Mat1a* and liver-specific *Phb1* knockout (KO) mice develop hepatocellular carcinoma (HCC) spontaneously (11, 12). While the suppressive role of MATα1 in liver cancer tumorigenesis is well characterized (13, 14), the role of PHB1 is controversial and unclear. The goals of the current work were to examine whether there is interplay between PHB1, MATα1 and c-MYC and define the role of PHB1 in the tumorigenesis of HCC and CCA, the two most common primary cancers of the liver.

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Experimental Procedures

Materials and reagents

α-^{32}P dCTP (6,000 Ci/mmol) was purchased from PerkinElmer (Boston, MA). Antibodies used for Western, ChIP, Seq-ChIP, and immunohistochemistry (IHC) to PHB1, MATα1, c-MYC, MAFG, c-MAF, β-ACTIN, and IgG were purchased from Abcam (Cambridge, MA). Lipofectamine 2000 and RNAi-Max were purchased from ThermoFisher (Carlsbad, CA). siRNAs to *PHB1* (Cat# 4392422), *c-MYC* (5′-CGAUUCCUUCUAACAGAAtt-3′), *MAFG* (5′-CGGACUAGAGAGAGUUGCGtt-3′), *c-MAF* (5′-GCAUCGUGUACUUACCAGUtt-3′) and *MAT1A* (5′-GCACAACGAAGACAUCACGtt-3′) were purchased from ThermoFisher. Bile acids and interleukin-6 (IL-6) were from Sigma (St. Louis, MS).

Source of normal liver, human HCC and CCA with adjacent non-tumorous specimens

Specimens were obtained from three hospitals, two in the United States (Cedars-Sinai Medical Center, Los Angeles, CA and the Mayo Clinic, Rochester, MN) and one in China (Xiangya Hospital Central South University, Changsha, Hunan province, China). From China, 27 pairs of fresh-frozen HCC and three pairs of fresh CCA and adjacent benign tissue samples were obtained from patients undergoing surgical liver resection. All fresh-frozen samples, which were collected from 2013 to 2016, stored in the biobank, and kept in liquid nitrogen tank for long-term storage. In addition, five other paraffin embedded normal liver tissues and five CCA specimens were used for IHC. All the samples were verified by three pathologists, Department of Pathology, Central South University. The approval for the use of human samples was obtained from the Institutional Review Board of the Central South University, Xiangya Hospital Authority. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Medical Ethical Committee of Xiangya Hospital Central South University.

From Cedars-Sinai Medical Center, six pairs of archived fresh-frozen HCC, seven pairs of CCA and adjacent benign tissue samples were obtained from patients undergoing surgical liver resection. From the Mayo Clinic seven pairs of CCA and adjacent benign tissue samples were accessed and provided as de-identified samples. Protocols were approved by the Institutional Review Boards of Cedars-Sinai Medical Center and the Mayo Clinic, Rochester, MN. Both tumor and normal tissue were evaluated histologically to confirm presence or absence of neoplasm. These samples were used to measure *PHB1* mRNA levels (see below).

Animal experiments

Both *Mat1a* KO and liver-specific *Phb1* KO mice were previously described (11, 12). Five months old male liver-specific *Phb1* heterozygotes and *Phb1* flox controls (n=8 per group) were subjected to left and median bile duct ligation (LMBDL) as we described (9) and observed for up to 3.5 months afterwards. Parts of the liver tissues were used for RNA and protein analysis, the rest were fixed in 4% formalin for histology and IHC. Murine cholestasis-associated CCA model was previously described (9). Briefly, Balb/c mice were given 2 weekly intraperitoneal injections of diethylnitrosamine (DEN, 100mg/kg); 2 weeks

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later, some mice also received LMBDL, and then 1 week later, were fed DEN (25mg/kg in corn oil), weekly by oral gavage (DLD). CCA developed at week 28 in the DLD group but not in groups that received only DEN or LMBDL (9). Effect of c-MYC was evaluated using shRNA as described (9). CCA specimens from our previous study (9) were used for measurement of mRNA levels. All procedure protocols, use, and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center (Los Angeles, CA).

Cell lines and treatments to vary gene expression

Human CCA KMCH and Huh28, and HCC HepG2 and Hep3B cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mmol/L L-glutamine. H69 cells, SV40 transformed normal human biliary epithelial cells, were cultured as described previously (15). To examine the interplay between PHB1 and MATα1, c-MYC/MAFG/c-MAF, 1×10^5 KMCH, Huh28, HepG2 or Hep3B cells per well of 6-well plates were transfected with vectors overexpressing PHB1 (Origene, Rockville, MD), MATα1, c-MYC, MAFG, c-MAF or empty vectors (GeneCopoeia, Rockville, MD) for 24 hours using Lipofectamine 2000 according to the manufacturer's protocol. For gene knockdown studies, 10nM siRNA against *PHB1*, *MAT1A*, *c-MYC*, *c-MAF* or *MAFG* and equivalent scramble control were delivered into KMCH, Huh28, HepG2 or Hep3B cells for 24 hours by Lipofectamine RNAiMAX following the manufacturer's protocol.

Effects of cholic acid (CA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), glycodeoxycholic acid (GDCA) on gene expression were examined following treatment with 50-100μM for 16 hours in H69 cells. Effect of IL-6 (10ng/ml) treatment was assessed after 4 hours in H69 cells.

SAMe-D cells are derived from HCC obtained from *Mat1a* KO mouse and was cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mmol/L L-glutamine (16). Transfection of SAMe-D cells with PHB1 or empty vector was performed using the jetPRIME® reagent (Polyplus-transfection®, Radnor PA) according to the manufacturer's protocol. After 24 hours of transfection, cells were grown in the presence of 250 (μg/ml G418 for 3 weeks to generate stably transfected cells. Passage 2 or 3 stably transfected cells were used for further experiments.

Anchorage-independent growth assay

2×10^3 PHB1 overexpressing or empty vector stably transfected SAMe-D cells were grown in 0.7% soft agar on a 0.5% base soft agar layer in 6-well plate for two weeks in DMEM supplemented with 10% FBS and G418. Plates with only soft agar layers and no cells served as negative controls. Colonies formed at the end of two weeks were stained with 0.005% crystal violet for 1 hour, washed thoroughly with water and images were acquired using Evos Advanced Microscopy Group (AMG) transmitted light microscope coupled with Evos *xl* software (AMG, Bothell, Washington, USA).

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Promoter constructs and luciferase assay

1.2 kb Human *PHB1* promoter was generated using the upstream primer AGGTAAAAACTAGAATGAGAGCTGAGC (bp -1150 to - 1124) and the reverse downstream PCR primer ACACCTGCTTCCACTCTGACCTC (bp +28 to +50, NM-002634) from a human genomic DNA (BioLINE, Taunton, MA). The fragment was cloned into a pEZX-PG02.1 vector between *EcoR*I sites from GeneCopoeia. The wild type E-box-luc and mutant E-box-luc where the core motif of E-box CACGTG was changed to CTCGTC were previously described (8). The promoter constructs and pGL4.13/SV40 (1 ug) were co-transfected into KMCH, Huh28, HepG2 and Hep3B cells with Lipofectamine 2000 following the manufacturer's instructions. Luciferase assays were performed 24 hours later using the Pierce™ Gaussia Luciferase Flash Assay Kit (ThermoFisher) and the Dual Luciferase Reporter Assay System (Promega, Madison, WI) as directed by the manufacturer suggested protocol.

Histology and immunohistochemistry (IHC)

Formalin-fixed liver and CCA tissues embedded in paraffin were cut and stained with hematoxylin and eosin (H&E) for routine histology. IHC staining of PHB1, MATα1, c-MYC, MAFG, c-MAF and IgG was performed with kit from Dako (Carpinteria, CA) or Abcam according to the manufacturer's method. Control with normal mouse IgG showed no staining (not shown). The OxyIHC kit (Millipore, Billerica, MA) was used to determine oxidative stress in liver tissues following manufacturer's protocol.

RNA isolation and gene expression analysis

Total RNA was isolated by using Quick-RNA miniPrep kit (Zymo Research, Irvine, CA) from cell lines and murine and human liver tissues, HCC, CCA, and adjacent non-tumorous tissues. Gene expression was assessed using real-time PCR. Total RNA was subjected to reverse transcription (RT) by using M-MLV Reverse transcriptase (Lucigen, Middleton, WI). TaqMan probes for human and murine *PHB1*, *MAT1A*, *c-MYC*, *MAFG*, *c-MAF*, glutamate-cysteine ligase catalytic and modifier subunits (*Gclc* and *Gclm*), and the Universal PCR Master Mix were purchased from Bio-Rad (Hercules, CA). Hypoxanthine phosphoribosyltransferase 1 or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, for human CCA) was used as a housekeeping gene. The thermal profile consisted of an initial denaturation at 95°C for 3 minutes followed by 40 cycles at 95°C for 3 seconds and at 60°C for 30 seconds. The cycle threshold (Ct value) of the target genes was normalized to that of the housekeeping gene to obtain the delta Ct (ΔCt). The ΔCt was used to find the relative expression of target genes according to the formula: relative expression= $2^{-\Delta\Delta Ct}$, where ΔΔCt= ΔCt of target genes in experimental condition – ΔCt of target gene under control condition.

Chromatin immunoprecipitation (ChIP) and sequential-ChIP (Seq-ChIP) assay

ChIP and Seq-ChIP were done to examine changes in protein binding to the E-box region of the human *PHB1* promoter in an endogenous chromatin configuration using the manufacturer's suggested protocol from the Pierce agarose ChIP kit (ThermoFisher). Briefly, DNA immunoprecipitated by MAX antibody was processed for a second round of

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immunoprecipitation using anti-MATα1, anti-c-MYC, anti-MAFG, anti-c-MAF, anti-PHB1 or anti-MNT antibodies. The purified DNA was detected by PCR analysis. PCR of the human *PHB1* promoter region containing E-box used forward primer 5′-AGTTCCACCTGTCCTCTTCATCAG-3′ (bp -388 to -365) and reverse primer 5′-TTGGTGATCAACGCGAGGATGTTG-3′ (-208 to -185 relative to transcriptional start site) (GenBank® accession no. NM-002624). All PCR products were run on 2% agarose gels. The PCR conditions consisted of an initial denaturation at 94°C for 3 minutes followed by 25 cycles at 94°C for 30 seconds, the annealing and extension at 67°C for 90 seconds using the Advantage GC 2 PCR kit (Clontech, Mountain View, CA), in accordance to their suggested protocol.

Western blot and co-immunoprecipitation (co-IP) analysis

Total protein extracts from cells expressing varying amounts of PHB1, MATα1, C-MYC, MAFG and c-MAF were subjected to Western blot analysis as described (14). Equal amounts of total protein extracts (15 μg/well) were resolved on 12.5% SDS–polyacrylamide gels. Membranes were probed with antibodies to PHB1, MATα1, c-MYC, MAFG and c-MAF. To ensure equal loading, membranes were stripped and reprobed with anti-ACTIN antibodies. Blots were developed by enhanced chemiluminescence (Millipore Corporation). For co-IP, proteins were immunoprecipitated by anti-PHB1 antibody and then subjected to Western blotting with antibody to MATα1, c-MYC, MAFG, c-MAF or GAPDH.

Direct protein-protein interaction

Recombinant human PHB1, MATα1, and MAFG proteins were from Prospec (East Brunswick, NJ). 2 μg of MATα1, MAFG, or PHB1 protein was immobilized to agarose beads by their respective antibody. After washing, beads were mixed with 1 μg PHB1, MATα1, or MAFG protein and rotated for 4 hours at 4°C. Beads were then washed 6 times, boiled in SDS sample buffer and proteins separated on SDS-PAGE and subjected to Western blotting.

Electrophorectic mobility shift assay (EMSA) with recombinant proteins

Binding of recombinant human PHB1, MATα1, MAX, and c-MYC (200ng) alone or in different combinations to the E-box of the human *PHB1* promoter (bp – 260 to -245 relative to transcriptional start site) was examined with EMSA as we described (8).

5-Bromo-2′-deoxyuridine (BrdU) incorporation

BrdU incorporation was measured with BrdU Detection Kit according to the manufacturer's protocol (BD Biosciences, Pharmingen, San Jose, CA).

Statistical analysis

Data are expressed as mean±SEM. Statistical analysis was performed using ANOVA and Fisher's test. For mRNA and protein levels, ratios of genes and proteins to respective housekeeping densitometric values were compared. Significance was defined by $p<0.05$.

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Results

PHB1 interacts with MATα1, MAFG, c-MAF and c-MYC

We previously showed PHB1 directly interacts with c-MYC (6), which in turn directly interacts with MATα1, MAFG and c-MAF (8). To see if PHB1 interacts with MATα1, MAFG and c-MAF in the normal mouse liver, we used co-IP with anti-PHB1 antibody followed by Western blotting. This revealed MATα1, MAFG, c-MAF and c-MYC as interacting proteins with PHB1 (Fig. 1A). To examine whether the interactions are direct, we used recombinant proteins immobilized to beads and found PHB1 can directly interact with MATα1 (Fig. 1B) and MAFG (Fig. 1C).

PHB1 suppresses growth in HCC and CCA cells, is down-regulated in human HCC and CCA

We showed MATα1 is a tumor suppressor in HCC and CCA cells (8, 14) and c-MYC, MAFG and c-MAF all behave as oncoproteins in CCA cells (8). PHB1 is similar to MATα1 in both CCA and HCC cells as overexpression of either protein suppressed growth while knockdown increased growth (Fig. 2A). In contrast, c-MYC, MAFG and c-MAF all increased growth when overexpressed in both HCC and CCA cells (Fig. 2A). PHB1 is highly expressed in normal hepatocytes and bile duct epithelial cells, and its expression is markedly reduced in human HCC and CCA specimens, similar to MATα1 (Fig. 2B). We showed c-MYC, MAFG and c-MAF are induced in human CCA (8) and here we found they are induced in HCC as well (Fig. 2B). In four independent HCC microarray datasets from the GEO database, *PHB1* mRNA levels are reduced in HCC as compared to adjacent non-tumorous tissue in the majority (Fig. 2C). We found only one available CCA microarray dataset from the GEO database (GSE15765) and *PHB1* mRNA levels are lower in CCA as compared to HCC (Fig. 2D). We have verified these changes using our own available specimens. Consistent with these results, of the paired HCC and CCA samples (tumor and adjacent non-tumorous tissue from the same patients) available, 22 of 33 HCCs and 14 of 17 CCAs showed lower *PHB1* mRNA levels in the tumors to 44±6% and 30±5% of adjacent non-tumorous tissues, respectively.

Interplay among PHB1, MAT1A, c-MYC, MAFG and c-MAF

We next examined the influence of PHB1 on the expression of MAT1A, c-MYC, MAFG and c-MAF and found PHB1 positively regulates MAT1A but negatively regulates c-MYC, MAFG and c-MAF. Overexpressing PHB1 raised MAT1A mRNA and protein levels but it lowered the mRNA and protein levels of c-MYC/MAFG/c-MAF in both HCC and CCA cells (Fig. 3A). The opposite was true of PHB1 knockdown (Fig. 3B). In turn, PHB1 expression is regulated positively by MAT1A, but negatively by c-MYC, MAFG and c-MAF (Fig. 3C).

PHB1 and MATα1 heterodimerize with MAX, bind to the E-box and repress E-box driven promoter activity

We showed MATα1, c-MYC, MAFG and c-MAF all interact at the E-box with MATα1 repressing the E-box and the other three activating the E-box (8). Here we found both PHB1

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and MATα1 only need MAX in order to bind to the E-box (Fig. 4A). Figure 4B shows that PHB1 represses the E-box driven promoter activity as overexpression lowered while knockdown of PHB1 raised E-box driven promoter activity. This was true in both HCC and CCA cell lines and did not occur if the E-box was mutated (Fig. 4B).

Occupancy of PHB1 promoter E-box and its regulation by MATα1, c-MYC, MAFG and c-MAF

PHB1 promoter region contains an E-box close to the transcription start site (Fig. 5A). In non-malignant cholangiocytes H69, MNT, MATα1 and PHB1 are the predominant proteins that co-occupy this region. However, in malignant HCC and CCA cells, this is switched to c-MYC, c-MAF and MAFG (Fig. 5A). The same is true in human HCC and CCA, where co-occupancy of the human *PHB1* promoter E-box region is switched from MNT, MATα1 and PHB1 in non-cancerous tissues to c-MYC, c-MAF and MAFG in HCC or CCA (Fig. 5B). Regulation of the human *PHB1* promoter by these proteins is confirmed as overexpressing MAT1A increased *PHB1* promoter activity, while overexpressing c-MYC, MAFG or c-MAF lowered *PHB1* promoter activity. These effects were eliminated if the E-box site was mutated (Fig. 5C).

Liver-specific Phb1 KO mice develop HCC and CCA and heterozygotes are sensitized to develop CCA following LMBDL

Consistent with results obtained from the HCC and CCA cells, liver-specific *Phb1* KO livers have elevated *c-Myc*, *c-Maf*, *Mafg* but reduced *Maf1a* mRNA levels and protein expression (Fig. 6A-B). As we reported previously (12) all 8-month old KO mice develop HCC but most (6 of 8) also exhibit aberrant bile duct proliferation, and one developed CCA (Fig. 6C-E). Five months old liver-specific *Phb1* heterozygotes do not have any overt phenotype and have normal serum alanine transaminase levels (data not shown). However, they are sensitized to develop aberrant bile duct proliferation and one developed frank CCA with pancreas and lung metastasis only 3.5 months after LMBDL (Fig. 7A-C). This did not occur in any of the *Phb1* flox mice subjected to LMBDL. LMBDL resulted in lowering of *Mat1a*, *Phb1*, but higher *c-Myc*, *c-Maf* and *Mafg* mRNA levels in both flox controls and heterozygotes, with the heterozygotes exhibiting greater degree of change as compared to flox controls (Fig. 7D). Changes in PHB1 protein levels were verified using Western blotting (Fig. 7E). Since PHB1 is also important as a co-activator of NRF2 for ARE-dependent genes (6), we examined expression of *Gclc* and *Gclm*. Consistent with our previous findings (6), *Phb1* heterozygotes have lower baseline *Gclc* and *Gclm* mRNA levels that fell further after LMBDL (Supplemental Fig. 1). This translated to higher level of oxidative stress in the liver tissue, particularly around the periportal region, in both hepatocytes and biliary epithelial cells (Supplemental Fig. 2).

PHB1 expression correlates inversely with growth in murine CCA model

We examined the murine CCA model we described (9) where c-MYC is a key driver for tumor growth. CCA development required both DEN and LMBDL (DLD) in wild type mice in this model (9). We found *Phb1* mRNA levels were 51% lower in the DLD group as compared to DEN or LMBDL only groups (Supplemental Fig. 3A). Reducing c-Myc mRNA

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level with shRNA suppressed tumor growth (9) and consistently, raised Phb1 mRNA levels more than 100% (Supplemental Fig. 3B).

PHB1 is a tumor suppressor in SAMe-D cells

SAMe-D cells are HCC cells derived from the *Mat1a* KO mouse (16). SAMe-D cells express much lower PHB1 as compared to normal mouse hepatocytes (Fig. 8A). SAMe-D cells overexpressing PHB1 have lower c-MYC, c-MAF, MAFG protein levels (Fig. 8B). SAMe-D cells exhibit anchorage independent growth, which is markedly inhibited when PHB1 is stably expressed (Fig. 8C).

Role of bile acids and IL-6 on suppressing PHB1 expression during cholestasis

We examined the effects of CA, CDCA and GDCA on PHB1 expression in H69 cells as compared to LCA as positive control. CA and CDCA had no effects on PHB1 expression at 50 or 100μM. GDCA at 100μM lowered *MAT1A* and *PHB1* mRNA levels by 20 and 15%, respectively, while raising *MAFG*, *c-MYC* and *c-MAF* by 30, 51 and 71%, respectively (Supplemental Fig. 4A). No effect occurred at 50μM GDCA. We also examined IL-6 (10ng/ml for 4 hours) and found that it lowered *MAT1A* and *PHB1* mRNA levels by 21 and 26% while raising *c-MYC*, *MAFG* and *c-MAF* mRNA levels by 39, 46, and 53%, respectively (Supplemental Fig. 4B).

Discussion

PHB1 is best known for its role in the mitochondria but there is increasing evidence that it is involved in multiple other pathways related to its subcellular localization that include the nucleus and the lipid rafts of the plasma membrane (17). Within the mitochondria two homologous PHB proteins, PHB1 and PHB2, form a large multimeric complex 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 (18). Within the nucleus PHB1 interacts with multiple proteins to modulate the transcriptional activity of transcription factors such as p53 and E2F (17). We recently added NRF2 and c-MYC to the list of transcription factors that interact with PHB1 (6). We found PHB1 and c-MYC function to enhance or repress NRF2 binding to the ARE and ARE-dependent gene expression, respectively (6). Importantly PHB1 expression falls during chronic cholestatic liver injury in experimental mouse models as well as in humans with primary biliary cirrhosis, biliary atresia and Alagille syndrome (6, 7). In contrast, c-MYC expression is rapidly induced following cholestatic liver injury and these changes contributed significantly to the down-regulation of two ARE-dependent genes, GCLC and GCLM, and lower GSH levels (6). In addition to positively regulating NRF2-ARE activity, PHB1 also interacts with histone deacetylase 4 (HDAC4) and keeps it in the cytosol. During cholestasis the fall in PHB1 expression allows HDAC4 to translocate to the nucleus to alter gene expression via epigenetics (7). Thus, the fall in PHB1 expression during cholestasis can negatively affect mitochondrial function, expression of GSH synthetic enzymes and lead to HDAC4-mediated epigenetic changes. Whether decreased PHB1 expression participates in cholestasis-associated CCA development has never been examined.

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The role of PHB1 in cancer is mixed and controversial. PHB1 was originally cloned from regenerating livers where its expression was found to be nearly absent shortly after 2/3 partial hepatectomy and was thought to be a tumor suppressor (hence its name) (19). However, the tumor suppressive role of PHB1 has been controversial as PHB1 expression is higher in many cancers (2), which was thought to be related to the fact that its promoter region contains many c-MYC binding sites and c-MYC is often induced in cancer (20). Nevertheless, a clear tumor suppressive role of PHB1 has been demonstrated in breast, gastric and prostate cancers (17, 21). Overexpressing PHB1 in intestinal epithelial cells was also found to attenuate colitis-associated colon cancer tumorigenesis in mice (22). Importantly PHB1 expression falls in intestinal inflammation, including patients with inflammatory bowel disease (22), making this observation very relevant for the increased susceptibility of these patients to colon cancer.

We reported liver-specific *Phb1* KO mice developed multi-focal HCC (12). Although this may have occurred in part due to the regenerative response to severe liver injury, we found in AML12 cells (immortalized cell line from normal murine hepatocytes) PHB1 expression inversely correlated with cell proliferation, suggesting a tumor suppressive role in hepatocytes (12). This, in addition to our recent observation that PHB1 expression falls in chronic cholestasis, prompted us to investigate whether it may play a role in HCC and in cholestasis-associated CCA development.

We focused on the interplay between PHB1, c-MYC and MATα1 in HCC and CCA because PHB1 expression is lower in *Mat1a* KO mice (10) and we found c-MYC also interacts with MATα1 and they regulate each other in reciprocal manner via the E-box in both HCC and CCA cells (8). In addition, two other transcription factors MAFG and c-MAF also interact with c-MYC and MATα1 and like c-MYC, they act as oncoproteins in CCA (8). We found PHB1 can directly interact with MATα1, MAFG, and MAX. MAX is a basic helix–loop–helix–leucine zipper (bHLH-LZ) transcription factor that can heterodimerize with c-MYC, MNT or MGA (the latter two are referred to as MAD proteins) and these heterodimers compete for binding to the E-box (23). c-MYC/MAX heterodimer typically activates E-box, whereas MNT/MAX turns off E-box (24). We found PHB1 and MATα1 are similar to MNT and MGA in their ability to heterodimerize with MAX in order to bind to the E-box and repress E-box-driven promoter activity. While *c-MYC*, *MAFG* and *c-MAF* have E-boxes in their promoter regions that act as enhancers (8), E-boxes in the human *MAT1A* and *PHB1* promoter regions act to repress their gene transcription. In addition, c-MYC, MAFG and c-MAF activate E-box but MATα1 and PHB1 repress E-box. This results in PHB1 and MAT1A positively regulating each other, whereas PHB1 and MAT1A exert reciprocal regulation against c-MYC/MAFG/c-MAF.

PHB1 is growth suppressive in two HCC and two CCA cell lines. It is down-regulated in the majority of both human HCC and CCA at the mRNA level. Since PHB1's effect may be mediated via MAT1A, which we showed behaves as a tumor suppressor in HCC and CCA (8), we used SAMe-D cells, which are derived from HCC of a *Mat1a* KO mouse (16), to examine the effect of PHB1 on in vitro tumorigenicity. We found increased PHB1 expression lowered the expression of c-MYC, MAFG and c-MAF and inhibited anchorage independent growth, which is consistent with its role as a tumor suppressor in hepatocytes.

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Since SAMe-D cells do not express MAT1A, the effect of PHB1 is not mediated by raising MAT1A expression in these cells.

c-MYC expression is elevated or deregulated in up to 70% of human cancers by different mechanisms and is linked to aggressive cancer phenotype, including liver cancer (23-25). We showed a critical role for c-MYC induction in a mouse model of cholestasis-associated CCA (9). Multiple mechanisms participate in the induction of c-MYC, including down-regulation of miRNA (miR)-34a and MAT1A expression during cholestasis (8, 9). Our current work adds down-regulation of PHB1 expression as a feed forward mechanism. The reciprocal regulation between PHB1 and c-MYC illustrates a complex cross-talk between these two proteins that participates in the progression of cholestatic liver injury and likely also the development of liver cancers. We previously showed during chronic cholestasis by bile duct ligation or treatment with lithocholic acid that c-MYC lowered PHB1 expression by a mechanism that involves miR-27a and miR-27b, which are c-MYC target genes that target PHB1 (6). Our current work shows c-MYC also represses PHB1 transcriptional activity, which in turn facilitates sustained c-MYC induction. In hepatocytes and cholangiocytes the effect of PHB1 on c-MYC transcriptional activity involves direct repression by PHB1/MAX, as well as indirect mechanism since PHB1 positively regulates MAT1A expression, another repressor of c-MYC, while negatively regulates MAFG and c-MAF, two activators of c-MYC (8). Indeed, liver-specific *Phb1* KO mice have very low MAT1A but markedly induced c-MYC expression. These mice develop HCC and abnormal bile duct proliferations by 8 months of age. Since these mice have baseline liver injury, we further examined whether reduced PHB1 expression could sensitize the animal to develop cholestasis-associated CCA. We previously established the mouse CCA model using DEN and LMBDL, as LMBDL alone did not result in CCA in wild type mice and even with the combination of DEN and LMBDL, CCA development required a long time (28 weeks) (9). Liver-specific *Phb1* heterozygotes exhibit no overt phenotype, have normal liver histology and biochemistry but are sensitized to develop abnormal bile duct proliferation and CCA, only 3.5 months after LMBDL alone. This strongly supports PHB1's role as a tumor suppressor in cholestasis-associated CCA. Consistently, PHB1 expression is reduced in the murine CCA model and its expression inversely correlate with c-MYC expression and tumor growth. While our current work is focused on the interplay between PHB1 with MATα1 and c-MYC on the E-box, the increased sensitivity of *Phb1* heterozygotes to cholestasis-induced CCA development is likely multi-factorial. LMBDL reduced the expression of PHB1 and GSH synthetic enzymes and these were accentuated in *Phb1* heterozygotes. Reduced PHB1 expression can lower the expression of GSH synthetic enzymes as well as impair mitochondrial function, resulting in higher oxidative stress that can cause DNA damage. These mechanisms likely also participate in the cholestasis-induced CCA.

Although cholestasis raises the hepatic and serum levels of multiple bile acids, there is controversy as to whether these bile acids are important in the development of cholestatic liver injury as the toxic bile acids do not increase to high enough levels (26). In patients with advanced primary biliary cirrhosis, elevated serum CA and CDCA (30-40μM) have been reported (glycoconjugate represents 50-55%, taurine conjugate 40-45%) (27). Based on these reports we examined the effects of 50-100μM of GCDA, CA, CDCA as compared to LCA (as positive control) in H69 cells. CA and CDCA had no effects on PHB1 or c-MYC

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expression even at 100μM after 16 hours. GDCA had an effect but only at 100μM. Since this is unlikely to be relevant during BDL, we then explored the possibility that IL-6 may be involved in triggering the observed changes in gene expressions during BDL. We chose IL-6 because its level is higher after BDL and also in patients with HCC and CCA (28-30). We found IL-6 (10ng/ml) treatment of H69 cells for only 4 hours significantly reduced *MAT1A* and *PHB1* mRNA levels by 22-26%, respectively and raised *c-MYC*, *MAFG* and *c-MAF* by 40-53%. These results suggest bile acids are not likely to be responsible for causing the changes in these genes, but rather pro-inflammatory cytokines such as IL-6. How this occurs and whether other cytokines are also involved will be a subject of future investigation.

Given the heterogeneity of HCC and CCA, it is no surprise that not all human specimens showed down-regulation of PHB1. However, the majority of available paired samples (CCA and adjacent non-tumorous tissue) from three different institutions showed lowering of *PHB1* mRNA levels in the CCA, with an average of 70% lowering in 14 out of 17 samples. This is also true for HCC, where 22 of 33 showed an average of 56% lowering of *PHB1* mRNA levels. Our results are consistent with the publically available microarray datasets from the GEO database, where *PHB1* mRNA levels appear to be lower in CCA as compared to HCC. Future work will examine differences in the tumors that may explain why some express lower PBH1 but others do not.

In summary, our current work shows an important role for PHB1 in both HCC and CCA, where it acts as a transcription factor that heterodimerize with MAX and represses the E-box. In so doing it negatively regulates three transcription factors, namely c-MYC, MAFG and c-MAF, which we showed exhibit oncogenic activity in HCC and CCA; whereas it positively regulates MAT1A, a tumor suppressor in both cancers. Although the current work extends the complex interplay between MATα1, c-MYC, MAFG and c-MAF we showed recently (8), findings are important for PHB1 biology and establishes PHB1 as a tumor suppressor in liver cancer. In addition, we show that both MATα1 and PHB1 behave like MAD proteins in forming heterodimers with MAX to repress the E-box driven promoter activity. Finally, the finding that reduced PHB1 expression predisposes the animal to cholestasis-associated CCA has important clinical implication as we have shown PHB1 expression is reduced in patients with primary biliary cirrhosis and Alagille syndrome (7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used (alphabetical order)

ARE	anti-oxidant response element
bHLH-LZ	basic helix–loop–helix–leucine zipper
BrdU	5-bromo-2′-deoxyuridine
CA	cholic acid
CCA	cholangiocarcinoma
CDCA	chenodeoxycholic acid
ChIP	Chromatin Immunoprecipitation
Co-IP	co-immunoprecipitation
DEN	diethylnitrosamine
DLD	DEN+LMBDL+DEN

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EMSA	Electrophoretic mobility shift assay
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GDCA	glycodeoxycholic acid
HDAC4	histone deacetylase 4
H&E	hematoxylin and eosin
HCC	hepatocellular carcinoma
IHC	immunohistochemistry
IL-6	interleukin-6
IP	immunoprecipitation
KO	knockout
LCA	lithocholic acid
LMBDL	left and median bile duct ligation
MAT	methionine adenosyltransferase
miRNAs	MicroRNAs
MS	mass spectrometry
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PHB1	prohibitin 1
SAMe	S-adenosylmethionine
SC	scramble siRNA
Seq-ChIP	sequential ChIP
shRNA	small hairpin RNA
siRNA	small interfering RNA

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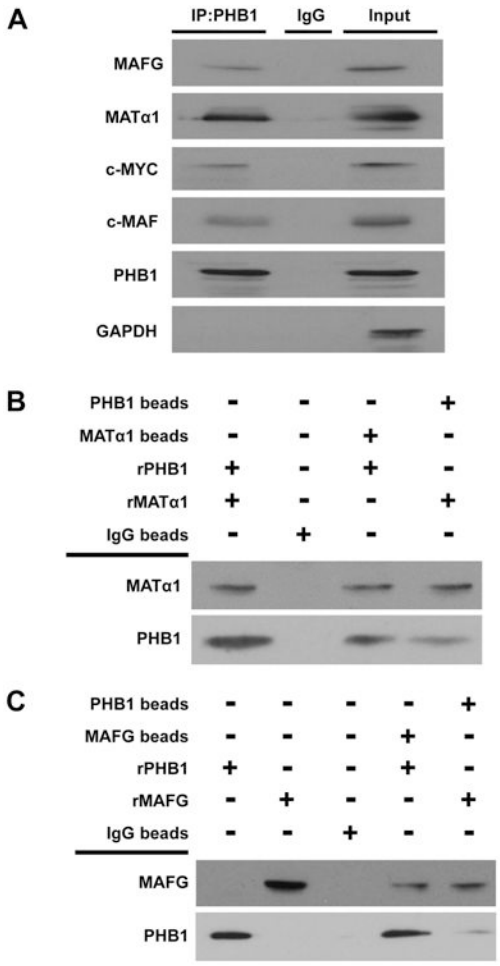

Figure 1. PHB1 interacts with MATα1, c-MYC, MAFG and c-MAF

(A) The liver protein lysates from a 3-month old *Phb1* flox male mouse liver (12) were subjected to immunoprecipitation (IP) using anti-PHB1 or nonspecific IgG, followed by Western blotting for MAFG, MATα1, c-MYC, c-MAF, PHB1, and GAPDH (as negative control). **(B)** In vitro pull down assay using immobilized recombinant PHB1 or MATα1 demonstrates direct interaction between PHB1 and MATα1. **(C)** In vitro pull down assay using immobilized recombinant PHB1 or MAFG demonstrates direct interaction between PHB1 and MAFG. Results represent a total of at least 3 independent experiments.

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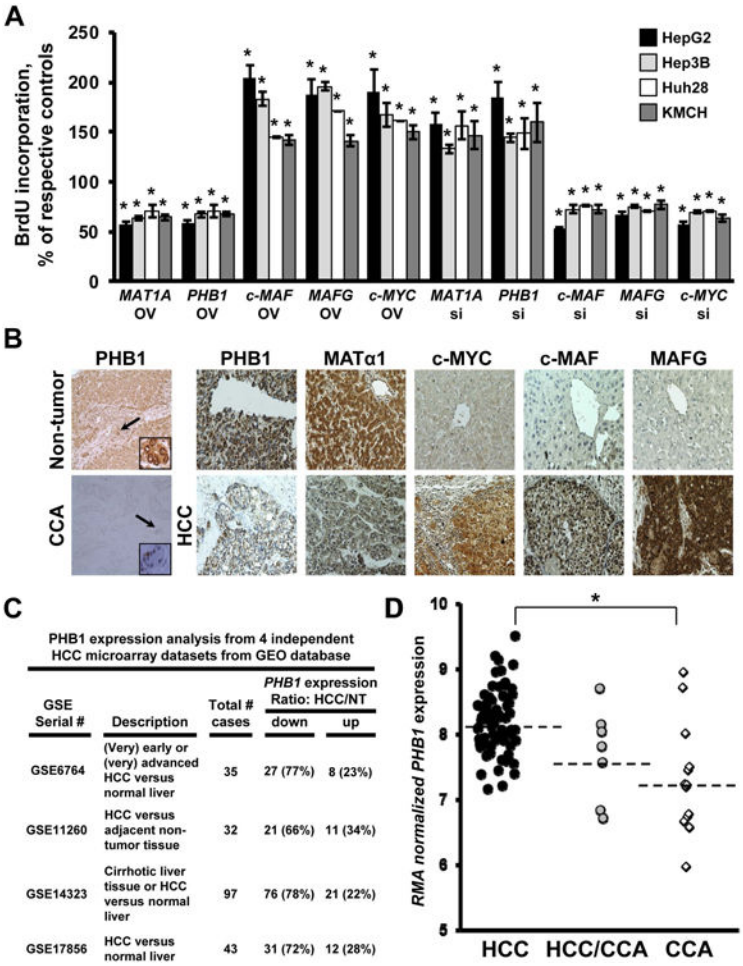

Figure 2. PHB1 suppresses growth in HCC and CCA cells, is down-regulated in human HCC and CCA

Effect of varying PHB1 expression on growth of two HCC (HepG2 and Hep3B) and two CCA (KMCH and Huh28) cell lines was assessed using BrdU as described in Experimental Procedures. **(A)** Overexpression of PHB1 suppressed BrdU incorporation, similar to overexpression of MAT1A or knocking down c-MAF, MAFG or c-MYC. In contrast, overexpression of c-MAF, MAFG, and c-MYC all increased BrdU incorporation, similar to knocking down MAT1A or PHB1. *$p < 0.05$ versus respective controls. **(B)** Immunohistochemical (IHC) analysis of PHB1, MATα1, c-MYC, MAFG and c-MAF in paraffin-embedded specimens from human HCC and CCA. In normal liver, PHB1 is highly expressed in hepatocytes and bile duct epithelial cells but it is down-regulated in CCA. PHB1 and MATα1 are highly expressed in non-tumorous livers and down-regulated in HCC. In contrast, c-MYC, MAFG and c-MAF have low expression in non-tumorous livers and are up-regulated in HCC. Arrows point to the small bile duct (insets at the bottom right of the IHC image). Original magnification, ×200. **(C)** *PHB1* mRNA levels in 4 independent HCC microarray from GEO database as compared to adjacent non-tumor tissue. **(D)** *PHB1* mRNA levels from the only GEO database that contains information on CCA (GSE15765) show lower expression in CCA as compared to HCC. The database normalized PHB1 expression by the RMA (robust multi-array average) method (31).

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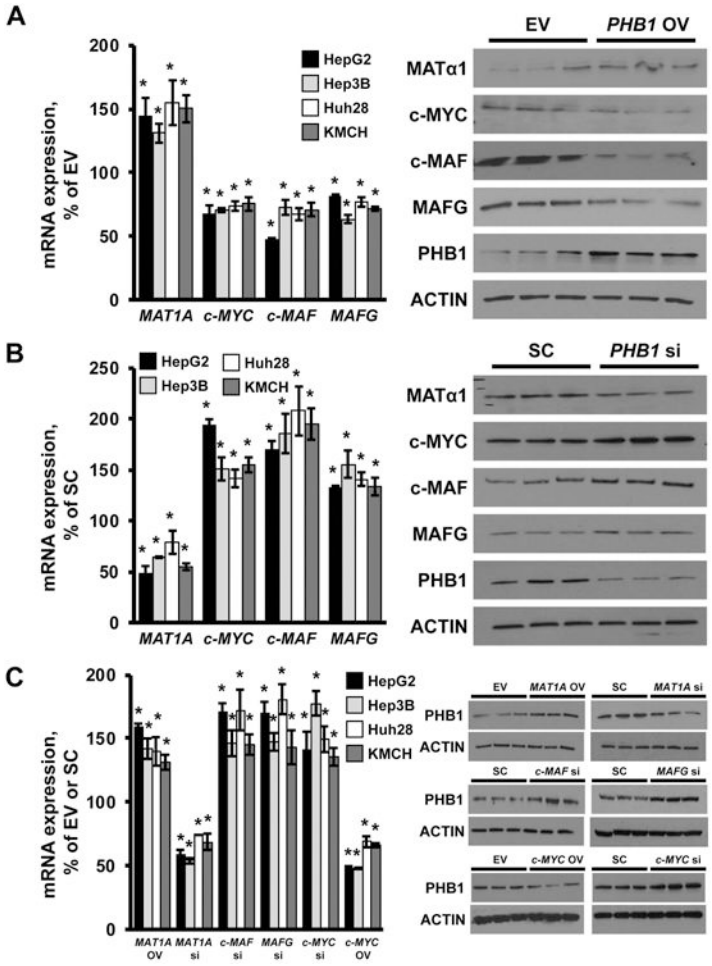

Figure 3. Interplay among PHB1, MAT1A, c-MYC, MAFG and c-MAF

PHB1 expression was varied by overexpression **(A)** or knockdown **(B)** in HepG2, Hep3B, KMCH and Huh28 cells and the expression of MAT1A, c-MYC, MAFG and c-MAF was measured as described in Experimental Procedures. **(C)** PHB1 expression was measured in the same four cell lines after varying MAT1A, c-MYC, MAFG and c-MAF expression. Results are expressed as mean ± SEM from at least 3 independent experiments, *$p < 0.05$ versus respective controls. Protein expression was measured in HepG2 and Huh28 cells (results shown are for HepG2 cells, with similar results found in Huh28 cells).

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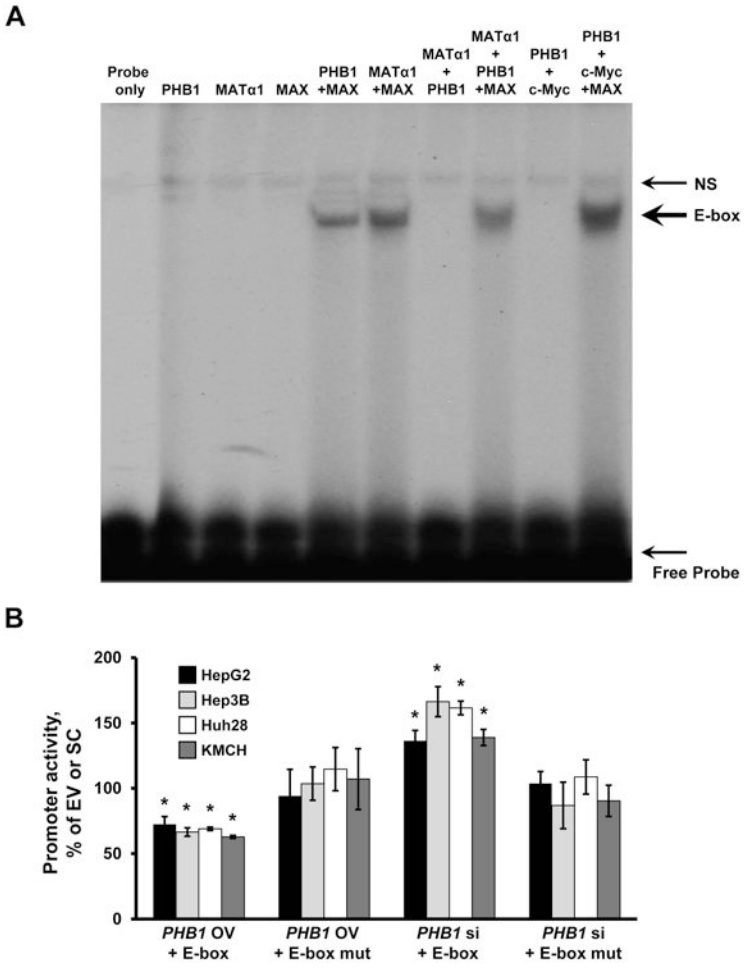

Figure 4. PHB1 and MATα1 heterodimerize with MAX, bind to the E-box and repress E-box driven promoter activity

(A) EMSA analysis of a multimerized human PHB1 E-box element (X3) using recombinant PHB1, MATα1, c-MYC and MAX proteins in different combinations. PHB1 cannot bind to E-box alone or with c-MYC but can bind to E-box in the presence of MAX, similar to MATα1. **(B)** Promoter activity of a consensus E-box-driven luciferase reporter construct and its mutant after overexpression or knockdown of PHB1 in two HCC and two CCA cell lines. *p< 0.05 vs respective controls. Results represent a total of at least 3 independent experiments done in duplicate.

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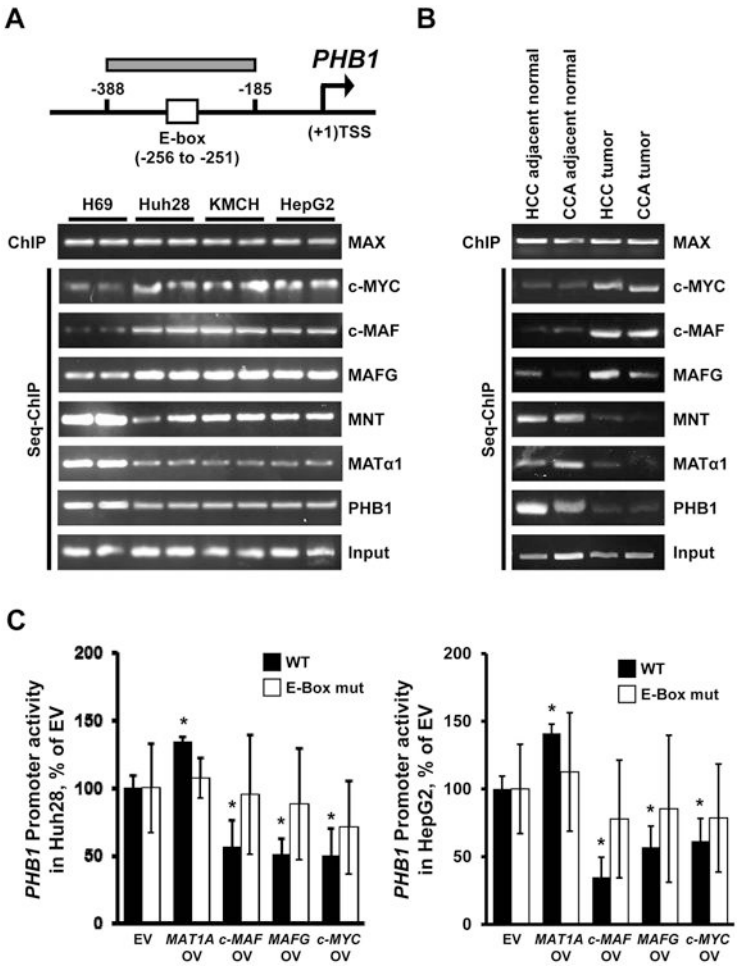

Figure 5. Occupancy of *PHB1* promoter E-box and its regulation by MATα1, c-MYC, MAFG and c-MAF

(A) H69 (benign bile duct epithelial cells), Huh28 and KMCH (CCA cells) and HepG2 (HCC cells) were subjected to ChIP analysis with MAX followed by Seq-ChIP with c-MYC, MAFG, c-MAF, MNT, MATα1, or PHB1 spanning the E-box containing human *PHB1* promoter (shown on top) as described in Experimental Procedures. **(B)** ChIP was done using human HCC, CCA and their adjacent non-tumorous tissues with MAX followed by Seq-ChIP with c-MYC, MAFG, c-MAF, MNT, MATα1, or PHB1 spanning the E-box containing human *PHB1* promoter as in **(A)**. Representatives from three pairs of HCC and CCA are shown. **(C)** Effect of overexpressing MAT1A, c-MYC, MAFG or c-MAF on human *PHB1* promoter (wild type or E-box mutant) was examined in Huh28 and HepG2 cells. *p< 0.05 vs control (empty vector). Results represent a total of 3 independent experiments done in duplicate.

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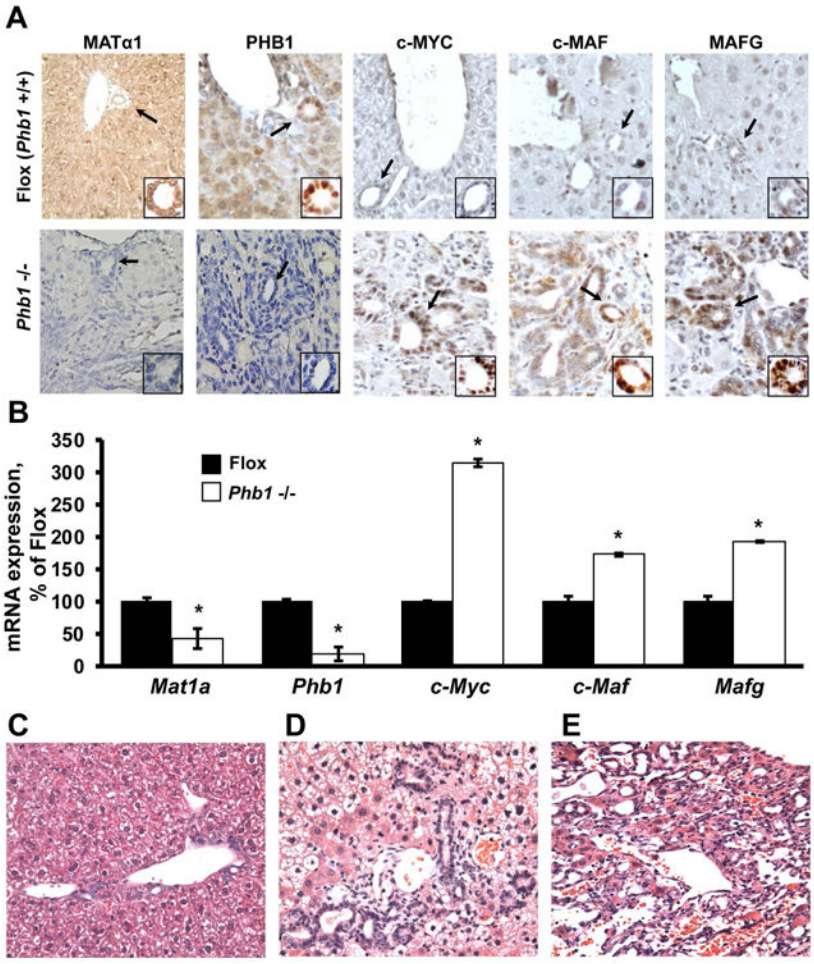

Figure 6. Liver-specific *Phb1* KO mice have altered MAT1A, c-MYC, MAFG and c-MAF expression, develop HCC and CCA

Expression of PHB1, MATα1, c-MYC, MAFG and c-MAF was measured using **(A)** IHC (arrows point to the bile duct in the insets at the bottom right of each IHC image) and **(B)** real-time PCR in liver-specific *Phb1* KO mice livers (n=3 for each group). Comparison of liver histology on hematoxylin and eosin (H&E) between **(C)** 8-months old flox controls (normal histology) and liver-specific *Phb1* KO mice (all 8 mice have HCC, not shown) show **(D)** atypical hyperplasia of bile ducts in 6 out of 8 mice, and **(E)** CCA in 1 out of 8. Original magnification, X200 for all.

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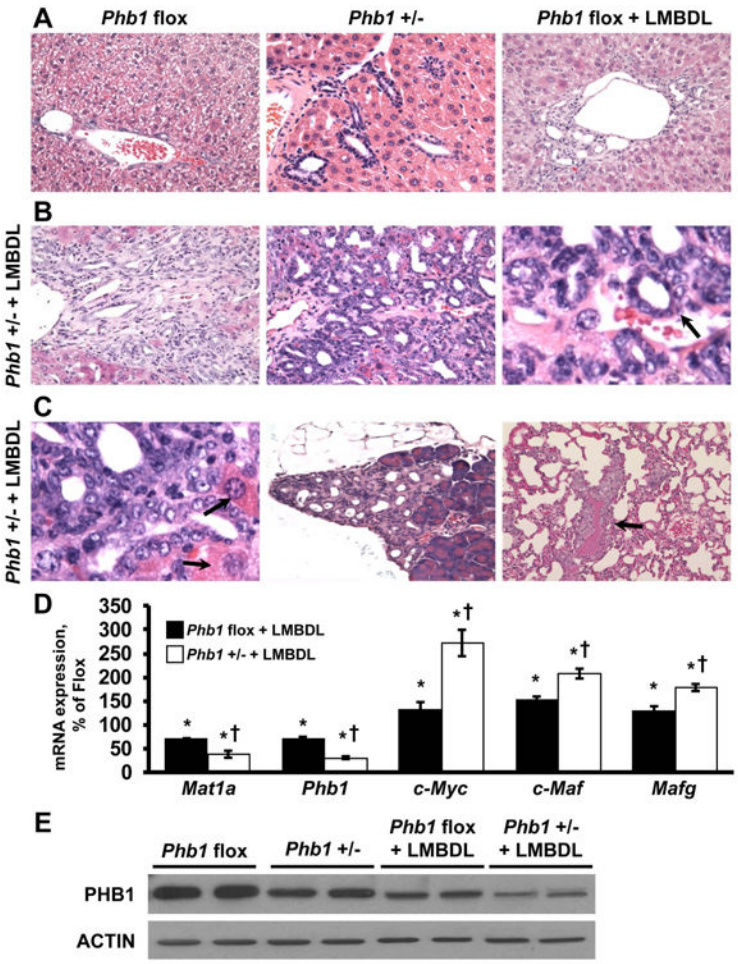

Figure 7. Liver-specific *Phb1* heterozygotes are sensitized to develop LMBDL-induced CCA

5-month old liver-specific *Phb1* heterozygotes and flox controls (n=8 each group) were subjected to LMBDL as described in Experimental Procedures and followed for 3.5 months. **(A)** Flox control (left panel); heterozygous control (middle panel), showing proliferation of bile duct epithelial cells in 5/8 at baseline; flox LMBDL control (right panel), where bile duct proliferation was found in portal tract in 8/8; **(B)** heterozygous LMBDL mice exhibited aberrant bile duct proliferation (left panel) in 8/8; CCA (middle panel) was found in 1/8 heterozygotes after LMBDL with vascular invasion (right panel, arrow), **(C)** invasion to adjacent tissue (left panel, arrows), pancreas (middle panel) and lung metastasis (right panel, arrow). Representative H&E are shown, original magnification, X200 for all except X400 for F and G. **(D)** Changes in *Phb1*, *Mat1a*, *c-Myc*, *Mafg* and *c-Maf* expression after LMBDL, expressed as % of *Phb1* flox control. Real-time PCR measured mRNA levels of these genes from n=3 in each group. *p< 0.05 vs Flox, †p < 0.05 vs *Phb1* flox + LMBDL. **(E)** Western blotting showed comparable changes in PHB1 protein levels.

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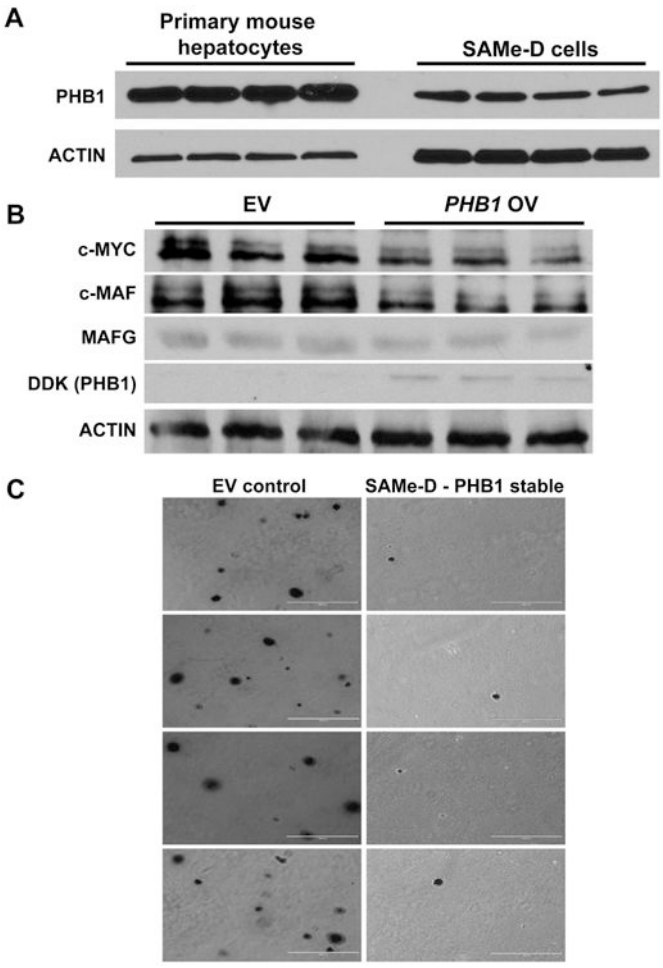

Figure 8. PHB1 is a tumor suppressor in SAMe-D cells

(A) PHB1 expression is lower in SAMe-D cells, which are derived from HCC of a *Mat1a* KO mouse. **(B)** Effect of PHB1 overexpression on expression of c-MYC, c-MAF, and MAFG in SAMe-D cells. **(C)** Anchorage-independent growth in SAMe-D cells. SAMe-D cells were stably transfected with PHB1 overexpression vector as described in Experimental Procedures and anchorage-independent growth was measured. Overexpression of PHB1 had a profound inhibitory effect on anchorage-independent growth.