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Hepatic Prohibitin 1 and Methionine Adenosyltransferase α1 Defend Against Primary and Secondary Liver Cancer Metastasis

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Abstract

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WF - reviewed the literature, drafted the manuscript, data collection and figure preparation
DC, JW, HY - data collection, analysis, and interpretation
BY, TWHL - assisted in the literature review and figure preparation
SS, ES, MLT, JMM - critical reading of the manuscript
AG, EP – provided human CRLM tissue array
SY – provided bioinformatics support and mutation analysis of the TCGA database
DDV, DK – assisted with characterization of extracellular vesicles
XL – assisted with immunohistochemistry
SCL - study concept and design, data analysis and interpretation, edited the manuscript, obtained funding, and provided overall study supervision

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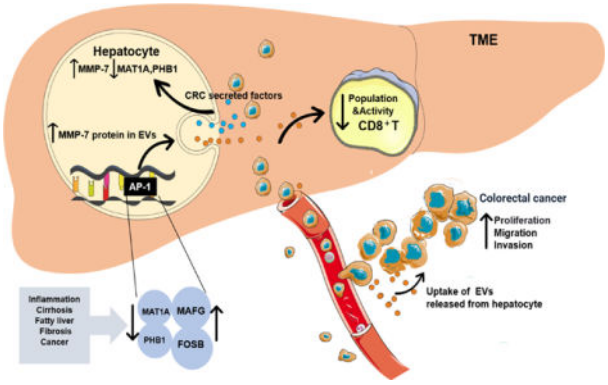
Background & Aims: Liver is a common site of cancer metastasis (a.k.a. secondary liver cancer, most commonly from colorectal cancer (CRC)) and primary liver cancers that have metastasized have poor prognosis. The underlying mechanisms of how the liver defends against these processes are largely unknown. Prohibitin 1 (PHB1) and methionine adenosyltransferase 1A (MAT1A) are highly expressed in the liver. They positively regulate each other and their deletion results in primary liver cancer. Here we investigated their roles in primary and secondary liver cancer metastasis.

Methods: We identified common target genes of *PHB1* and *MAT1A* using metastasis array, used luciferase reporter assay and chromatin immunoprecipitation to measure promoter activity and transcription factors binding. We examined how PHB1 or MAT1A loss promotes liver cancer metastasis and whether their loss sensitizes to CRC liver metastasis (CRLM).

Results: Matrix metalloproteinase-7 (MMP-7) is a common target of MAT1A and PHB1 and its induction is responsible for increased migration and invasion when MAT1A or PHB1 is silenced. Mechanistically, PHB1 and MAT1A negatively regulate the *MMP-7* promoter activity via an AP-1 site by repressing the MAFG-FOSB complex. Loss of *MAT1A* or *PHB1* also increased MMP-7 in extracellular vesicles, which was internalized by colon and pancreatic cancer cells to enhance their oncogenicity. Low hepatic MAT1A or PHB1 expression sensitized to CRLM, but not if endogenous hepatic MMP-7 was knocked down first, which lowered $CD4^+$ T cells while increasing $CD8^+$ T cells in the tumor microenvironment. Hepatocytes co-cultured with CRC express less MAT1A/PHB1 but higher MMP-7. Consistently, CRLM raised distant hepatocytes' MMP-7 expression in mice and humans.

Conclusion: We have identified an PHB1/MAT1A-MAFG/FOSB-MMP-7 axis that controls primary liver cancer metastasis and sensitization to CRLM.

Graphical Abstract



In hepatocytes, decreased MAT1A and PHB1 or increased MAFG and FOSB expression promote transcriptional activation of *MMP-7* via a critical AP-1 site. EVs from hepatocytes containing high MMP-7 (brown circles) may be taken up by CRCs, resulting in more aggressive characteristics. Meanwhile, CRCs can release factors (blue circles) that lower hepatocytes' MAT1A/PHB1 expression while upregulating MMP-7, which inhibits the activity of cytotoxic $CD8^+$ T cells.

Keywords

Prohibitin 1; methionine adenosyltransferase 1A; matrix metalloproteinase-7; MAFG; FOSB; hepatocellular carcinoma; cholangiocarcinoma; colorectal cancer; metastasis

INTRODUCTION

Primary liver cancers are a leading cause of cancer death worldwide, with 5-year survival rates of 33% for hepatocellular carcinoma (HCC) and 10–15% for cholangiocarcinoma (CCA) if diagnosed early. However, if either cancer has spread beyond regional lymph nodes, the survival rate drops to just 3%. Secondary liver cancers are cancers that originate elsewhere and metastasis to the liver, with colorectal liver metastasis (CRLM) being the most common is a major cause of death. The liver's ability to support metastasis has been linked to its dual blood supply and immune-tolerant environment,[1] but whether the liver has mechanisms that defend against primary and secondary liver cancer metastasis is unknown.

Here we unveil a previously unrecognized axis involving prohibitin 1 (PHB1) and methionine adenosyltransferase 1A (MAT1A) that is important for both primary and secondary liver cancer growth and metastasis. PHB1, a mitochondrial chaperone that is evolutionary conserved and ubiquitously expressed, is involved in multiple essential cellular functions including apoptosis, survival, and cellular signaling.[2,3] We reported PHB1 acts as tumor suppressor in the liver and its expression is lower in most human HCC and CCA patients.[4] Consistently, liver-specific *Phb1* knockout (KO) mice developed HCC and heterozygotes are predisposed to aberrant bile duct proliferation and CCA after left and median bile duct ligation.[4,5]

MAT1A encodes for MATα1, which is mainly expressed in the liver and is responsible for S-adenosylmethionine (SAMe) synthesis.[6] MAT1A is mainly expressed in normal differentiated liver, its expression is downregulated in most cirrhotic patients and HCC.[7] We showed mice lacking *Mat1a* spontaneously develop steatohepatitis[6] and HCC.[8] Interestingly, PHB1 and MAT1A exert reciprocal positive regulation on each other.[4,9] Furthermore, they interact with each other and with MAX, c-MYC, and MAFG at the E-box element to repress E-box-dependent gene expression.[4,10,11] These observations prompted us to investigate whether they might cooperate and whether there are common target genes that may be involved in liver cancer metastasis.

In this study, we identified that MAT1A and PHB1 negatively regulate matrix metalloproteinase-7 (MMP-7) expression at the transcriptional level. We defined the underlying molecular mechanisms and identified an PHB1/MAT1A-MAFG/FOSB-MMP-7 axis that is important in primary liver cancer metastasis. We also found that reduced expression of hepatic MAT1A or PHB1 sensitizes the liver to CRC metastatic growth, but this was abolished if the hepatic endogenous MMP-7 was silenced first. Taken together, these results suggest hepatic MAT1A and PHB1 defend against metastasis of primary and secondary liver cancers.

MATERIALS AND METHODS

See Supplementary CTAT Table and Supplemental Materials & Methods for details.

RESULTS

PHB1 and *MAT1A* mRNA levels are lower in metastatic HCC and CCA

Low *MAT1A* mRNA levels correlated with worse survival in HCC patients.[12] Lower *PHB1* mRNA levels also correlated with poorer survival in advanced HCC patients (Fig. S2A) and in HCC with metastasis inclined microenvironment (Fig. S2B). Similarly, decreased *MAT1A* mRNA levels were observed in HCC with vascular invasion (Fig. S2C) and advanced-stage HCC (Fig. S2D). Consistently, PHB1 and MAT1A expression in HCC that metastasized to the lung is lower than primary HCC, which is lower than adjacent non-tumorous liver (Fig. S2E).

PHB1 and MAT1A regulate tumor metastasis related genes in liver cancer cell lines

To identify common target genes regulated by PHB1 and MAT1A, we silenced both genes and examined changes using a tumor metastasis array. We found the expression of 75 tumor related genes were altered (Table S1) and focused on MMP-7, the most significantly upregulated oncoprotein (Fig. S3A). *MMP-7* mRNA levels are negatively correlated with *MAT1A* and *PHB1* mRNA levels in HCC and CCA GEO databases (Fig. S3B). Of the top nine genes upregulated, only MMP-7 was consistently induced in multiple cell lines (Fig. S3C). The others were either inconsistent or too low for detection (HGF and MMP-10) (Fig. S4). MMP-7 expression is higher in 1-month old liver-specific *Phb1* KO mice liver (Fig. S3D), 4-months old *Mat11a* KO hepatocytes (Fig. S3E), and *Mat1a* KO HCCs (Fig. S3F). MMP-7 expression and activity are upregulated in HCC lines from *Mat1a* and *Phb1* KOs compared to primary mouse hepatocytes (PMH) (Fig. S3G–J). Since *MAT1A* encodes the enzyme responsible for SAMe synthesis and SAMe can inhibit genes by an epigenetic pathway,[16] we next examined whether the catalytic activity of MAT is required to inhibit MMP-7 expression. We found no difference between overexpressing WT MAT1A and catalytic mutant of MAT1A, which indicates MAT1A's effect is SAMe-independent (Fig. S3K).

PHB1 and MAT1A negatively regulate MMP-7 expression in liver cancer cell lines

Next, we varied the expression of PHB1 and MAT1A in multiple cell lines and found overexpressing either PHB1 or MAT1A suppressed while knocking them down increased *MMP-7* mRNA (Fig. S3C, 1A) and protein levels in the cell (Fig. 1B) and in the medium (Fig. 1C). Since PHB1 and MATα1 interact with each other and positively regulate each other's expression,[4,9] we examined whether they exert additive or synergistic effects on MMP-7 expression. Double knockdown or overexpression of PHB1 and MAT1A in MzChA-1A (Fig. 1D–E) and Hep3B (Fig. S5A–B) cells exerted additive effects on *MMP-7* mRNA levels and promoter activity compared to single knockdown or overexpression. However, combining MAT1A overexpression with PHB1 knockdown or vice versa had minimal effects on *MMP-7* mRNA levels and promoter activity (Fig. S5C–D). These results

support the notion that PHB1 and MAT1A cooperate to suppress MMP-7 expression at the transcriptional level.

MMP-7 expression profile in metastatic liver cancer and correlation with HCC patient survival

MMP-7 mRNA levels are higher in HCC with metastasis (Fig. S6A–B). In a more advanced HCC dataset (GSE45114), MMP-7 expression correlated inversely with PHB1 and MAT1A, while PHB1 and MAT1A correlated positively with each other (Fig. S6C–E). This is also true in the TCGA database of HCC with microvascular invasion (Fig. S6F–G). Consistently, high MMP-7 expression correlated with poor survival in the TCGA database (Fig. S6H). We also examined using the TCGA database whether there are certain mutations that correlated with lower PHB1 and MAT1A expression. Of the most frequently and significantly mutated genes (26 genes),[15] tumors that have RPS6KA3 or EEF1A1 mutations exhibit reduced expression of PHB1 whereas MAT1A expression was reduced in the tumors with RB1 or TP53 mutations (Fig. S7).

Interplay between PHB1/MAT1A and MAFG/FOSB in regulating MMP-7 expression

We reported PHB1 and MAT1A suppress MAFG expression in liver cancer cells.[4,10] MAFG can form heterodimers with FOSB and transactivate *MMP-1* promoter via an AP-1 element.[17] The human *MMP-7* promoter has a functional enhancer AP-1 element.[18] To see if MAFG and FOSB regulate MMP-7, we varied their expression in HCC and CCA cell lines and found they positively regulate MMP-7 expression (Fig. 2A). Meanwhile, PHB1 and MAT1A negatively regulate FOSB expression (Fig. 2B). Lastly, SAMe-D and PHB1-D cells express much higher levels of FOSB and MAFG compared to PMH (Fig. 2C). Similar to MAT1A and PHB1, MAFG and FOSB also cooperate to regulate MMP-7 expression at the transcriptional level as demonstrated by double overexpression or knockdown (Fig. 2D–E).

PHB1 and MAT1A negatively while MAFG and FOSB positively regulate MMP-7 promoter activity via AP-1

We next measured reporter activity of the full-length human *MMP-7* promoter (−1270 to +20bp) (Fig. S8A) as the expression of PHB1, MAT1A, MAFG and FOSB was varied. We found overexpressing PHB1 and MAT1A or silencing MAFG and FOSB suppressed *MMP-7* promoter activity, while silencing PHB1 and MAT1A, or overexpressing MAFG and FOSB had the opposite effect (Fig. 3A). Consistently, *MafG* and *FosB* mRNA levels are upregulated in rat CCA peritoneal metastasis samples and positively correlated with *Mmp-7* expression (Fig. S8B–C). We next investigated the role of the AP-1 site in the *MMP-7* promoter using site-directed mutagenesis and found that MAT1A, PHB1, MAFG or FOSB had no effect on the *MMP-7* promoter activity when the AP-1 site of the full-length promoter was mutated (Fig. 3B).

To further examine whether PHB1, MATα1, MAFG and FOSB bind to the AP-1 site, we used ChIP, Seq-ChIP and EMSA assays. None of these proteins can bind directly to the promoter region that includes the AP-1 site alone on ChIP (not shown). On Seq-ChIP following ChIP using anti-c-JUN antibody, all were able to bind (Fig. 3C). Overexpressing

MAFG and FOSB or silencing MAT1A and PHB1 increased FOSB and MAFG binding, whereas silencing MAFG/FOSB or overexpressing MAT1A/PHB1 had the opposite effects (Fig. 3C). c-JUN is not required to bind to the AP-1 site, as although MAFG, FOSB, MATα1, and PHB1 recombinant proteins individually could not bind to the AP-1 site on EMSA, complexes of MATα1 with PHB1, MAFG or FOSB were able to bind. In addition, complexes of MATα1, PHB1 with MAFG or FOSB and all four proteins were able to bind as indicated by "supershifting" the band's position (Fig. 3D). Lastly, silencing c-JUN did not prevent MAFG overexpression from inducing MMP-7 expression (Fig. S5E). These findings suggest the PHB1/MATα1-MAFG/FOSB complex can bind to the AP-1 site of the *MMP-7* promoter to regulate its activity, with PHB1/MATα1 repressing while MAFG/FOSB activating the promoter.

Loss of PHB1 and MAT1A increase invasion and migration via MMP-7 in liver cancer cells

To examine the effects of PHB1, MAT1A and MMP-7 on cell migration and invasion, we varied their expression in Hep3B and MzChA-1 cells for 48 hours. We found MMP-7 overexpression or PHB1 and MAT1A knockdown significantly increased cell migration and invasion, whereas MMP-7 knockdown or overexpression of either PHB1 or MAT1A had the opposite effect (Fig. 4A–C, Fig. S9A–B). Combining MMP-7 with PHB1 or MAT1A overexpression eliminated MMP-7's migration and invasion inductive effects, but silencing MMP-7 largely eliminated PHB1 and MAT1A knockdown-mediated inductive effect on migration and invasion (Fig. 4A–C, Fig. S9A–B).

Effects of PHB1, MAT1A, and MMP-7 on tumorigenicity and metastasis in vivo

To further confirm MMP-7 as a key downstream target that enhances metastasis when MAT1A or PHB1 expression is lost in vivo, we used CRISPR KO of MMP-7 in MATα1-D and PHB1-D cells (Fig. S1C). Growth and metastasis were assessed by small animal imaging after intrahepatic implantation of MATα1-D cells with stable MMP-7 knockdown or scramble control (SC) in nude mice for up to 40 days, or PHB1-D cells in WT immunocompetent C57BL/6 mice for up to 10 days. We found silencing MMP-7 strongly inhibited HCC cell growth and metastasis in both models (Fig. 4D–E).

Effects of MMP-7 containing EVs on MC38 and UN-KPC cells' oncogenic activity

MMP-7 is released in EVs.[19] To evaluate the effects of EVs extracted from MATα1-D and PHB1-D cell culture media, and sera of *Mat1a* and *Phb1* KO mice on MC38 (murine CRC) and UN-KPC (murine PDAC) cells' oncogenic activity, we first characterized isolated EVs by electron microscopy and NanoSight. We found increased secretion of EVs ranging in size from 20nm to 1000nm in the culture media of MATα1-D and PHB1-D cells and sera of liver-specific *Phb1* KO mice compared to PMH and sera from *Phb1* flox mice, respectively (Fig. 5A–B, S10A–B). We further characterized the EVs and found that although MMP-7 is found in both large and small EVs, it is more abundant in small EVs (S10C-E). MMP-7 expression in EVs secreted by MATα1-D, PHB1-D cells, and *Mat1a, Phb1* KO, *Phb1* heterozygous mice is higher as compared to AML12 cells and WT or flox mice, respectively (Fig. 5C, E). Furthermore, EVs of *Mat1a and Phb1* KO mice increased MMP-7 expression in MC38 cells (Fig. 5D) and promoted cells proliferation, migration and invasion (Fig. 5F–G). Importantly, the enhanced proliferation, migration and invasion in MC38 and UN-KPC

cells treated with MATα1-D, PHB1-D cells derived EVs (20μg/ml) were eliminated by knocking down MMP-7 in these cells first (Fig. 5H–I, S9C–D).

Mat1a KO and liver-specific Phb1 heterozygotes are sensitized to CRLM via an MMP-7-dependnt mechanism

We next examined the effect of hepatic MAT1A and PHB1 on liver metastasis from CRC using the CRLM human tissue array and mouse model. First, we found normal mouse hepatocytes have low to no MMP-7 expression (Fig. 6A top). However, in human CRLM tissue array, MMP-7 expression is highly induced in hepatocytes surrounding CRLM, both adjacent and many cells away from the CRLM (Fig. 6A bottom, Fig. S11–S12). In the mouse CRLM model, four-months old *Mat1a* KO mice are sensitized to CRLM growth and distant metastasis to lung and pancreas (Fig. 6B–C, S13A). At this age *Mat1a* KO livers are histologically normal.[6] In contrast, liver-specific *Phb1* KO mice have liver injury at an early age[5] so we used *Phb1* heterozygotes for the CRLM experiment instead. We found *Phb1* heterozygotes are also sensitized to CRLM (Fig. 6B–C). *Phb1* heterozygotes also have increased MMP-7, MAFG and FOSB expression (Fig. S13B–E), and higher MMP-7 content in serum EVs (Fig. S13F). To examine if hepatic MMP-7 expression could be contributing to the sensitization to CRLM, we silenced hepatic MMP-7 using adenovirus CRIPSR mMmp-7 vector four days prior to injecting MC38 cells. We found silencing hepatic MMP-7 reduced CRLM growth in WT mice and completely eliminated the heightened sensitivity of the *Mat1a* KO and *Phb1* heterozygotes to CRLM growth (Fig. 6B–C) without causing liver toxicity as measured by ALT and AST (Fig. S13G). To determine if colon cancer cells can induce MMP-7 expression in hepatocytes, we co-cultured human hepatocytes with RKO cells and found presence of RKO cells in the insert increased MMP-7 while decreasing MAT1A and PHB1 expression in human hepatocytes (Fig. 6D).

Effects of MMP-7 knockdown on the tumor microenvironment (TME) in CRLM

To better understand the influence of MMP-7 on the immune cells of the TME, we measured $CD4^+$, and $CD8^+$ T-lymphocyte content in isolated CRLM samples by flow cytometry (see Table S2 for antibodies used). We found that the $CD4^+$ T cell number was consistently decreased while $CD8^+$ T cell number increased in WT, *Mat1a* KO, and *Phb1* heterozygous mice when MMP-7 was silenced (Fig. 7A, S14A). To determine the $CD8^+$ T cell activation status, we further examined T cell activation markers - Ki-67, IFN-γ, perforin, and granzyme B (GrB) levels (Fig 7B, S14B) and found increased T cell activity in the MMP-7 KO group. Immunofluorescence staining validated elevated Ki67 expression in $CD8^+$ T cells in the MMP-7 KO group (Fig. S15A). Co-staining of the CRLM tissue also confirmed less than 50% of the $CD3^+$ cells also stained for CD4 or CD8 in *Mat1a* KO mice (Fig. S15B).

DISCUSSION

PHB1 is a ubiquitously expressed protein that exerts different biological functions depending on its subcellular localization.[20] MATα1 is also found in multiple subcellular locations.[16] Nuclear PHB1 and MATα1 can act as transcription co-factor to repress multiple oncogenes.[16,20] Although both PHB1 and MAT1A are downregulated in HCC, they are

associated with different mutations, suggesting they are regulated by distinct pathways. While we have reported on how loss of MAT1A and PHB1 leads to HCC and CCA, whether they are important for primary liver cancers to metastasize has not been examined. Furthermore, whether they can influence CRC liver metastasis is also unknown. In the current work we tested the central hypothesis that MAT1A and PHB1 form a major defense against primary and secondary liver cancer metastasis.

Analysis of publicly available liver cancer metastasis datasets supports the notion that lower MAT1A and PHB1 expression favor primary liver cancer metastasis. Consistently, low MAT1A expression correlated with increased metastasis and lower recurrence-free survival in HCC patients.[16] Moreover, MAT1A was identified as a downstream target of ARIDIA to suppress HCC metastasis.[21] However, the underlying mechanism(s) of how MAT1A and PHB1 suppress liver cancer metastasis remains incompletely understood.

Searching for common targets of PHB1 and MAT1A led to the identification of MMP-7 as a key downstream target that is inhibited by both. Consistently, multiple publicly available databases show inverse correlation of *MAT1A* and *PHB1* mRNA levels with *MMP-7* mRNA levels. MMP-7 belongs to a family of proteolytic enzymes that degrades components of the extracellular matrix (ECM) and non-matrix proteins. Unlike most MMPs, MMP-7 is constitutively expressed by many epithelial cell types, including the liver, but is minimally expressed in normal hepatocytes.[22] MMP-7 is the smallest but highly potent metalloproteinase that is implicated in cancer invasion.[23,24] While the action of MMP-7 in cancer is well documented, how its expression is regulated in normal liver and whether hepatocyte's MMP-7 expression participates in determining susceptibility to CRC metastatic growth in the liver has not been examined to the best of our knowledge.

MMP-7 is a key downstream target in primary liver cancer metastasis when either MAT1A or PHB1 is downregulated in vitro and in vivo. However, MAT1A and PHB1 have other targets because overexpressing MMP-7 could not overcome their suppressive effects. For MAT1A, we identified LIN28B, oncogene that is associated with HCC invasion, is suppressed when MAT1A is overexpressed.[25] We also found MAT1A negatively regulates FOXM1[12] and YWHAZ,[26] oncogenes that are linked to cancer metastasis. For PHB1, we showed PHB1 reduces IL-8 expression by lowering nuclear NF-κB and c-JUN content[27] and IL-8 is known to promote cancer invasion and metastasis.[28] PHB1 also negatively regulates WNT-beta-catenin signaling pathway, which is important in liver cancer metastasis.[29] Taken together, when either MAT1A or PHB1 is lost in liver cancer cells, upregulation of MMP-7 is the key event that triggers metastasis. However, MAT1A and PHB1 can suppress primary liver cancer metastasis by multiple mechanisms. MMP-7 is also essential for liver cancer growth in our study. MMP-7 is known to promote cancer growth, in part by degrading all insulin-like growth factor binding proteins (IGFBPs) and promoting IGF-1 signaling and inhibiting FAS-mediated apoptosis.[30]

We next investigated how MAT1A and PHB1 inhibit MMP-7 expression. The AP-1 site at −67 bp upstream of the transcriptional start site has long been thought to play a dominant role in the transcriptional activation of the *MMP-7* promoter.[31] The MAFG-FOSB complex activates *MMP-1* promoter[17] but whether it can activate *MMP-7* promoter has not been

investigated. Like their suppressive effect on MAFG expression[10], both MAT1A and PHB1 also inhibit the expression of FOSB. We found that MAFG-FOSB complex binds to the AP-1 site to *trans*-activate the *MMP-7* promoter. Using EMSA, ChIP, and Seq-ChIP, we derived a complex regulation of the *MMP-7* promoter activity where MATα1 and PHB1 are repressors of the AP-1 site whereas MAFG and FOSB are enhancers. Furthermore, we found while MATα1 and PHB1 alone cannot bind to the AP-1 element, the complex of MATα1 and PHB1 can bind without any other proteins on EMSA. This broadens the implication of these two proteins in transcriptional regulation of genes that harbor functional AP-1 elements in the liver, where both proteins are highly expressed. Thus, the *MMP-7* promoter activity and expression will depend on the relative abundance of the four proteins, with MATα1/PHB1 suppressing and MAFG/FOSB activating.

Since MMP-7 is released into the ECM and in EVs, we asked whether loss of hepatic MAT1A or PHB1 could sensitize the liver to cancer metastasis via MMP-7 in the EVs. Indeed, we found higher MMP-7 content in EVs isolated from culture media of MATα1-D and PHB1-D cells and sera of the corresponding KOs. Further, when these EVs are internalized by CRCs and PDACs, they promoted growth, migration, and invasion. However, if MMP-7 was silenced first in these cells, the EVs no longer had any oncogenic activity. Taken together, these results support a scenario where MMP-7 in the EVs released by the liver or liver cell when either MAT1A or PHB1 is downregulated can promote oncogenic activity of circulating tumor cells. This concept was further demonstrated when we found *Mat1a* KO and liver-specific *Phb1* heterozygotes were sensitized to CRLM and distant metastasis, and this sensitization was eliminated when we knocked down hepatocytes' MMP-7 expression four days prior to injecting the CRC cells intrasplenically. The adenovirus CRISPR delivery efficiently blocked hepatocytes' MMP-7 expression in the CRLM model, confirming this vector targets hepatocytes well.

An interesting observation from our study is induction of distant hepatocytes' MMP-7 expression when there is CRC in the liver. This is true in humans and mice. Our co-culture experiments suggest a scenario where colon cancer cells release factors that lower MAT1A and PHB1 and induce MMP-7 expression and release. The nature of these factors will be the subject of future investigation.

Degradation of ECM by MMPs creates an optimal microenvironment for tumor metastasis. Some tumor infiltrating lymphocytes (TILs) can contribute to tumor progression in CRC and many solid tumors.[32] MMPs also play an essential role in TILs.[33,34] Importantly, high $CD8^+$ T cell staining in CRLM correlated with good survival and high $CD4^+$ T cell number correlated with poor survival.[35] Our findings that of MMP-7 knockdown was associated with a higher $CD8^+$ T cells and lower $CD4^+$ T cells are consistent with these reports. Perforin, granzymes, IFN-γ, and $Ki67^+$ cells levels are a measurement of the effector T cell activation.[36] Our results showed MMP-7 could also inhibit effector T cell activation. The underlying mechanisms, however, will require further investigation.

In summary, we have demonstrated that loss of PHB1 and MAT1A induce MMP-7 expression and release in EVs, which can promote primary and secondary liver cancer growth and metastasis. This process is mediated by activation of the MAFG-FOSB-MM-7

axis that is normally suppressed by PHB1 and MAT1A. Thus, normal expression of hepatic MAT1A and PHB1 serves as a key defense mechanism to protect the liver from cancer metastasis. However, once cancer arrives in the liver, factors are released from the cancer that lower this defense and raise MMP-7 expression to drive the metastatic process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

All data associated with this study are included within the main text or the supplementary section and CTAT methods table. Further data supporting the findings of this study is available upon request to the corresponding author.

Abbreviations:

AFP	Alpha-fetoprotein
CCA	Cholangiocarcinoma
ChIP	Chromatin immunoprecipitation
CRC	Colorectal cancer
CRLM	Colorectal cancer liver metastasis
ECM	Extracellular matrix
EMSA	Electrophoretic mobility shift
EV	Extracellular vesicle
Granzyme B	GrB
H&E	Hematoxylin and Eosin
HCC	Hepatocellular carcinoma
IHC	Immunohistochemistry
KO	knocked down
Luc	Luciferase
MAT1A	Methionine adenosyltransferase 1A

Mets	Cancer metastasis
MMP-7	Matrix metalloproteinase-7
NL	Normal liver
OV	Overexpressing
PDAC	Pancreatic ductal adenocarcinoma
PHB1	Prohibitin 1
PMH	Primary mouse hepatocytes
SAMe	S-adenosylmethionine
SC	scramble control
Seq-ChIP	sequential - Chromatin immunoprecipitation
TILs	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
WT	Wild type

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Impact and Implications

Primary and secondary liver cancer metastasis is associated is poor outcome but whether the liver has underlying defense mechanism(s) against metastasis is unknown. Here we examined the hypothesis that hepatic prohibitin 1 (PHB1) and methionine adenosyltransferase 1A (MAT1A) cooperate to defend the liver against metastasis. Our studies found PHB1 and MAT1A form a complex that suppresses matrix metalloproteinase-7 (MMP-7) at the transcriptional level and loss of either PHB1 or MAT1A sensitizes the liver to metastasis via MMP-7 induction. Strategies that target the PHB1/MAT1A-MMP-7 axis may be a promising approach for the treatment of primary and secondary liver cancer metastasis.

Highlights

- Hepatic MMP-7 is induced when either PHB1 or MAT1A is downregulated.
- PHB1-MAT1A repress while MAFG-FOSB activate *MMP-7* transcription via an AP-1 site.
- MMP-7 is secreted in EVs and taken up by cancer cells to increase oncogenic activity.
- Silencing MMP-7 blocked increased CRC liver metastasis in *Mat1a* KO and *Phb1*$^{+/-}$ mice.
- Presence of CRC in liver lowered hepatocytes' MAT1A but raised MMP-7 expression.

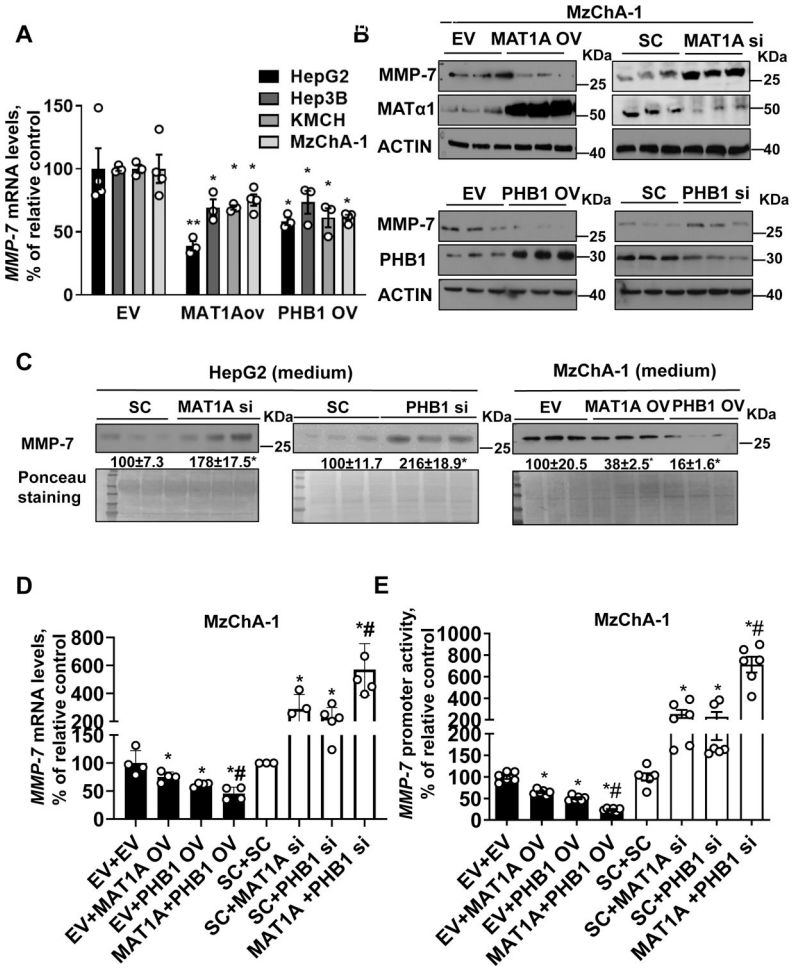

Figure 1. PHB1 and MAT1A cooperate to negatively regulate MMP-7 expression at the transcriptional level.

Cells were transfected with MAT1A or PHB1 overexpression vector (OV) or treated with siRNA (si) for 48 hours prior to measuring MMP-7 expression in the cells and medium. **A**) *MMP-7* mRNA levels, ANOVA test, *p = 0.01, 0.05, 0.025, 0.01, 0.04, 0.04, 0.05, and 0.014 vs EV. **B**) intracellular and **C**) secreted MMP-7 protein levels in MzChA-1 and HepG2 cells. Student's t-test, ANOVA test, *p = 0.028, 0.013, 0.03, and 0.014. **D**) *MMP-7* mRNA levels and **E**) promoter activity were examined after MAT1A or PHB1 OV alone or combined, and MAT1A or PHB1 siRNA alone or together in MzChA-1 cells. Results are expressed as mean ± SEM from at least three independent experiments, ANOVA test, *p = 0.05, 0.014, 0.01, 0.026, 0.029, and 0.011 vs. respective controls (EV+EV, and SC+SC), #p = 0.006, 0.037, 0.03, and 0.004 vs. single OV or si.

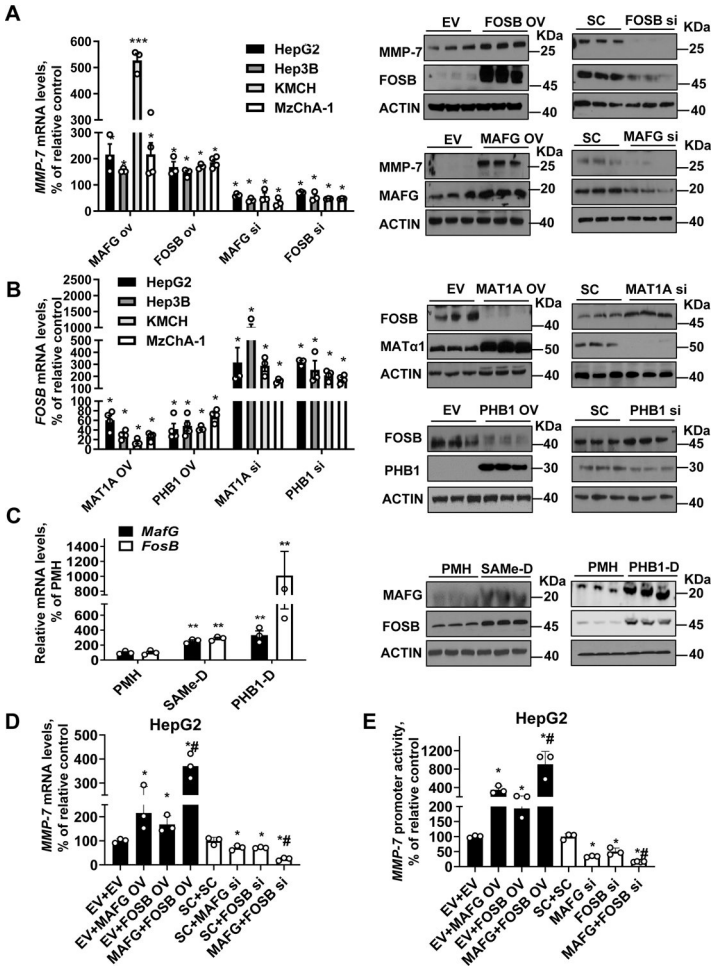

Figure 2. MAFG and FOSB positively regulate MMP-7 while PHB1 and MAT1A negatively regulate FOSB expression.

A) mRNA and protein levels of MMP-7 were measured by RT-qPCR and western blot after varying MAFG and FOSB expression for 48 hours. ANOVA test, *p = 0.047, 0.018, 0.05, 0.03, 0.01, 0.04, 0.01, 0.016, 0.04, 0.028, 0.017, 0.03, 0.014, 0.02, and 0.04, ***p = 0.000087 vs. EV or SC **B**) FOSB expression was examined after varying PHB1 and MAT1A expression for 48 hours. ANOVA test, *p = 0.034, 0.05, 0.018, 0.028, 0.024, 0.01, 0.018, 0.013, 0.015, 0.01, 0.012, 0.045, 0.011, 0.012, 0.024, and 0.026 vs. EV or SC **C**) MAFG and FOSB expression were measured in SAMe-D and PHB1-D cells as compared to primary mouse hepatocytes (PMH). ANOVA test, **p = 0.00151, 0.00157, 0.00137, and 0.0049 vs. PMH **D**) *MMP-7* mRNA levels and **E**) promoter activity were examined after MAFG or FOSB OV alone or combined, and MAFG or FOSB siRNA alone or together in HepG2 cells. ANOVA test, *p = 0.047, 0.031, 0.018, 0.037, 0.047, 0.029, 0.016, 0.04, 0.017, 0.013, 0.017, and 0.014 vs. EV+EV and SC+SC, $^{\#}$p = 0.037, 0.0048, 0.002, 0.0027, 0.029, 0.011, 0.0049, and 0.002 vs. single OV or si. Results are expressed as mean % of EV ± SEM from at least three independent experiments.

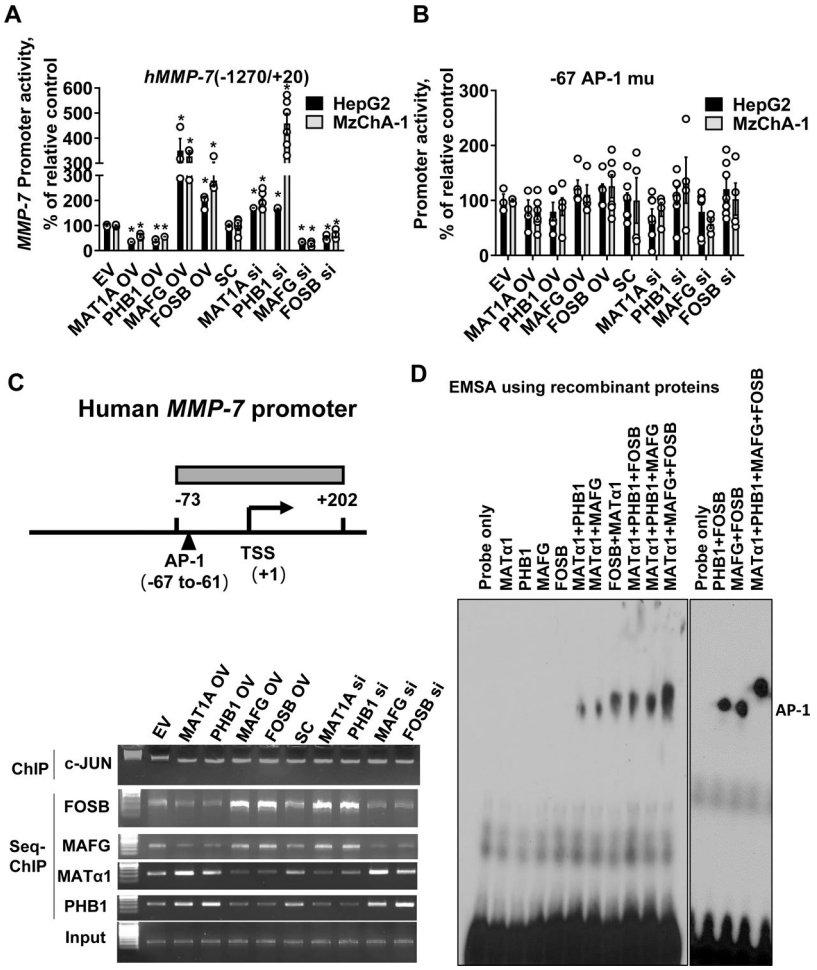

Figure 3. Effects of PHB1, MAT1A, MAFG and FOSB on *MMP-7* promoter activity.

A) Effects of varying PHB1, MAT1A, MAFG and FOSB expression on WT *MMP-7* (−1270 to +20bp) and **B**) *MMP-7* promoter activity with AP-1 site mutation. AP-1 site mutant MMP-7 promoter activity was ~ 20% of the WT promoter (data not shown). Results are expressed as mean % of EV or SC ± SEM from three experiments done in duplicates. **C**) ChIP was done using anti-c-JUN antibody followed by Seq-ChIP using antibodies to PHB1, MATα1, MAFG and FOSB to examine binding to the *MMP-7* promoter (−73 to +202 bp) containing the AP-1 site. **D**) EMSA was done using the consensus sequence of AP-1 using recombinant proteins (1μg) alone or combined. Results are representative of three independent experiments, ANOVA test, *p = 0.01, 0.025, 0.015, 0.018, 0.02, 0.015, 0.016, 0.04, 0.05, 0.042, 0.028, 0.04, 0.015, 0.017, 0.035, and 0.038 vs. EV or SC.

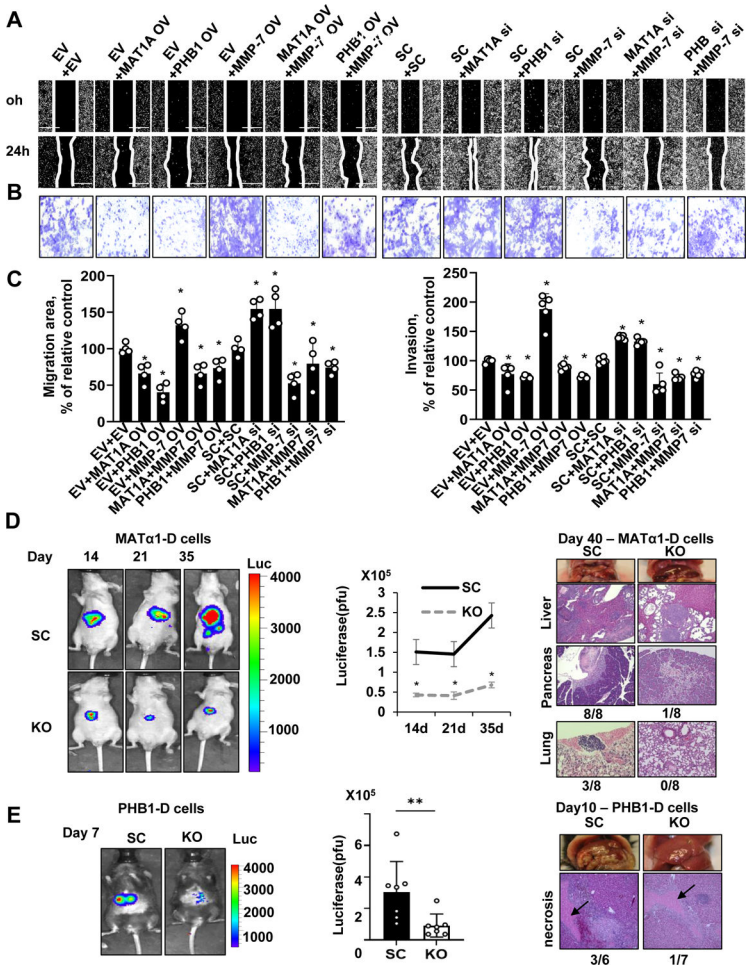

Figure 4. Effects of PHB1, MAT1A, and MMP-7 on migration and invasion.

A) Effects of varying PHB1, MAT1A and MMP-7 expressions alone or together on MzChA-1 cell migration and **B**) invasion**. C**) Results are expressed as mean % of EV+EV or SC+SC ± SEM from three experiments done in duplicates, ANOVA test, *p = 0.04, 0.01, 0.014, 0.04, 0.012, 0.017, 0.018, 0.02, 0.02, 0.01, 0.024, 0.01, 0.017, 0.014, 0.012, and 0.01,0.026, 0.019, 0.02, 0.024 vs. relative controls (EV+EV or SC+SC). **D**) MATα1-D and **E**) PHB1-D cells expressing SC or MMP-7 CRISPR stable knockdown and luciferase were injected into the liver of nude mice (MATα1-D) and WT C57BL/6 mice (PHB1-D), and tumor growth was monitored by small animal imaging as described in Methods. Representative small animal images showing luciferase measurement are shown and summarized in the graph to the right of the images. Representative H&E staining of liver, lung, and pancreas with numbers of mice that exhibited metastasis or liver necrosis are indicated below the pictures in nude mice or WT C57BL/6 mice. Results are expressed as mean ± SEM from n=7–8 per group, ANOVA test, Student's t-test, *p = 0.05, 0.016 and 0.01, **p = 0.001 vs SC.

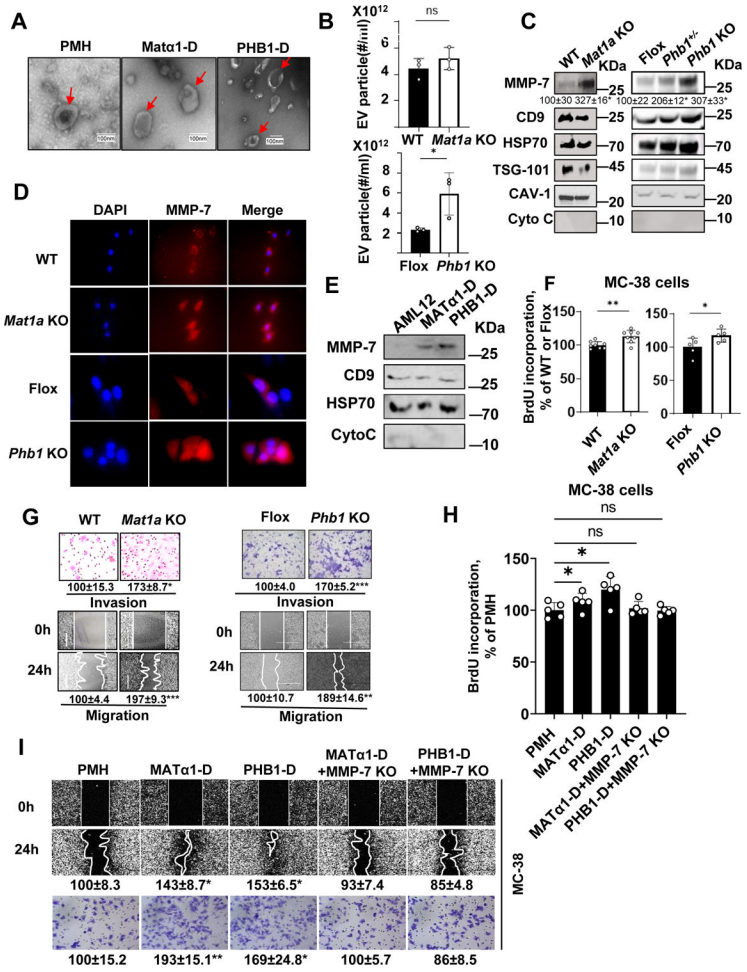

Figure 5. Mice and HCC cells lacking MAT1A or PHB1 release EVs containing more MMP-7 to induce cancer cell growth, migration and invasion.

A) EVs isolated from culture media examined under electron microscopy. **B**) EVs were isolated from sera were analyzed by NanoSight, Student's t-test, ^{ns} p = 0.319, *p = 0.043 vs. Flox from n=3 per group. **C**) MMP-7 and EV markers in EVs isolated from sera were evaluated by western blot. Student's t-test, ANOVA test, *p = 0.036, 0.029, and 0.014 vs. respective controls (WT, Flox, and PMH). **D**) Immunofluorescence staining of MMP-7 in MC38 cells treated with EVs from sera of the *Mat1a* and *Phb1* KO mice. **E**) MMP-7 and EV markers in EVs isolated from culture media were evaluated by western blot. **F)** BrdU incorporation, Student's t-test, *p = 0.004, and 0.049. **G**) invasion and migration of MC38 cells treated with EVs from sera of *Mat1a* KO and WT littermates, *Phb1* KO and flox littermates. Student's t-test, *p = 0.021, **p = 0.0083, ***p = 0.00018, 0.00077. vs. respective controls (WT, and Flox) **H**) BrdU incorporation, **I**) migration (top two rows) and invasion (bottom row) of MC38 cells treated with EVs from culture media of PMH, MATα1-D and PHB1-D, MATα1-D with MMP-7 CRISPR KO and PHB1-D with MMP-7 CRISPR KO cells. Results are expressed as mean % of respective controls ± SEM from three experiments done in duplicates, ANOVA test, *p = 0.05, 0.016, 0.05, and 0.011, **p = 0.0086, ^{ns} p = 0.691, and 0.900 vs. PMH.

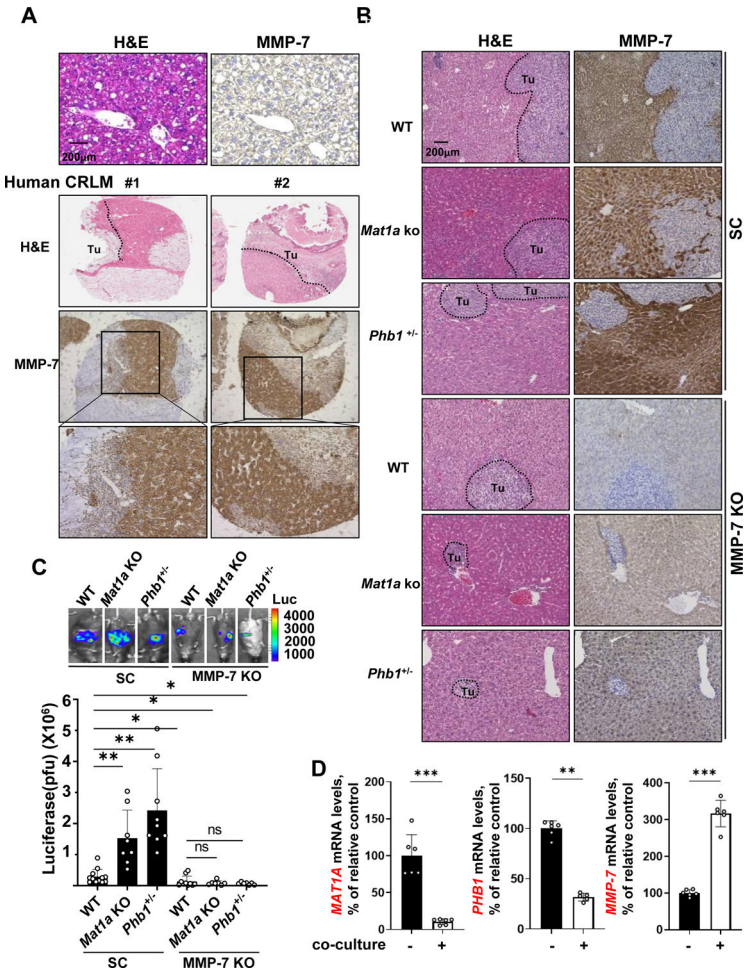

Figure 6. *Mat1a* KO and liver-specific *Phb1*$^{+/-}$ mice are sensitized to CRLM by a mechanism that requires hepatic MMP-7.

A) Representative H&E and IHC staining of MMP-7 in normal mouse liver (top) and human CRLM tissue array (bottom). **B)** Representative H&E and IHC staining of MMP-7 in murine CRLM model from different groups. **C**) Assessment of tumor growth in WT, *Mat1a* KO, *Phb1*$^{+/-}$ mice with or without silencing endogenous MMP-7 by in vivo bioluminescence imaging. Results are expressed as mean ± SEM from N=8–12 per group, ANOVA test, *p = 0.05, 0.03, and 0.018, **p = 0.004, and 0.006, ^{ns} p = 0.51 and 0.34 vs. WT+SC/KO. **D)** MAT1A, PHB1 and MMP-7 expression in human hepatocytes with and without co-culture of RKO cells. Results are expressed as mean % of control expression (without co-culture) from three experiments done in duplicates, Student's t-test, **p = 0.0024, ***p = 0.000019 and 0.000057 vs. without co-culture.

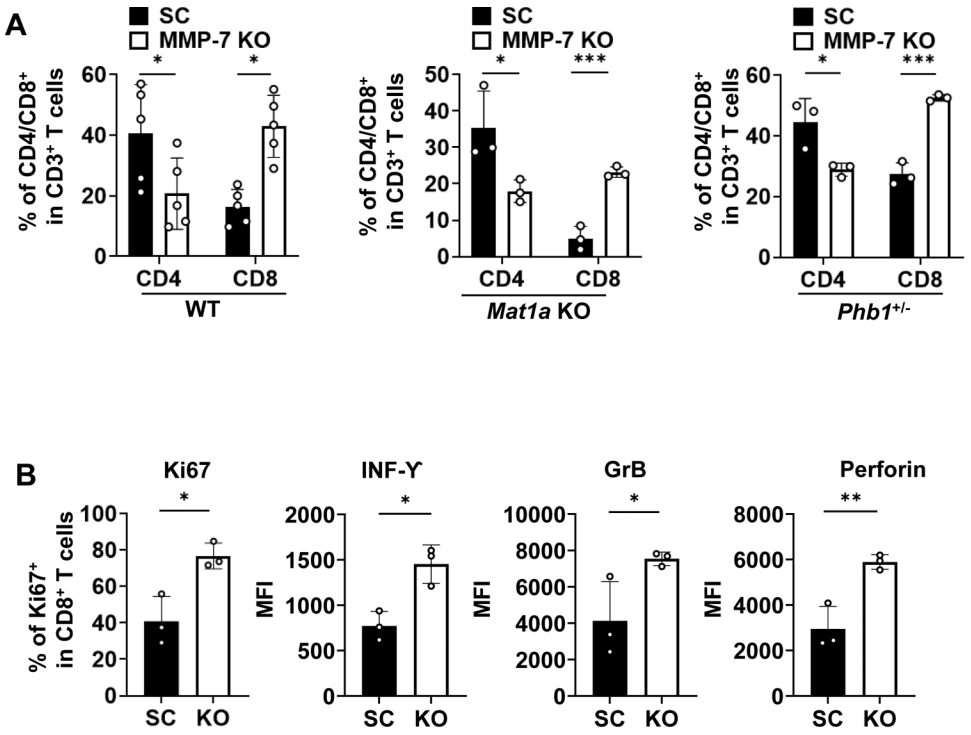

Figure 7. Features of CD4$^+$ and CD8$^+$ T Cell Infiltration in CRLM.

A) flow cytometry analysis of percentage for CD4$^+$ and CD8$^+$ subsets of CD3$^+$ T-lymphocytes in the CRLM from WT (left), *Mat1a* KO (middle), *Phb1*$^{+/-}$ (right) mice with SC or MMP-7 KO adenovirus injection (N=3–5). ANOVA test, *p = 0.048, 0.019, and 0.027, ***p = 0.00098 and 0.000377 vs. respective SC. **B)** Percentage of T cells activation marker - Ki67$^+$ in CD8$^+$ T cells (left) and quantified MFI (right) for T cells activation markers - INF-γ, GrB and Perforin in CD8$^+$ T cells in CRLM from WT injected with SC or MMP-7 KO adenovirus. Results are expressed as mean ± SEM from three experiments done in duplicates, Student's t-test, *p = 0.015, 0.011, and 0.05, **p = 0.0079 vs. respective SC.