LATS1 and LATS2 regulate mouse liver progenitor cell proliferation and maturation through antagonism of the coactivators YAP and TAZ

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Abstract

In the adult liver, the Hippo pathway kinases MST1/2 and LATS1/2 control activation of the transcriptional coactivators YAP and TAZ in hepatocytes and biliary epithelial cells (BECs) thereby regulating liver cell proliferation, differentiation and malignant transformation. Less is known about the contribution of Hippo signaling to liver development. Here we use conditional mutagenesis to show that the Hippo signaling pathway kinases LATS1 and LATS2 are redundantly

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required during mouse liver development to repress YAP and TAZ in both the biliary epithelial and hepatocyte lineages. In the absence of LATS1/2, BECs exhibit excess proliferation while hepatoblasts fail to mature into hepatocytes, defects that result in perinatal lethality. Using an *in vitro* hepatocyte differentiation assay we demonstrate that YAP activity decreases and Hippo pathway kinase activities increase upon differentiation. In addition we show that YAP activation *in vitro*, either resulting from depletion of its negative regulators LATS1/2 or by expression of a mutant form of YAP that is less efficiently phosphorylated by LATS1/2, results in transcriptional suppression of genes that normally accompany hepatocyte maturation. Moreover, we provide evidence that YAP activity is repressed by Hippo pathway activation upon hepatocytic maturation *in vitro*. Finally, we examine the localization of YAP protein during fetal liver development and find that higher levels of YAP protein are found in biliary epithelial cells, while in hepatocytes, YAP levels decrease with hepatocyte maturation.

Conclusion—Our data indicate that Hippo signaling, mediated by the LATS1 and LATS2 kinases, is required to restrict YAP and TAZ activation during both biliary and hepatocyte differentiation and suggest that dynamic regulation of the Hippo signaling pathway plays an important role in differentiation and functional maturation of the liver.

Introduction

The mammalian liver has essential roles in metabolism, energy homeostasis, detoxification, bile acid production and synthesis and secretion of plasma proteins such as albumin and clotting factors. Proper development of the liver is essential for normal liver function in the perinatal period and in the adult. Indeed, developmental defects can lead to a wide variety of liver dysfunction in children and adults resulting in increased morbidity and mortality[1].

Specification and differentiation of the liver begins during embryonic development and continues into the early postnatal period [2,3]. An important event during this process is the derivation of hepatocytes and biliary epithelial cells (BECs), two major cell types of the liver, from a common fetal progenitor termed the hepatoblast [4]. While there is significant knowledge regarding how hepatoblasts are specified and how they adopt either hepatocyte or BEC fates, our understanding of key aspects of liver progenitor cell development is lacking. For example, fetal hepatocytes must undergo a maturation process at birth that involves large changes in gene expression: hepatic transcription factors such as HNF4α, FoxA2, and C/EPBα are important regulators of this process[2,3].

In addition to these well studied transcription factors, emerging evidence indicates that the transcriptional coactivator YAP is essential for normal liver development [5] and controls a switch from a fetal to adult liver gene expression program in part by modulating the activities of HNF4α and FoxA2[6]. YAP activation is most commonly associated with regulation by the canonical Hippo pathway, a kinase cascade that phosphorylates and inactivates YAP [7-10]. However, there are several reports of YAP regulation by other pathways [11,12], and thus the mechanism by which YAP is regulated during liver development is unknown.

Based on these observations, we tested the hypothesis that the Hippo pathway kinases LATS1 and LATS2 are critical for differentiation and maturation during liver development.

Here we report that these two kinases are redundantly required to restrict the activity of the transcriptional co-activators YAP and TAZ during the process of hepatocyte maturation and BEC differentiation. Mice that have conditional deletion of both LATS1 and LATS2 in hepatic progenitor cells fail to properly regulate hepatoblast differentiation in a YAP and TAZ-dependent manner. The abnormally high activity of YAP and TAZ results in an inability of hepatocytes to adopt a mature phenotype and to defects in BEC development that both likely contribute to perinatal lethality. Furthermore, using an *in vitro* system to model hepatocyte maturation, we show that forced YAP activation alone is sufficient to inhibit hepatocyte maturation. Taken together, our results suggest a model in which high levels of Hippo signaling are required in the hepatocyte lineage and intermediate levels of Hippo signaling are required in committed biliary epithelial progenitor cells.

Materials and Methods

Animals

This study was carried out in strict accordance with the recommendations in the Institutional Animal Care and Use Committee (IACUC) for the University of Texas MD Anderson Cancer Center at Houston. Floxed alleles for *Lats1*[13], *Lats2*[14], *Yap*[15] and *Taz*[16] have been described previously. Albumin-cre [17] mice were obtained from the Jackson Laboratory. The protocol was approved by the Animal Welfare Committee. All efforts were made to minimize suffering.

Periodic acid–Schiff (PAS) staining

Liver tissues were dewaxed in Xylene and rehydrated through decreasing alcohol gradient until distilled water. They were then oxidized by 0.5% periodic acid for 5 minutes, followed by coloring reaction in Schiff's reagent at room temperature. After wash with tap water, tissues were counterstained with hematoxylin. After further wash, tissues were dehydrated, cleared and sealed for microscopy imaging.

RNA isolation

Fresh liver tissues were homogenized in TRIzol reagent. Total RNA was extracted by chloroform-isopropanol method. RNA was dissolved in distilled water and stored in -80°C.

Serum AST/ALT and blood glucose level measurement

Newborn mice were sacrificed and fresh blood was collected. After centrifuge out the blood cells, serum was transferred to new tubes and immediately sent to the MD Anderson Clinical Pathology Lab for chemistry analysis.

Microarray and bioinformatic analysis

RNA isolation, cRNA synthesis, hybridization to Illumina mouse BeadChip v.2 microarrays, data processing, statistical analysis and gene ontology analysis was done as previously described[18]. Complete datasets containing features differentially expressed in Wild-type versus Hippo pathway mutant liver tissues are contained in a supplementary excel spreadsheet.

Cell culture and Hepatocyte Differentiation assay

BMEL (Bipotential Mouse Embryonic Liver cell lines) were isolated from *Lats1/2* mice according to the standard protocol described before[19]. Cells were cultured in Advanced DMEM/F12 media (Invitrogen, 12634) containing 10% FBS, 1XGLutamax, 1% penicillin&streptomycin, 50ng/ml EGF (Peprotech AF-100-15), 30ng/ml IGFII (Peprotech 100-12), 10ug/ml Insulin (Invitrogen, 12585014) on dishes coated with Collagen I (Life Technologies, A1048301) in a humidified atmosphere with 5% CO_2 at 37°C. 6×10^5 cells/well were plated on gelatin-coated 6-well dishes for hepatocyte differentiation assay.

For the isolation of PMEL (Primary Mouse Embryonic Live cell lines), E14.5 livers were dissected out and dissociated into single cell suspension in William's media E (Invitrogen, 12551) by pipetting up and down. The cells were then plated on dishes coated with 0.1% Gelatin at a ratio of one liver to 8 wells of 6-well plates for hepatocyte differentiation assay.

Hepatocyte differentiation assay was conducted in basic media formulated by DMEM media, 10% FBS, 1% Penicillin&Streptomycin, 1× Nonessential Amino Acid, 2mmol/L L-glutamine, 1× insulin transferrin-selenium 10^{-7} mol/L Dexamethasone (Sigma). For the induction of fetal hepatic maturation, 10ng/ml Oncostatin M was added during the first 5 days, and 0.362mg/ml Matrigel (Corning, 356234) diluted in ice-cold basic media was then overlaid on day 5. Control cultures received basic media only. The media was then replaced every 2 days. Cells were harvested on day 7 for analysis of gene expression.

qRT-PCR and Western Blotting

Total RNA was extracted using RNeasy plus mini kit (Qiagen, 74136). cDNA was made using Superscript III first strand kit (Invitrogen, 18080-051). Predesigned Taqman primer&probe mix were used for QRT-PCR gene expression assay.

For Western Blot analysis, the cells were lysed in ice-cold RIPA lysis buffer (Pierce, 89901) containing protease inhibitors (Roche, 11697498001). The cell debris was pelleted by centrifugation at 16,000g for 10 min. The supernatants were mixed with 5XSDS loading buffer and incubated at 37oC for 1hr. The samples were analyzed by SDS-PAGE. The following antibodies were used: anti-pMST1/2 (Cell signaling, 3681), anti-MST1 (Cell signaling, 3682), anti-pYAP (Cell signaling, 4911), anti-YAP (Cell signaling, 4912), anti-actin (Cell signaling, 4967).

In vitro LATS1 kinase assay

GST fusion YAP proteins (2 μg) were used for the in vitro kinase assay. LATS1 kinase was immunoprecipitated (3477S, Cell Signaling Technology, 1:100 dilution for immunoprecipitation) from the indicated cell lysates and subjected to the kinase assay in the presence of cold ATP (500 μM) and GST-YAP fusion protein. The reaction mixture was incubated at 30 °C for 30 min, terminated with SDS loading buffer and subjected to SDS–PAGE. Phosphorylation of YAP at the S127 site was determined by YAP S127 phospho-antibody (4911S, Cell Signaling Technology, 1:1,000 dilution). GST antibody (sc-138, 1:1,000) was from Santa Cruz Biotechnology.

Immunohistochemistry and Immunofluorescence

Mouse liver tissues were fixed and sectioned following standard procedures. Prior to immunostaining, sections were deparaffinized in xylene for 15 min and rehydrated in a descending alcohol series. Antigen retrieval was carried out by boiling in citrate buffer pH6.0 (BioGenex, HK086-9K) for 20 min in a pressure cooker. Subsequently, slides were incubated overnight with primary SOX9 antibody (Millipore, AB5535, 1:5000) at 4°C. The anti-rabbit Vectastain ABC system (PK-6101) was used as secondary antibody and enhanced metal DAB (Thermo Scientific, #34065) was used as substrate. Counterstaining was performed with hematoxylin (Vector Laboratories, H-3404). The slides were mounted with permanent mounting media (Vector Laboratories, H-5000).

For immunofluorescence, paraffin sections were rehydrated following the protocol described above, and were incubated with primary antibodies after antigen retrieval. The primary antibodies used were anti-SOX9 (Millipore, AB5535, 1:1000), anti-KI67 (eBioscience, 14-5698-80, 1:1000), anti-HNF4α (Abcam, ab41898, 1:200), anti-YAP1 (Proteintech, 13581-1-AP, 1:200), anti-YAP (Santa Cruz, sc-101199, 1:250), anti-CD31 (cell signaling, 77699, 1:100), anti-CD45 (Abcam, ab10558, 1:1000), anti-desmin (Abcam, ab3362, 1:500), anti-Osteopontin (Protein tech, 22952-1-AP, 1:1000) and anti-Epcam (cell signaling, 2929, 1:500). Sections were counterstained with Dapi. Images were recorded and analyzed using confocal laser scanning microscope (Olympus Fluoview 1000) with Fluoview software.

For cell number measurement, Image J was used to record the number of positive stained cells. A minimum of three samples from each group were stained and counted, the results were analyzed in Prism 6.

TUNEL assay

TUNEL assay was carried out following the manufacturer's instructions (Millipore, 17-141).

Results

Deletion of the Hippo pathway kinases LATS1/2 in mouse embryonic liver progenitor cells results in perinatal lethality

To generate mice with liver-specific conditional deletion of LATS1 and LATS2, we intercrossed mice that were homozygous for floxed alleles of *Lats1*[13] and *Lats2*[14] (*Lats1/2*) with mice that contained an Alb-cre transgene[17] combined with homozygosity for the *Lats1* floxed allele[13] and heterozygous for the *Lats2* floxed allele. The Albumin-cre transgene directs recombination in cells that express albumin, including hepatoblasts in the embryonic liver[17] leading to deletion of floxed alleles in both hepatocyte and biliary epithelial cell lineages. Mice homozygous for the *Lats1* and *Lats2* floxed allele that were also positive for the Albumin-cre transgene were not recovered (0/19) at two weeks of age, suggesting that conditional deletion of *Lats1/2* using Albumin-cre is lethal prior to that age (Supplemental table 1). Efficient deletion of both *Lats1* and *Lats2* was achieved by P0 as evidenced by a significant reduction of *Lats1* and *Lats2* mRNA levels (Figure 1E). To determine more precisely the time of death of Albumin-cre; *Lats1/2* mice, we genotyped mice at embryonic and perinatal stages (Supplemental table 1). Albumin-cre; *Lats1/2* mice were recovered at

near normal mendelian frequencies at embryonic stages and at P0 (11/60, expected 15/60), but not at P1 (0/19) suggesting that conditional deletion of *Lats1/2* in the liver of mice is lethal at or around P1. Mice that contained at least one wild-type allele of *Lats1* or *Lats2* were viable and fertile, indicating that LATS1 and LATS2 are redundantly required for viability past P1.

Abnormal bile duct development and liver function in Albumin-cre; *Lats1/2* mutant mice

To investigate further the consequence of LATS1/2 deletion in the liver, we initially performed gross and histological analysis together with serum assays for liver function (Figure 1). Albumin-cre; *Lats1/2* mutant livers were easily recognized by their larger size at P0 and the presence of localized discoloration of the liver due to necrosis (Figure 1A,D). H&E staining revealed a pathology of bile duct malformation, with no obvious tubular bile ducts visible in sections of mutant tissues (Figure 1B), whereas in wild-type mice bile ducts were easily found adjacent to the portal vein (Figure 1B, for quantification see Supplemental figure 6). Elevated serum ALT and AST levels suggest liver injury in Albumin-cre; *Lats1/2* mutants (Figure 1F) and liver dysfunction was evidenced by a lack of glycogen accumulation as assayed by periodic acid Schiff (PAS) staining (Figure 1C) and lower blood glucose levels (Figure 1F). Taken together, these results demonstrate that LATS1 and LATS2 are required for normal BEC morphogenesis and for normal hepatocyte function in the early perinatal period.

Rescue of perinatal lethality in Albunin-cre; *Lats1/2* mutants by simultaneous depletion of the Hippo pathway transcriptional co-activators YAP and TAZ

The LATS1 and LATS2 kinases are well known negative regulators of the transcriptional co-activators YAP and TAZ[8,11]. Phosphorylation of YAP and TAZ by LATS1 or LATS2 results in their cytoplasmic retention [20]and destabilization[21,22]. To determine whether the phenotypes we observe in Albumin-cre; *Lats1/2* mutants were due to aberrant activation of YAP and/or TAZ, we generated mice with combined deletion of LATS1, LATS2, YAP[15] and TAZ[16] using conditional alleles of LATS1/2, YAP, TAZ and Albumin-cre. In contrast to Albumin-cre; *Lats1/2* mutants that do not survive past P0, Albumin-cre; *Lats1/2;Yap, Taz* mice are viable and fertile (Supplementary figure 1). However, mice that retain both alleles of either YAP or TAZ in the context of deletion of LATS1 and LATS2 were either not viable at weaning (YAP 0/5) or had reduced viability at weaning (TAZ 2/16) (Supplementary figure 1), indicating that *in vivo*, the LATS1/2 kinases actively inhibit the activities of both YAP and TAZ during development and in the early postnatal period. These findings are consistent with similar results that have recently been reported for the adult liver[23]. Further supporting the notion that the primary function of LATS1/2 in the liver is to inhibit YAP/TAZ, the gene expression profile of Albumin-cre; *Lats1/2;Yap, Taz* and Albumin-cre; *Yap, Taz* mutant livers are highly similar and cluster together when compared to wild-type gene expression profiles (Supplementary figure 2).

Loss of LATS1/2 leads to large changes in gene expression related to proliferation and metabolism

To gain further insight into the molecular mechanisms underlying the perinatal lethality of Albumin-cre; *Lats1/2* mutant mice, we compared the transcriptome of livers from wild-type

P0 mice with livers harvested from P0 Albumin-cre; *Lats1/2* mutants by microarray analysis. Approximately 1800 transcripts were significantly upregulated and 1500 transcripts were downregulated in Albumin-cre; *Lats1/2* livers versus wild-type livers at P0. Gene ontology analysis (Supplementary figure 3) of the up-regulated transcripts revealed a significant enrichment for pathways in cancer ($P<10^{-12}$) while metabolic pathways were enriched in the down-regulated transcripts ($P<10^{-40}$). These findings are consistent with known tumor suppressive roles for the LATS1/2 kinases [24]and further suggest a novel role for these kinases in regulating genes involved in liver metabolism. Several up-regulated and down-regulated transcripts were selected for confirmation by quantitative RT-PCR. As expected, metabolic genes (Gys2, Hsd17b6, Ugt1a1, Agxt) were significantly down regulated in mutant livers, as were transcripts for some members of the nuclear hormone receptor family (Fxrα, Fxrβ, Car) that contribute to regulation of liver metabolism (Figure 2). Significantly up regulated genes included several expressed by biliary epithelial cells (Sox9, CK19) suggesting either an increase in numbers of this cell type in mutant liver tissues or an increased level of mRNA for these transcripts. In contrast, mRNA levels of the liver transcription HNF4α and of the nuclear hormone receptor Pxr were not significantly different between wild-type and mutant livers (Figure 2) indicating that there was not a general disruption of transcriptional regulation in the Albumin-cre; *Lats1/2* livers.

LATS1/2 regulate biliary epithelial cell proliferation and differentiation

The apparent lack of immature bile ducts in Albumin-cre; *Lats1/2* mutant livers suggested a defect in the development of the BEC lineage. To explore the nature of this defect further, we performed immunohistochemistry and immunofluorescence with the BEC lineage marker Sox9. Sox9 is normally expressed in mature BECs as well as cells of the ductal plate that are committed to the biliary epithelial lineage and lie adjacent to the portal vein beginning at E13.5 dpc in the mouse[25]. These Sox9 positive cells undergo a complex series of morphogenetic rearrangements to give rise to patent bile ducts of the liver that maintain expression of Sox9[25]. At P0, scattered Sox9 positive cells that are the remnant of the ductal plate as well as newly forming bile ducts are apparent in wild-type tissue sections (Figure 3A). In contrast, Albumin-cre; *Lats1/2* mutant livers display a significant increase in Sox9 positive cells that surround the portal vein; however these cells do not form organized ductal structures (Figure 3A).

In principle, an increase in the number of Sox9 positive cells could arise from increased proliferation, from an increase in the number of cells specified to the biliary epithelial cell lineage, or a combination of both. To help distinguish between these possibilities, we initially performed double labeling with a proliferation marker Ki67 and with Sox9. In wild-type tissues at P0 there is little co-labeling of Ki67 and Sox9 (Figure 3B) as biliary epithelial cells exit the cell cycle as part of their normal development while undergoing morphogenesis to bile ducts[25]. However, in Albumin-cre; *Lats1/2* mutants there is a significant increase in the number of Ki67/Sox9 positive cells (Figure 3B) suggesting that many biliary epithelial cells fail to exit the cell cycle and continue to proliferate. Similar results were also obtained using other markers of biliary epithelial cells including cytokeratin 19 (Supplementary figure 4) and osteopontin (supplementary figure 7).

To explore the possibility that there might be an increase in the number of cells specified towards the biliary cell lineage in Albumin-cre; *Lats1/2* mutants, we performed double labeling with HNF4α and Sox9. In wild-type livers, HNF4α is expressed in bipotential hepatoblasts and in cells that are committed to the hepatocyte lineage but is not expressed by Sox9 positive cells that are committed to the biliary epithelial lineage[25] and hence there is no overlap in their expression (Figure 3C). In contrast, Albumin-cre; *Lats1/2* mutants have significant numbers of double labeled HNF4α/Sox9 cells (Figure 3C) suggesting that some hepatoblasts that would normally be adopting the hepatocyte fate are being diverted to the biliary epithelial cell fate. Alternative possibilities are that HNF4a positive hepatocyte progenitors are dedifferentiating to a bipotential progenitor state or that there is selective apoptosis of HNF4a hepatocyte progenitors in Albumin-cre;*Lats1/2* livers. To explore these possibilities we performed immunostaining for Epcam (Supplemental figure 7) that is expressed in liver progenitor cells as well as comparing apoptosis in wild-type and mutant livers by TUNEL staining (Supplemental figure 8). The majority of cells in Albumin-cre;*Lats1/2* livers that are Sox9 positive are also Epcam negative. Moreover, a majority of these Sox9 positive cells are also osteopontin positive (Supplementary figure 7). Additionally, apoptosis is not increased in Albumin-cre;*Lats1/2* livers compared to wild-type livers. Taken together these results suggest that the expanded Sox9 positive cell population is likely to be derived from overproliferation of committed biliary epithelial cells rather than representing dedifferentiation of more mature progenitors into a more primitive state.

A well-known inducer of the biliary cell fate is notch signaling[26] and notch signaling is known to be activated by YAP in adult hepatocytes[27] and in hepatocellular carcinoma at least in part by direct up-regulation of the notch ligand jagged-1[28]. To examine the status of notch signaling upon LATS1/2 deletion, we stained wild-type and *Lats1/2* mutant liver sections with antibodies directed against Hes1, a direct target of activated notch, and Jagged-1, a ligand for the notch receptor. Increased numbers of Hes1 positive cells were found in periportal regions of Albumin-cre; *Lats1/2* mutant liver sections relative to wild-type sections (Supplementary figure 5) suggesting an increase in notch signaling after LATS1/2 depletion. Similarly, Jagged protein was detected in a much larger field of cells in the Albumin-cre; *Lats1/2* mutants as compared to wild-type (Supplementary Figure 5). These results suggest that the LATS1/2 kinases, in addition to their roles in regulating expression of metabolic genes in maturing hepatocytes, also regulate the proliferation and morphogenesis of biliary epithelial cells during liver development possibly in part by repressing notch signaling.

Depletion of LATS1/2 and activation of YAP *in vitro* inhibits hepatocyte maturation

Our molecular and genetic analyses suggest that LATS1/2 functions to restrict YAP/TAZ activity in hepatocytes thereby allowing a fetal to perinatal switch in gene expression in these cells. To gain further support for a critical role of the LATS1/2 kinases and YAP/TAZ in this process, we employed an in vitro differentiation system[29] where either primary fetal liver progenitor cells (PMEL or primary mouse embryonic liver cells) or immortalized fetal liver progenitor cell lines[19] (BMEL or bipotential mouse embryonic liver cell lines) can be induced to express many genes found in mature hepatocytes. To that end, we cultured PMEL and generated BMEL cell lines from LATS1/2 conditional mutant liver progenitor cells and

subjected them to differentiation conditions[29]. As expected, upon culture at high density, addition of oncostatin M and matrigel, BMEL cells significantly up regulate transcripts of tyrosine aminotransferase (TAT) and tryptophan 2,3-dioxygenase (TDO2) relative to basal conditions (Figure 4A). Expression of a hyperactive form of YAP (YAP^{S127A}) that is less efficiently inhibited by the LATS1/2 kinases inhibits expression of both TAT and TDO2 under differentiation conditions in BMEL cells (Figure 4A). When the LATS1/2 kinases were deleted *in vitro* following transduction with adenovirus particles that direct expression of the cre recombinase (Ad-Cre) BMEL cells failed to up regulate TDO2 and Prlr under differentiation conditions (Figure 4B). TAT expression was slightly inhibited upon depletion of LATS1/2 in BMEL cells, but this reduction was not statistically significant. Similar results were observed for PMEL cells derived from *Lats1/2* mice (Figure 4C,D) with the exception that repression of TAT expression was significant upon depletion of LATS1/2 in PMEL cells. With expression of YAP^{S127A} , similar results were obtained from PMEL cells as that of BMEL cells (Figure 4E). These findings support our *in vivo* observations that LATS1/2 are required for proper induction of mature hepatocyte gene expression during liver progenitor cell maturation and demonstrate that YAP activation alone can likewise lead to similar effects.

Hippo signaling is increased upon hepatocyte differentiation *in vitro*

The *in vitro* hepatocyte differentiation assay also allowed us to address whether the Hippo signaling pathway is activated upon application of differentiation conditions to PMEL and BMEL cells. Hippo pathway activity can be monitored by the phosphorylation status of core components, including the MST1/2, LATS1/2 kinases and YAP. Indeed, we observe that compared to basal culture conditions, phosphorylation of both MST1/2 and YAP increase (Figure 5A) as well as phosphorylation of LATS1 (Figure 5B) upon differentiation, suggesting that the Hippo pathway is activated under these conditions leading to increased YAP phosphorylation that is correlated with decreased YAP activity. To further investigate this possibility, we employed an *in vitro* assay for LATS1 kinase activity using recombinant GST-YAP as substrate combined with LATS1 immunoprecipitated from BMEL cells under either basal or differentiation conditions (Figure 5B). LATS1 activity was clearly enhanced upon differentiation as indicated by an increased signal for phosphorylated YAP relative to basal conditions. In contrast, when LATS1/2 is depleted from BMEL cells, YAP phosphorylation is reduced (Figure 5C) while total YAP protein levels increase (Figure 5C). Similar results were obtained with PMEL cells with regard to YAP protein levels (Figure 5C) and to a lesser extent with regards to YAP phosphorylation (Figure 5C). Taken together, these results suggest that during hepatocyte maturation, as modeled in BMEL and PMEL cells, Hippo pathway activity increases resulting in increased YAP phosphorylation and decreased YAP activity. Moreover, the LATS1/2 kinases are required to prevent YAP protein accumulation and activation during this process.

Dynamic regulation of YAP protein levels and localization during fetal liver development

Our working model for Hippo pathway mediated regulation of liver progenitor cell development predicts that LATS1/2 functions to restrict YAP/TAZ activity in both fetal biliary epithelial cells and in fetal hepatocytes to ensure their proper development. To examine this prediction in more detail, we performed immunofluorescence to determine the

localization of YAP protein during normal fetal liver development and in Albumin-cre; *Lats1/2* mutant livers. At E15.5 we observe relatively high levels of YAP immunoreactivity in cells adjacent to the portal vein as well as cells distant from the portal vein (Figure 6A). Expression is variable with some cells showing high levels of immunofluorescence and other cells exhibiting low levels. Where YAP is expressed, it appears to be predominantly cytoplasmic, however, some cells also appear to have nuclear staining for YAP at this stage. These results were reproducible and the relative ratios of Yap negative:Yap cytoplasmic:Yap cytoplasmic and nuclear were approximately 3:3:1 (Supplementary figure 11). At E18.5 a different pattern of YAP staining emerges (Figure 6A). High levels of YAP immunostaining is evident in cells adjacent to the portal vein whereas cells outside of the periportal region show diminished YAP expression. Furthermore, some periportal cells display intense nuclear staining for YAP, some both cytoplasmic and nuclear staining, and others no or little staining in either the nucleus or cytoplasm. These results were also reproducible and quantification indicates a ratio of 15:1: 1:3 for cells with Yap negative:Yap cytoplasmic:Yap nuclear:Yap cytoplasmic and nuclear staining patterns at E18.5 (Supplementary figure 11).

To help determine whether these different YAP staining patterns correspond to specific liver cell types, we performed dual immunostaining for YAP and markers of the hepatocyte (HNF4α) and biliary epithelial (Sox9) lineages at E18.5. At this stage, HNF4α positive cells were largely YAP negative (Figure 6B), while Sox9 positive cells generally exhibited both nuclear and cytoplasmic YAP staining (Figure 6B). Cells that exhibited intense nuclear staining for YAP were neither positive for HNF4α nor for Sox9 and are therefore neither hepatocytes nor biliary epithelial cells. To further define the population of cells that exhibit high levels of nuclear Yap staining we performed double immunolocalization of Yap with endothelial (CD31), leukocyte (CD45), and stellate cell (Desmin) markers (Supplementary figure 10). Cells that express high nuclear Yap are found to co-express each of these markers in equal numbers suggesting that these cells are not a single population but rather represent heterogenous cell types. These results suggest that both YAP levels and nuclear/cytoplasmic localization are tightly regulated according to developmental stage and cell type in the mouse liver. To determine whether LATS1/2 contributes to the localization and levels of YAP in the mouse liver in vivo we carried out immunostaining of YAP in Albumin-cre; *Lats1/2* mutant liver sections (Figure 6C). In contrast to wild-type tissues, Albumin-cre; *Lats1/2* mutant tissues display widespread YAP immunoreactivity in both periportal cells and in cells distant from the periportal regions. In periportal regions YAP staining is largely both cytoplasmic and nuclear and co-localizes with Sox9 immunoreactivity. However, in parenchymal cells distant from periportal regions, YAP staining is largely nuclear in Albumin-cre; *Lats1/2* tissues. Our immunolocalization studies indicate a highly dynamic regulation of both the levels and subcellular localization of YAP during mouse liver development that correlate with maturation of specific cell types. In the biliary epithelial lineage, moderate levels of cytoplasmic and nuclear YAP are evident, while in maturing hepatocytes there appears to be a general down regulation of YAP protein levels. Furthermore, our results highlight the role of the LATS1/2 kinases in ensuring proper levels and localization of YAP protein during hepatocyte and biliary epithelial cell development.

Discussion

In mature hepatocytes, activation of YAP has been shown to result in dedifferentiation, adoption of a progenitor-like state, and activation of a biliary-type gene expression program[30]. This switch involves the repression of an adult gene expression program and activation of a progenitor/biliary epithelial program[27]. However, the role of Hippo signaling and YAP activities in embryonic liver development is less well understood. Our results demonstrate that during mouse development the Hippo pathway kinases LATS1/2 act to restrict YAP and TAZ activity in both hepatocyte and biliary epithelial cell lineages. During normal development, YAP protein is down-regulated in differentiating hepatocytes, whereas in developing biliary epithelial cells it is maintained at relatively higher levels. Our results show that in both cell types, YAP protein levels and activation are constrained in a redundant fashion by the inhibitory LATS1 and LATS2 kinases.

Control of hepatocyte maturation by the LATS1/2 kinases

The main consequence of LATS1/2 deletion in the hepatocyte lineage is an inability of immature hepatocytes to execute a switch to a mature gene expression program that is necessary for perinatal liver function. Mechanistically, this results from hyperactivation of the transcriptional coactivators YAP and TAZ. YAP is known to directly interfere with the activities of key transcriptional regulators of hepatocyte specification and function, including HNF4α, FoxA2, and C/EBPα [6,31] and this is likely to contribute to impaired hepatocyte function upon LATS1/2 depletion. Whether TAZ functions in a similar manner has not been directly addressed. Mechanistically it is unclear how Yap interferes with hepatocyte transcription factor function, but there is evidence that in mature hepatocytes this is accomplished in part by interfering with access of transcription factors to regulatory regions of genes expressed in mature hepatocytes[6]. Our results in vivo are consistent with this observation in that we do not detect significant alterations in HNF4α levels in Albumin-cre;*Lats1/2* hepatocytes. However, in Lats1/2 mutant BMEL cells we observe decreased HNF4α levels at both the transcriptional and protein levels upon differentiation (Supplementary figure 11) suggesting that in addition to interfering with access of key hepatocyte transcription factors to DNA, high levels of Yap expression may prevent induction of a hepatocyte gene expression program. Additional experiments are required to explore this possibility. At present the reasons for the differences that we observe in vivo and in vitro for HNF4α expression in lats1/2 mutant hepatocyte progenitors is unclear but may reflect differences in the timing and duration of lats1/2 inactivation relative to hepatocyte differentiation *in vivo* versus *in vitro*.

YAP protein levels and subcellular localization are dynamically modulated during hepatocyte maturation *in vivo*. As hepatocytes mature, YAP protein levels decrease and nuclear YAP staining is diminished in a LATS1/2 dependent manner. One possible explanation for this observation is that LATS1/2 kinase activity increases in the hepatocyte lineage as these cells undergo maturation. Indeed, factors that induce hepatocyte differentiation *in vitro* (high cell density, extracellular matrix)[29] activate the Hippo pathway[32] in a variety of cell types including in cultured liver progenitor cells as we have shown here. Additionally, YAP activity may be down regulated selectively in mature

hepatocytes by LATS1/2 independent mechanisms. It is well known that regulation of YAP activity and levels are complex, with both Hippo pathway dependent and Hippo pathway independent modes of regulation[11,33]. Improved methods for monitoring Hippo pathway and YAP activity *in vivo* with single cell resolution will be required to distinguish between these possibilities.

LATS1/2 regulate biliary differentiation and proliferation

In contrast to their function in hepatocyte maturation, the role of the LATS1/2 kinases in the biliary cell lineage is to dampen, but not to completely eliminate YAP and TAZ activation. Two main lines of evidence support this conclusion. First, deletion of YAP in hepatoblasts results in fewer bile ducts[34,35] suggesting that some YAP activity is required for normal BEC development. Second, as we have shown here, hyperactivation of YAP results in a failure of BECs to exit the cell cycle and to undergo proper morphogenesis. Hence intermediate YAP levels are necessary in biliary progenitor cells to ensure proper numbers of BECs. Current evidence suggests a key role for YAP in both the control of biliary cell progenitor proliferation and specification. YAP directly regulates the expression of key factors that promote biliary epithelial development, including Sox9[36] and components of the notch signaling pathway[27,28]. Indeed when YAP is activated by LATS1/2 depletion, we observe an increase in the number of HNF4α/Sox9 double positive cells and an increase in notch signaling activity suggesting that more cells are being specified towards the BEC lineage. YAP activation in Albumin-cre; *Lats1/2* mutants also leads to increased biliary progenitor proliferation and this may contribute to the increased numbers of Sox9 positive cells that we observe in this mutant. Whether distinct levels of YAP activity in BECs versus hepatocytes is controlled by modulating Hippo pathway kinase activity or by other mechanisms is not currently known.

Deletion of LATS1/2 in hepatoblasts results in phenotypes distinct from deletion of other Hippo pathway components

The perinatal lethality resulting from LATS1/2 deletion in the mouse liver is unique among phenotypes that have been observed upon liver-specific deletion of Hippo pathway components. Liver-specific ablation of upstream Hippo pathway components MST1/2[18,37,38], Sav1[18,39], or NF2[5,40] results in viable animals, although they share common defects, such as injury, progenitor cell expansion, and susceptibility to tumor formation. One possible explanation for the difference in the phenotype we observe in LATS1/2 mutant livers relative to depletion of other Hippo pathway components is that the mammalian LATS1/2 kinases may have activities that function independently of other Hippo pathway components. That the perinatal lethality of Albumin-cre; *Lats1/2* mutants can be completely suppressed by simultaneous deletion of YAP and TAZ suggests that while LATS1/2 has many other well documented substrates[24], the main targets of LATS1/2 in the fetal and postnatal liver are the Hippo pathway components YAP and TAZ. Another possibility is that YAP/TAZ activity might be increased to a higher level in LATS1/2 mutant liver cells relative to that of MST1/2, Sav1, or NF2 mutant liver cells and that this higher level exceeds a threshold that is compatible with viability. Consistent with this possibility is that in Albumin-cre;*Mst1/2* mutant hepatocytes, there is residual phosphorylation of YAP at LATS1/2 phosphorylation sites[18,38], suggesting that LATS1/2 is still active to some degree

in the absence of the upstream MST1/2 kinases. Moreover, YAP and TAZ themselves induce a negative feedback loop through directly enhancing the transcription of LATS1/2[23,41]. As depletion of LATS1/2 would be predicted to disrupt this negative feedback loop, higher levels of YAP/TAZ activation in LATS1/2 mutant livers would be anticipated relative to depletion of other upstream Hippo pathway components and this may be responsible for the perinatal lethality in Albumin-cre: *Lats1/2* mutant mice.

In summary, we have shown a critical role for Hippo signaling and the LATS1/2 kinases in regulating the development of liver progenitor cells through inhibition of the transcriptional coactivators YAP and TAZ. Our results also suggest that differential activation of YAP and TAZ is critical for proper liver progenitor cell differentiation and maturation in both the hepatocyte and biliary epithelial cell lineages. While our study focused on the role of Hippo signaling in embryonic and perinatal liver development, our findings are also likely relevant to adult liver homeostasis and repair. Hippo signaling and LATS1/2 kinases have been shown to be important in maintaining quiescence and in inhibiting de-differentiation of several adult cell types including cardiomyocytes[42] and hepatocytes[27] suggesting that pharmacological manipulation of LATS1/2 kinase activity might be an effective means to expand these populations in vitro and to contribute to tissue repair following injury or disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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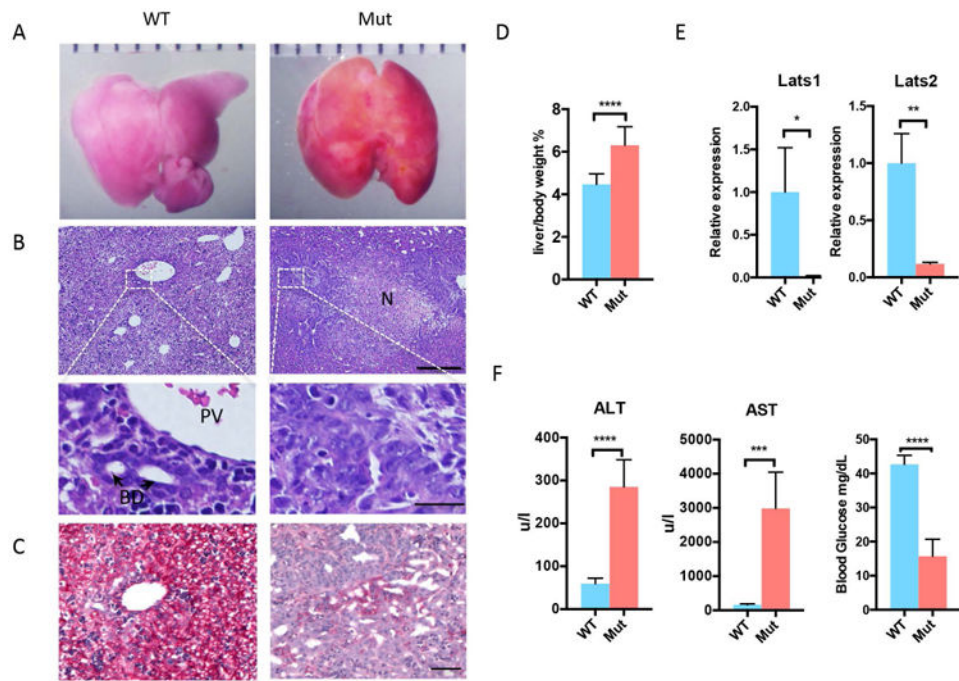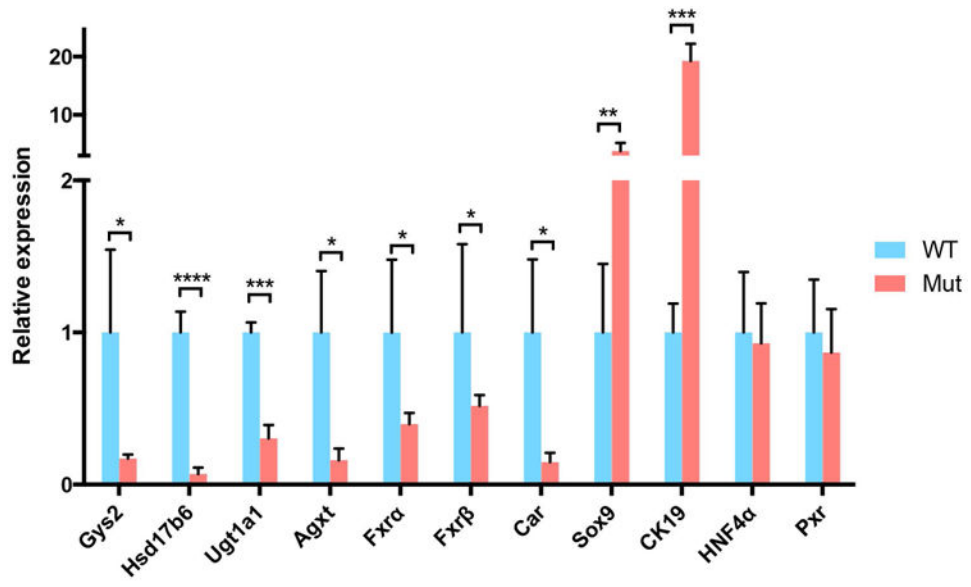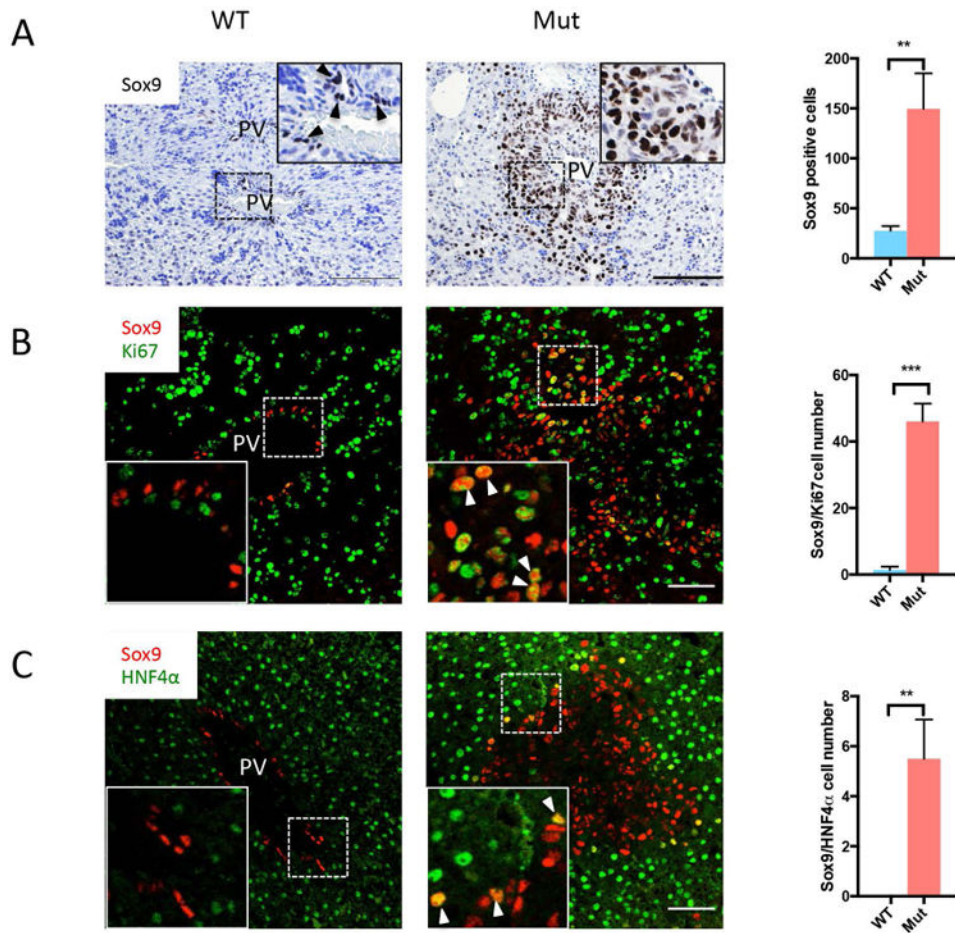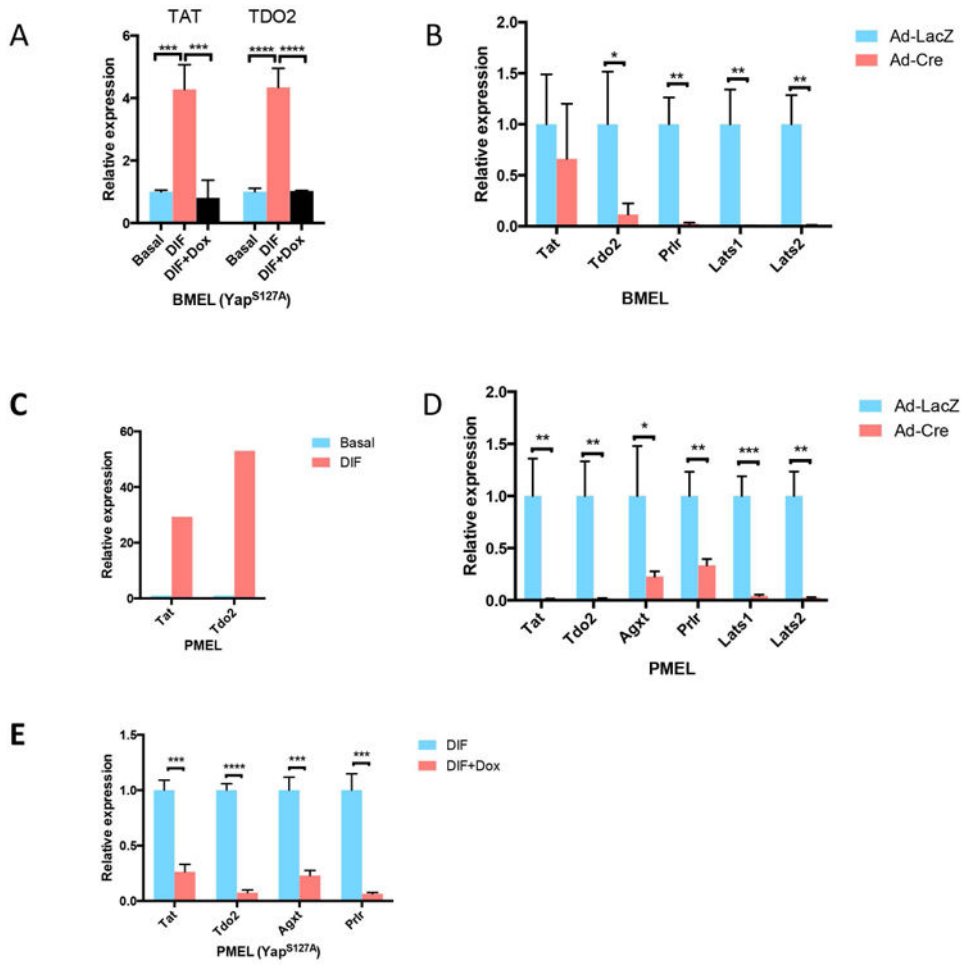
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