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Hippo Signaling in the Liver Regulates Organ Size, Cell Fate, and Carcinogenesis

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Abstract

The Hippo signaling pathway, also known as the Salvador–Warts–Hippo pathway, is a regulator of organ size. The pathway takes its name from the Drosophila protein kinase, Hippo (STK4/MST1 and STK3/MST2 in mammals), which, when inactivated, leads to considerable tissue overgrowth. In mammals, MST1 and MST2 negatively regulate the transcriptional co-activators yes-associated protein 1 (YAP) and WW domain containing transcription regulator 1 (WWTR1/TAZ), which together regulate the expression of genes that control proliferation, survival, and differentiation. YAP and TAZ activation have been associated with liver development, regeneration, and tumorigenesis. How their activity is dynamically regulated in these contexts, however, is just beginning to be elucidated. We review the mechanisms of Hippo signaling in the liver and explore outstanding questions for future research.

The ability of the liver to regenerate after injury is widely known and was even noted by the ancient Greeks. As punishment for helping mankind, Prometheus from Greek mythology was chained to a rock and had his liver eaten by an eagle, only for it to regenerate throughout the night and the cycle to be repeated the next day. Indeed, within hours of a partial hepatectomy, the remaining lobes rapidly grow larger through a combination of hepatocyte hypertrophy and replication[1, 2]. Ninety-five percent of the mass lost from a hepatectomy can be recovered within a month, thus enabling procedures such as split-liver transplantation[3, 4]. Conversely, transplantation of an oversized liver graft into a recipient leads to a gradual reduction of the graft through apoptosis[5–7]. How organs such as the liver sense and regulate their size is central to our understanding of development, regeneration, and disease.

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The Hippo signaling pathway has emerged as an important biochemical pathway in this context. Originally described in the fruit fly, *Drosophila melanogaster,* this pathway is named for the serine/threonine kinase Hippo (STK3/MST2 and STK4/MST1, in mammals), which when lost, results in enlarged organs from excessive proliferation and decreased apoptosis[8]. MST1 and MST2, in partnership with its scaffolding molecule salvador family WW domain containing protein 1 (SAV1), regulate the activity of the large tumor suppressor kinases 1 and 2 (LATS1 and 2). In turn, LATS1 and 2 and their partners, the MOB kinase activators 1A and B (MOB1A and MOB1B), phosphorylate the co-transcriptional activators, Yes-associated protein 1 (YAP1) or its paralog, WW domain containing transcription regulator 1 (WWTR1/TAZ), at several serine residues (Figure 1A, Hippo ON and YAP inactive).

These phosphorylation sites regulate the position and activity of YAP1 in the cell. The most well-studied serine phosphorylation site is S127 in the human protein (S112 in the mouse protein). When phosphorylated at this amino acid, YAP binds the scaffolding molecule 14-3-3 and is eventually shuttled to the proteasome for degradation[9–13]. When not phosphorylated at this amino acid, YAP translocates into the nucleus to activate various gene expression programs (Figure 1A, Hippo OFF).

Although slightly smaller, TAZ has similar sites of regulation and activity. YAP and TAZ seem to have redundant roles in the liver, supported by the fact that they bind the same genomic targets[14, 15], but whether there are nuances in how they regulate their target genes remains to be explored.

Reports that *yorkie*, the Drosophila homolog of *YAP*, promotes proliferation and prevents apoptosis[16] spurred researchers to investigate whether manipulation of *YAP* and other homologues in the mammalian Hippo pathway produced similar results in vertebrates. Simultaneously, the genomic region containing *YAP* was found to be amplified in breast and liver cancer[17, 18], supporting the idea that increased levels of this protein could contribute to excessive growth and oncogenesis. Shortly thereafter, transgenic overexpression of YAP in mice confirmed this hypothesis: these mice developed rapid hepatomegaly and, over time, hepatocellular carcinoma (HCC)[19, 20]. Notably, YAP-induced hepatomegaly is reversible. Restoring endogenous YAP levels after a period of overexpression leads to a rapid decrease in liver size (Figure 3A) and normalization of the parenchymal architecture, suggesting that the Hippo pathway appears to have an important role in maintaining liver size.

Since then, multiple groups have reported that genetic liver deletion of the upstream Hippo kinases such as MST1 and MST2[21–23] and LATS1 and LATS2[24], or their scaffold molecules SAV[23, 25], MOB1A, and MOB1B,[26, 27] decreases phosphorylation of YAP, causing its translocation to the nucleus, hepatomegaly, and liver cancer (Figure 1A, Hippo OFF). Because overall YAP levels and localization within the cell correlate with the transcriptional output of the Hippo Pathway, it can be thought of as the gatekeeper of the pathway. Often, YAP cellular localization is interpreted to be a proxy for Hippo activity (Hippo ON and YAP inactive or Hippo OFF and YAP active), which may be an oversimplification of YAP's more complex regulation.

Nuclear localization of YAP or TAZ is associated with the activation of target genes. Genes important for liver growth and regeneration, such as CTGF[28], JAG1,[29, 30] and NOTCH2,[30] are direct targets of Hippo signaling. As transcriptional co-activators, YAP and TAZ do not bind to DNA directly but instead act to recruit machinery to their transcription factor partners. In most cases, the TEAD family of transcription factors that bind YAP and TAZ are required for the phenotypes controlled by Hippo signaling[28, 31–34].

The kinases, scaffolding proteins, and transcriptional partners described above form the core of the canonical Hippo pathway with its construction and downstream activity highly conserved throughout evolution (Figure 1A, blue). During homeostasis, Hippo signaling is thought to be active, resulting in high phosphorylation of YAP and low levels of nuclear YAP. The Hippo pathway is therefore considered tumor suppressive, as loss of Hippo signaling results in YAP accumulation, translocation into the nucleus, and activation of genes that promote proliferation and prevent apoptosis (Figure 1B).

Pathway Regulation

The Hippo pathway responds not only to the biochemical but also the physical milieu of the cell[35, 36]. Plating cells at low-density results in YAP activation and proliferation[35, 36]. Once these cells reach confluence, YAP is shuttled out of the nucleus and cell proliferation ceases. Furthermore, increasing the tension experienced by confluent epithelial cells can reactivate YAP/TAZ and restart cell proliferation[37, 38].

Junctional complexes between cells are important sensors that coordinate tissue integrity and growth. These complexes are ideally positioned to detect changes in tissue tension or density and transduce these signals into various signaling pathways, so it would be logical to identify a role for YAP/TAZ at these sites. Whether similar themes are also observed in the liver following injury remains to be determined: indeed, partial hepatectomy results in a dramatic change in the shear stress experienced by hepatocytes due to increased portal venous pressure[39]. The ability of YAP and TAZ to sense changes in tissue architecture and rapidly promote expression of genes that promote organ growth requires further exploration into the cytoskeletal elements that may transduce these external cues.

Cytoskeletal regulators

One of the first upstream regulators identified was NF2 (merlin), mutations in which are associated with the spontaneous development of schwannomas and meningiomas in humans[40]. A member of the FERM family of plasma membrane-associated proteins[41], NF2, when deleted, leads to potent YAP activation and liver overgrowth. This overgrowth phenotype is reduced by hemizygous deletion of *Yap*, indicating that much of the resulting proliferation is due to YAP activity[42, 43]. Recent work has elucidated how NF2 interacts with downstream Hippo components: in *Drosophila* and mammals, NF2 complexes with LATS1 and 2 at the plasma membrane—an event that facilitates phosphorylation of LATS1 and 2 by MST1 and MST2 in complex with SAV[44]. Intriguingly, disruption of the actin cytoskeleton promotes interactions among NF2 and LATS1 and 2,[44] indicating the importance of the plasma membrane as a site of Hippo signal transduction.

A small interfering RNA (siRNA) screen has identified human kinases associated with junctional complexes that affect YAP phosphorylation and activity[45]. Several microtubule affinity-regulating kinases (MARKs) were found to tightly associated with YAP as well as scribble, LGL, DLG and LKB1, all members of the adherens junction (AJ). In prior reports, scribble and LKB1 have been reported to regulate cell growth and proliferation[46, 47]. Accordingly, disruption of *Lkb1* in mice activates YAP, resulting in liver overgrowth[48].

α-catenin, another intracellular member of the AJ complex, results in excessive growth and cancer when deleted in the skin[49, 50]. α-catenin directly binds to YAP, with its loss resulting in YAP nuclear localization and cellular proliferation[35]. In the liver, α-catenin helps organize the liver parenchyma. When α-catenin is reduced through siRNA knockdown, livers are significantly larger in size after injury than controls; moreover, liver sinusoids and bile canaliculi are disorganized, resulting in increased serum bile[51]. In keratinocytes, α-catenin physically interacts with NF2[52], but studies are needed to determine whether a similar regulatory complex exists in the liver.

Angiomotin (AMOT) is yet another protein found at the AJ that strongly binds YAP and TAZ[53–55]. AMOT also binds NF2, regulating its tumor suppressive functions[56]. Because NF2 loss leads to liver overgrowth, it was proposed that AMOT loss would lead to a similar phenotype. In contrast, livers of AMOT-knockout mice are indistinguishable from controls and have reduced regenerative responses after injury, indicating that AMOT instead facilitates YAP mediated gene activation[57].

It is not clear how these membrane complexes interact and integrate various cues into functional transcriptional changes. Liver epithelial cells have apical, basolateral, and tight junction complexes, all of which contain YAP and TAZ, and to varying degrees, associated regulatory proteins. At the subcellular level, how do these various plasma membrane compartments transmit extracellular signals through YAP and TAZ—particularly since they each serve slightly different roles? The example of AMOT facilitating downstream YAP activation in certain contexts despite its ability to also bind NF2 illustrates the complexity of YAP and TAZ regulation at the plasma membrane.

Studies have provided evidence for the differential regulation of *Yki* by AJ and basolateral complexes. AJ loss is predominantly associated with non-cell autonomous accumulation but a cell-autonomous decrease of *Yki*, whereas basolateral loss results in cell autonomous accumulation of *Yki*[58]. That cytoskeletal components can dynamically alter the cell state of a neighboring cell could help explain, in part, how tissues coordinate maintenance or regenerative programs.

Notably, these interactions are not unidirectional: YAP, through its close association with actin filaments, can also regulate cytoskeletal proteins. Recent work has shown that YAP overexpression in hepatocytes promotes the formation of a contractile actin structure that destabilizes E-cadherin-mediated cell-cell junctions[59]. In a similar vein, TAZ knockdown *in vitro* reduces cellular invasion and results in upregulation of E-cadherin[60]. This evolving crosstalk between YAP/TAZ and the cellular cytoskeleton has important implications for cellular organization and tissue homeostasis[61].

Receptor-mediated signaling

Bile duct ligation often leads to rapid biliary proliferation, suggesting that one of bile's components directly stimulates cell proliferation[62, 63]. Mouse knockout models of the nuclear receptor farnesoid X receptor (FXR) and the small hetero-dimer partner (SHP) result in a marked accumulation of hepatic bile acids without biliary obstruction. FXR- or SHP-knockout mice have enlarged livers and develop liver tumors through a YAP-dependent process that requires the scaffolding molecule IQ motif containing GTPase activating protein 1 (IQGAP1)[64]. Notably, IQGAP1 is greatly upregulated in these mice following the disruption of the plasma membrane through chronic bile acid exposure.

While their role in activating YAP within the liver is not well understood, G protein-coupled receptors (GPCRs) have been recently identified as important modulators of the Hippo pathway in other tissues[65, 66]. Small molecule screening has identified several serum-bound ligands that can modulate Hippo signaling, including epinephrine, estrogen, lyosphosphatidic acid, sphingosine 1-phosphate[65, 66], thrombin,[67] Wnt3a, Wnt5a, and Wnt5b[68]. Moreover, mutant GPCR signaling in uveal melanoma is one example in which normal Hippo signaling has been coopted[63,64], but studies are needed to determine whether HCC can develop via similar mechanisms.

Development, Homeostasis and Regeneration

YAP and TAZ have critical roles during development. YAP-knockout mice die at embryonic day 8.5 and present with defects in yolk sac vasculogenesis, chorioallantoic attachment, and body axis elongation[69]; TAZ-knockout mice develop renal cysts and emphysema[70, 71].

YAP is found throughout the adult liver, although biliary cells demonstrate the highest levels of YAP protein and activity (Figure 2A)[30, 72, 73]. The remainder of the liver parenchyma has a graded distribution of YAP, highest in the portal area and lowest in the central venous region (Figure 2B)[74]. The biliary ducts are most profoundly affected by liver-specific YAP deletion: these mice are born with hypoplastic biliary ducts that are progressively lost over time[42]. Gradually, hepatitis and fibrosis develop, likely due to cholestatic liver injury from the immature biliary system and hepatocyte hypersensitivity to injury[42].

Studies of the role of the Hippo pathway components during early liver development are limited. The Albumin-Cre mouse line that is commonly used to inactivate components of the Hippo pathway has moderate but limited activity in embryonic hepatoblasts/hepatocytes, which makes interpretation for a role of this pathway during development difficult. Using this model, inactivation of LATS1 and 2 during development leads to a prominent expansion of the ductal plate (the precursor of mature bile ducts) at E17.5 and an increase in immature biliary epithelial cells at the expense of mature hepatocytes at P1. Additionally, LATS1 and 2-knockout hepatoblasts are more likely to differentiate into biliary-like cells in vitro[75], a result consistent with our observation that YAP overexpression in adult hepatocytes causes transdifferentiation into biliary epithelium[30].

In the adult liver, YAP deletion in hepatocytes is inconsequential during homeostasis (Figure 2A, Ad-cre *Yap* fl/fl) as there appears to be compensation through increased expression of

TAZ[76]. Adult YAP liver knockouts do not acutely develop biliary duct loss or hepatocyte necrosis. However, mice with hepatocyte-specific deletion of YAP have extensive hepatic necrosis and high mortality after bile duct ligation, indicating that the compensatory increase in TAZ is insufficient to promote normal regeneration[30, 77].

After partial hepatectomy, an injury associated with a rapid and widespread proliferative response, overall YAP protein levels increase, YAP phosphorylation decreases, and Hippo target genes are upregulated[78–80]. YAP becomes localized to the hepatocyte nucleus immediately after injury, with overall YAP levels eventually decreasing several days later (Figure 2C). The coordination of hepatocyte proliferation after partial hepatectomy may be partially due to hedgehog pathway ligands derived from hepatic stellate cells (HepSC). YAP was recently reported to be a downstream target of the hedgehog pathway. By blocking hedgehog signaling in HepSCs, hepatocyte proliferation is blunted and nuclear YAP accumulation in neighboring hepatocytes does not occur[81], evidence of the critical contribution of stromal cells in promoting regeneration.

HepSCs often have high levels of nuclear YAP, indicating that their activity is particularly sensitive to the Hippo pathway. Moreover, YAP is important in promoting their activation into myofibroblasts, which secrete factors that help coordinate the regenerative response following liver injury[82]. Protracted YAP signaling may lock these HepSCs into a myofibroblastic fate, resulting in defective repair mechanisms that contribute to liver cirrhosis. A small molecule inhibitor of YAP[82], as well as omega-3 polyunsaturated acids (which accelerate YAP and TAZ degradation[83]), reduce development of fibrosis in mice, so agents that target this pathway may slow the development of cirrhosis.

Emerging Roles in Liver Zonation

Homeostasis and regeneration

Nuclear localization of YAP is mostly restricted to the periportal zone of the liver, with biliary cells demonstrating the highest levels[23]. Conversely, the pericentral zone has little to no nuclear YAP. Interestingly, this Hippo signaling gradient appears in opposition to that of WNT signaling, which is highest in the central venous area and diminishes towards the portal area[84]. The presence of these opposing gradients, which help define specific liver zones, raises the question as to how these 2 pathways interact in regulating liver development, homeostasis, and regeneration.

Liver growth can be driven through activation of both pathways independently. Injection of R-spondin 1 (RSPO1), which potentiates WNT signaling, causes increased proliferation and increased overall liver size[80]; YAP overexpression in hepatocytes likewise leads to hepatomegaly. However, whether hepatocytes from different zones effect these changes remains to be determined. With regards to YAP, we observed that YAP overexpression specifically activates periportal hepatocytes[30]. During homeostasis, all 3 zones (periportal, pericentral, and midlobular) have equivalent proliferation rates, measured by EDU incorporation[85], indicating that zonal dominance is not a particular feature of liver homeostasis.

In the context of regeneration, the type and duration of injury seem to dictate which cells are recruited for proliferation[85–87]. Partial hepatectomy, an acute injury that affects the liver indiscriminately, leads to the approximately equal expansion of all 3 zones. Regeneration post-PH seems to progress in multiple waves, with periportal hepatocytes proliferating first (possibly due to a shorter G1 phase)[88], followed by midlobular and pericentral hepatocytes. Chronic CCl_4 administration, conversely, which predominantly damages the central venous zone and results in fibrosis, leads to the activation and expansion of periportal hybrid hepatocytes (hybrid because they also express biliary markers in addition to hepatocyte markers). These hepatocytes reconstituted approximately 67% of the new hepatocytes generated in response to chronic CCl_4 injury[73]. Fundamentally, hepatocytes in all 3 zones have the capacity to replicate in response to injury; when this capacity in blocked in one zone, hepatocytes from other zones can seemingly compensate.

Within these contexts, it is not known how Hippo and WNT interact. The glutamine synthase zone (a marker of WNT activity that serves to define the pericentral zone) increases following YAP knockout and decreases following knockout of MST1 and MST2[74]. Additionally, the hepatocytes with increased YAP activity (in MST1 and MST2-knockout mice) have a decreased nuclear localization of β-catenin[74], so the 2 pathways may inhibit each other. Further studies are required to resolve how these pathways interact during homeostasis and regeneration.

Metabolism

In addition to variable proliferative capacity, hepatocytes have different metabolic functions depending on their position on the pericentral–periportal axis: pericentral hepatocytes are largely involved in glycolysis, bile synthesis, and glutaminogenesis, whereas periportal hepatocytes function mainly in gluconeogenesis and ammonia clearance. It is not clear whether the opposing Hippo and WNT signaling gradients also help define a particular metabolic state.

A study of a YAP-overexpressing zebrafish model found, surprisingly, that YAP can directly increase expression of glutamine synthase, independent of the WNT pathway. The resultant increase in glutamine increases nucleotide biosynthesis, expanding the available nucleotide pool that fuels YAP-mediated liver growth[89]. In mice, however, liver-specific deletion of MST1 or MST2 reduces the glutamine synthase zone[74], suggesting that upstream Hippo kinases might regulate other substrates that help to determine regulation of gene expression by YAP. Nevertheless, it is clear that YAP can modify the expression of genes that regulate metabolism within a cell. Determining the mechanisms of this process will require a more clear definition of the specific contexts in which gene expression is controlled.

Modifications of other components of the Hippo pathway can also affect liver metabolism. Mice that have a liver-specific disruption of *Lats2* have increased expression of SREBP target genes, resulting in increased cholesterol synthesis and the development of steatosis[90]. This phenotype occurs independently of YAP, indicating that the Hippo pathway can regulate metabolic homeostasis through multiple effectors.

Mechanisms that Moderate YAP/TAZ Activity

Persistent high-level expression of YAP leads to the development of liver cancer within 1–2 months (Fig 3A), whereas mutations in factors upstream of Hippo often require a much longer timescale (9–12 months) to develop. Additional YAP inhibitory and cell protective mechanisms are likely responsible for this disparity, with loss of such feedback contributing to cancer development.

One potential mechanism is inhibiting the binding of YAP and TAZ to their transcriptional partner TEAD, either through peptidomimetics or small molecular inhibitors[91, 92]. Vestigial-like4 (VGLL4) is an important competitive inhibitor of TEAD activation, acting to partially mask the YAP–TEAD binding site and interfering with its ability to activate downstream target genes[93, 94]. Peptides that mimic VGLL4 and interfere with YAP–TEAD interaction slow the growth of YAP-dependent gastric tumors[93].

MicroRNAs offer a mechanism to downregulate RNA expression. Depending on the Hippo pathway component that is targeted, this can up- or down-regulate YAP/TAZ activity. miR-9-3p has been reported to specifically target TAZ in hepatoma cell lines[95], an activity which will need to be validated *in vivo*. Several other microRNAs to be considered with respect to the liver include LATS2 targeting miR-135b[96] and miR-31[97], and miR-375 which regulates YAP1[98]. These microRNAs are reported to have activity in other tissues.

Gene expression signatures associated with high YAP activity often include altered expression of its upstream regulators[37, 45]: as YAP and TAZ activity increases, expression of proteins that reduce their activity also increase, generating a negative-feedback loop. One such target is the protein LATS2, a kinase that inactivates YAP and TAZ[76]. Knockout of YAP from livers of mice reduces expression of LATS2, resulting in reduced inactivation of TAZ. This would help explain the compensatory increase in levels of TAZ in YAP-knockout mice.

YAP has multiple potential regulatory sites, although serine 127 has garnered the most focus, because of its role in facilitating nuclear YAP localization. Mice that express transgenic YapS112A (mouse homolog of S127) do not have liver overgrowth and have livers with a normal appearance. Although Yap is predominantly localized to the nucleus, the total amount is decreased. These mice are particularly prone to injury—exposure to diethylnitrosoamine results in hepatomegaly and increased development of liver tumors. This demonstrates that additional mechanisms exist that maintain the total transcriptional output of the Hippo pathway. Further work has identified serine 366 as a phosphodegron site that can be exploited by the cell to downregulate total levels of YAP[24].

Additionally, SIRTUIN1 (SIRT1), a protein associated with longevity and tumor suppressive activity is associated with YAP levels in the liver. Using knockout and overexpressing mice as well as complementary cell lines, the expression of YAP directly correlates well with SIRT1 expression. Whether YAP is a direct enzymatic target of SIRT1 or a secondary downstream target has yet to be resolved[99].

These mechanisms highlight the extent to which the Hippo pathway has developed multiple means to temporally limit YAP activity. Understanding and measuring their overall contribution to Hippo signaling will be useful for designing targeted cancer therapies.

Liver Cancer Development

YAP is overexpressed in a number of solid tumors, including those of the colon[100], breast[17], lung[101, 102], and ovary[103, 104], as well as in medulloblastoma[105], cholangiocarcinoma, hepatoblastoma, and HCC (Figure 3B)[21, 72] indicating that it promotes cancer progression. In HCC, the core Hippo constituents are typically not mutated; instead, the increase in YAP levels is largely due to gene amplification and post-transcriptional regulation[18].

Increased YAP activity is an early event in liver cancer development[92]. In mice, overexpression of YAP alone results in HCCs[20]. Hepatocytes that express high levels of YAP dedifferentiate and acquire features of hepatic progenitor cells[30], which could allow them to rapidly accumulate oncogenic mutations through increased proliferation. Knockouts of Hippo pathway components such as MST1, MST2[21], SAV[23, 25], LATS[24], or NF2[42, 43] lead to similar phenotypes in mice (See Table 1), but there is little evidence that they contribute to HCC in humans[21]. Consequently, analyses of downstream factors in the Hippo pathway could provide more information about the role of YAP in HCC development and progression.

In this vein, a gene expression signature associated with loss of Hippo signaling has been identified that predicts reduced survival time of patients with HCC. This silence of Hippo signaling (SOH) signature was developed and validated in 4 independent international cohorts of patients with HCC. The SOH gene expression signature can be used to aid pathology staging criteria[106]. Notably, the SOH tumor signature overlaps partially with a previously reported hepatic stem cell gene signature[107] associated with an aggressive clinical course. There is evidence that increased YAP can lead to dedifferentiation and progenitor cell expansion.

Testing for components of the Hippo pathway by immunohistochemistry or mRNA expression is likely to be the most accessible means of delivering prognostic information to patients with HCC. The presence of nuclear YAP staining was strongly associated with a shorter disease-free survival time in a European cohort[108]. Studies from smaller Asian cohorts demonstrated that high levels of *YAP, TAZ* and *TAZ* mRNA were each independently associated with a lower overall rate of survival[60, 109].

YAP seems to be involved in several pathways that control liver development, regeneration, and disease. One of its signaling partners is NOTCH, which is required for biliary expansion and specification during liver development[110–112]. During tumorigenesis, YAP increases transcription of jagged1 and notch2 which promote hepatocyte dedifferentiation and cancer growth[29, 30].

HCC and HB also commonly have active WNT signaling[113, 114]. Mice with hepatocytes that express activated forms of YAP and β-catenin (but not either protein alone) rapidly develop hepatoblastoma[115]. Moreover, WNT signaling can stabilize YAP to potentiate its

downstream effects, through upregulation of TRIB2, which downregulates a ubiquitin ligase that normally targets YAP for degradation[116]. Additionally, in certain subtypes of HCC, loss of Hippo signaling correlates with mTOR pathway activation[117, 118], which is thought to proceed through activation of the PI3K pathway via a YAP-induced microRNA that reduces expression of PTEN[119]. YAP has also been shown to activate the mTOR pathway through the upregulation of amino acid transporters, SLC38A1 and SLC7A5[118]. This mechanism, in part, sustains YAP1-mediated proliferation of HCC cells. Interestingly, reduction of *SLC38A1* (which encodes a glutamine transporter) reduces proliferation of HCC cells. Studies of this dependency on glutamine and the potential role of YAP in effecting this metabolic change (also supported by[89]) could help identify new targets for HCC therapy.

YAP activity is also associated with human cholangiocarcinoma and reduction in its activity *in vitro* limits cholangiocarcinoma growth[120]. About 12% of cholangiocarcinomas are associated with focal deletions at the *SAV1* locus, providing a mechanism by which YAP can become activated[121]. Mice that express transgenic YAP and activated PI3K, or its downstream effector AKT, in liver develop cholangiocarcinomas. Transcriptional profiles of these cholangiocarcinomas are similar to those of patient tumor tissues[122]. Patients with cholangiocarcinoma have high mortality and few therapeutic options, so studies of this mouse model are important.

Targeting the Hippo pathway may be an effective strategy for treating HCC. Liposome-mediated administration of a YAP siRNA to HCC causes cells to redifferentiate into hepatocytes, supporting the observation that high expression of YAP increases the stemness of cells[74]. As these experiments were performed in cells with mutations in the Hippo pathway, further work is needed to determine whether hepatocyte redifferentiation is effective in HCC cells with other driver mutations (in WNT, PI3K, p53, etc.), although there is evidence that the Hippo pathway interacts with these other pathways during liver carcinogenesis.

It is unclear if alterations in the activity of TAZ, the other co-activator of the Hippo pathway, contribute to the development or progression of liver cancer and should be explored as therapeutic targets. TAZ is an important transactivator of the Hippo pathway in liver cancer cells[95, 109], although there has been no in-depth study to determine the specific roles for YAP and TAZ in the liver. Several genome-wide, chromatin immunoprecipitation studies of YAP and TAZ binding found these molecules to be interchangeable, binding to the same sites across the genome and presumably regulating the expression of similar genes[14, 15].

An exome sequencing study of 243 HCCs identified 161 genes that promote tumorigenesis, via 11 different pathways[123], but none were in the Hippo pathway. Small studies have shown that YAP amplification[18], deletion of STK3 or STK4[21], or deletions in SAV1[121] contribute to development of HCCs and cholangiocarcinomas. High tumor levels of YAP and TAZ have been associated with shorter disease-free survival times[21, 72, 101, 106, 108, 109, 115, 124–126]. Studies are needed to determine the mechanisms that activate YAP and TAZ, and other features of their regulation.

Altogether, these studies suggest that inactivation of Hippo signaling forms a baseline upon which other signaling systems affect cancer phenotype. Undoubtedly, understanding and targeting each of these pathways during therapy could significantly advance our ability to develop effective and durable liver cancer therapy.

Future Directions

The Hippo signaling pathway has been progressively recognized as a potent growth regulator. Over the last several years, we have increased our understanding of the function of this pathway during development and regeneration, and now have rationale for design of therapeutic agents. A large number of signaling inputs into the Hippo pathway have been identified, but it is not clear how these affect YAP activity to regulate disparate phenotypes (Figure 3C).

Many questions remain and continue to emerge from the study of this pathway. These include: What determines the zonation of YAP and TAZ expression in the liver? What is the consequence of hippo pathway zonation? Does this pattern of signaling affect zonation defined by Wnt signaling? How do junction complexes differ in their regulation of YAP and TAZ? How is YAP activity maintained during homeostasis? What factors increase YAP activity after liver injury and then subsequently down regulate it? Although YAP is often overexpressed in cancers, why are so few mutations found in the hippo pathway? How does hippo signaling interact with other biochemical pathways, such as NOTCH and WNT signaling? And finally, what are the downstream targets of YAP/TAZ that mediate their biological functions?

Answers to these and other questions will facilitate a better understanding of liver homeostasis and pathogenesis.

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Author names in bold designate shared co-first authorship.

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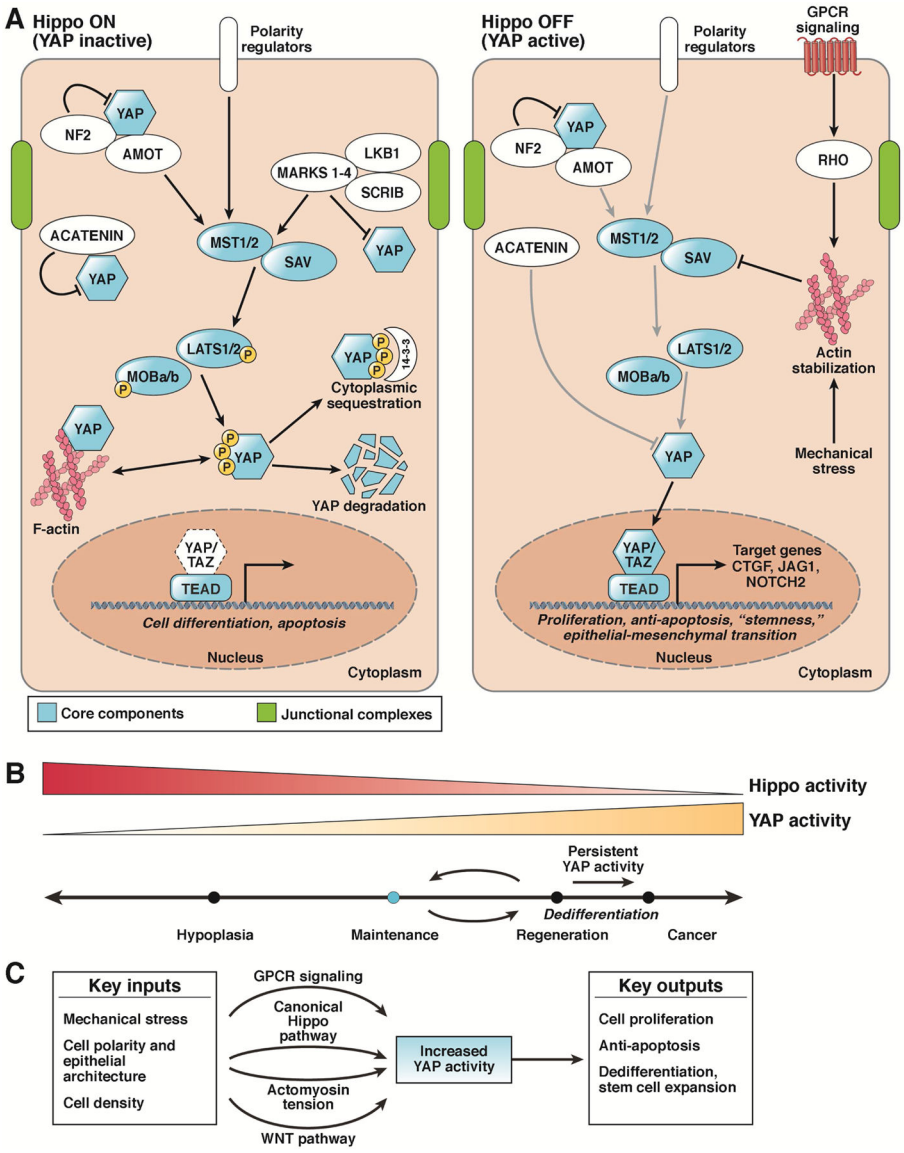

Figure 1. Regulation of the Mammalian Hippo Signaling Pathway

A. The Hippo pathway (mammalian) consists of the core components STK3 and STK4, SAV, LATS1 and 2, MOB1A and B, YAP, TAZ, and TEAD. Upon activation of the canonical Hippo pathway, STK3/4 phosphorylates and activates Lats1/2, which subsequently phosphorylates cytoplasmic Yap. During homeostasis, Hippo signaling is ON resulting in Yap phosphorylation (S112 in mice, S127 in humans) causing 14-3-3 binding and cytoplasmic sequestration. Phosphorylation of Yap can also lead to proteasomal degradation. When Hippo is off, YAP (in the unphosphorylated for) translocates to the nucleus and binds to the TEAD family of transcription factors, leading to the transcription of genes involved in cell survival, growth, and proliferation. Proposed cell activities for each state are found beneath the gene in italics. Arrows indicate positive relationships, while bars indicate negative activity.

B. YAP activity at various levels and for various time periods differentially modulates cell state and phenotype.

C. Multiple physiologic and pathologic inputs modulate YAP activity. Mechanical stress, cell polarity, and cell density are all factors that have been shown to modulate Yap activity. Additionally, knowledge of these different states is communicated via various signaling modalities, including the aforementioned canonical Hippo pathway, the Wnt pathway, GPCRs, and changes in cytoskeletal tension.

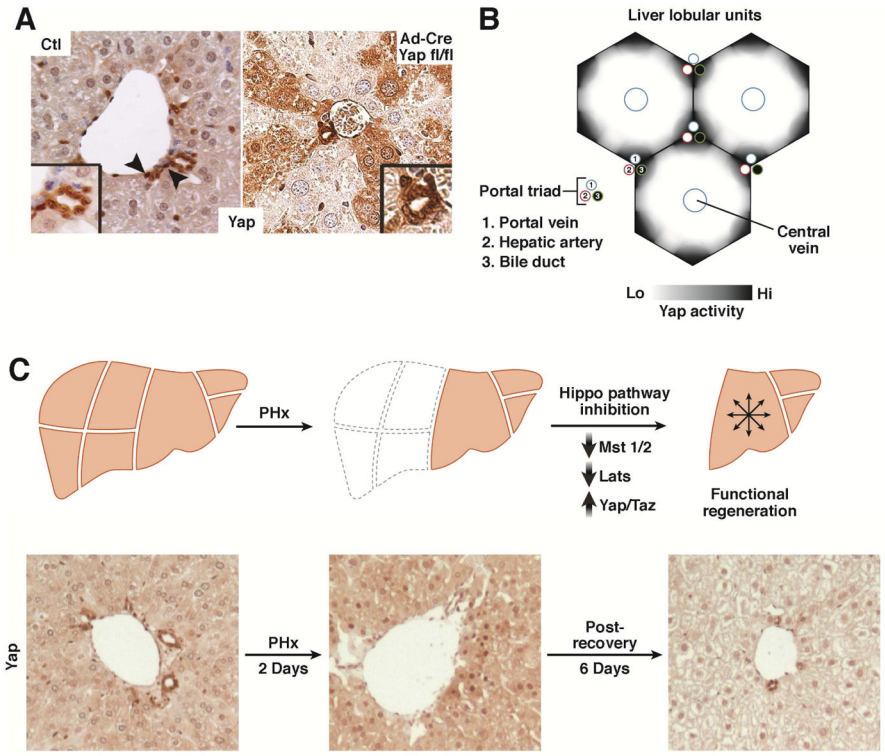

Figure 2. Yap Expression During Homeostasis and Regeneration

A. YAP is present in the epithelial cells of mouse liver (hepatocytes and biliary cells). YAP expression and nuclear-localization is more prominent in biliary cells (arrowhead) as compared to hepatocytes. Ad-Cre Yap fl/fl illustrates that YAP is present in hepatocytes as documented by mosaic Yap staining after deletion[30].

B. Schematic of YAP activity in the liver. YAP activity is highest in the biliary cells/portal hepatocytes, diminishing in the hepatocytes toward the central vein.

C. Hippo/Yap activity dynamically changes after partial hepatectomy. Yap levels increase with an associated decrease in MST1, MST2, LATS1 and LATS2 activity. These return to their normal levels as the liver reaches its appropriate size. Partial hepatectomy in mice results in YAP enrichment and an increase in nuclear localization (Day 2). After 8 days of recovery, YAP expression is reduced to below baseline levels.

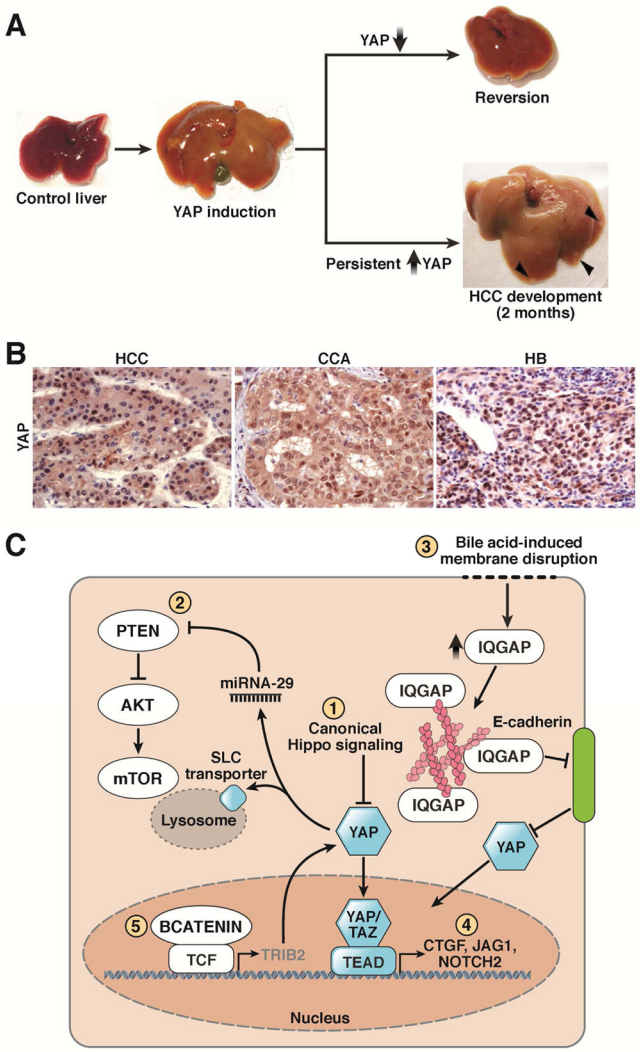

Figure 3. Effects of Yap Overexpression in the Liver

A. Liver-specific overexpression of YAP leads to massive hepatomegaly with livers approaching 4–5x their original size. Upon restoration of endogenous levels of YAP, the liver returns to its usual size[19]. Persistent YAP activation for 2 months frequently results in HCC development (Arrowheads).

B. Increased overall YAP and nuclear YAP is a feature of several liver cancers, including HCC, CCA, and HB[115].

C. YAP can mediate its tumorigenic effects either autonomously or through synergy with other pathways. YAP can be activated through canonical Hippo inactivation (1) or non-canonical membrane-associated signaling (2). YAP can also interact with the PI3K–Akt–mTOR pathway through a microRNA-mediated mechanism or via upregulation of lysosomal SLC transporters (3). Finally, YAP can interact with the NOTCH and Wnt pathways, as evidenced through upregulation of NOTCH ligands and receptors (4) and YAP's stabilization by the Wnt target gene *TRIB2* (5).

Table I

Phenotypes of Hippo Pathway Mutants in the Liver

Mutation	Genotype	Phenotype	References
YAP overexpression	ApoE-rtTA; TRE Yap	hepatomegaly, HCC	20
	LAP1-tTA;TetO-Yap(S127A)	hepatomegaly, HCC	19
	Ck19-CreERT2;TetO-Yap(S127A)	ductal hyperplasia	30
	AAV-Cre; TetO-Yap(S127A)	dedifferentiation, hepatomegaly, HCC	30
	Yap(S112A)/Yap(S112A)	no change at baseline, hypertrophy after injury	24
YAP knockout	AAV-TBG-Cre; Yap fl/fl	hepatocyte sensitivity to stress	30
	Alb-Cre; Yap fl/fl	biliary and hepatocyte hypoplasia	42
MOB1A and MOB1B	Mob1a Δ/Δ 1b tr/+	HCC development	26, 27
Knockout of MST1 and MST2	Ad-Cre; Mst1−/−; Mst fl/fl	HCC development	21
	Alb-Cre; Mst1−/− Mst fl/-		22
	Alb-Cre; Mst1−/− Mst fl/fl		23
Knockout of NF2	Alb-Cre; Nf2 fl/fl	biliary ductular reaction, HCC	42
	Ad-Cre; Nf2 fl/fl		43
Knockout of SAV	Alb-Cre; Sav1 fl/fl	Hepatic progenitor expansion, HCC, CC	25
Knockout of NF2 and SAV	Alb-Cre; NF2 fl/fl Sav1 fl/fl	Biliary cell hyperplasia	44
Knockout of LATS1 and LATS2	Ad-Cre; Lats1−/−; Lats 2 fl/fl	Hepatomegaly, biliary cell expansion	24
Knockout of α-catenin	ΛΝΠ α-catenin siRNA	Hepatomegaly, disorganized sinusoids, cholestasis,	51