



HHS Public Access

Author manuscript

J Hepatol. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

J Hepatol. 2015 December ; 63(6): 1491–1501. doi:10.1016/j.jhep.2015.07.008.

Emerging evidence on the role of the Hippo/YAP pathway in liver physiology and cancer

Dean Yimlamai, MD, PhD^{1,2}, Brendan H. Fowl^{1,2}, and Fernando D. Camargo, PhD^{1,3,4}

¹The Stem Cell Program, Department of Medicine, Boston Children's Hospital, Boston, MA 02115

²Division of Gastroenterology and Nutrition, Department of Medicine, Boston Children's Hospital, Boston, MA, 02115

³Harvard Stem Cell Institute, Cambridge, MA 02138

⁴Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138

SUMMARY

The Hippo pathway and its regulatory target, YAP, has recently emerged as an important biochemical signaling pathway that tightly governs epithelial tissue growth. Initially defined in *Drosophila*, this pathway has shown remarkable conservation in vertebrate systems with many components of the Hippo/YAP pathway showing biochemical and functional conservation. The liver is particularly sensitive to changes in Hippo/YAP signaling with rapid increases in liver size becoming manifest on the order of days to weeks after perturbation. The first identified direct targets of Hippo/YAP signaling were pro-proliferative and anti-apoptotic gene programs, but recent work has now implicated this pathway in cell fate choice, stem cell maintenance/renewal, epithelial to mesenchymal transition, and oncogenesis. The mechanisms by which Hippo/YAP signaling is changed endogenously are beginning to come to light as well as how this pathway interacts with other signaling pathways, and important details for designing new therapeutic interventions. This review focuses on the known roles for Hippo/YAP signaling in the liver and promising avenues for future study.

The Core Hippo Pathway Members in Biology

A fundamental question in biology is defining the underpinnings of how organisms and their constituent parts “know” the size they are to grow and when to stop. What are these mechanisms that restrict tissue and organism growth, and if this tight regulatory control is lost, could this then lead to the development of cancer? The liver in particular has the ability to regrow to its original mass within a few weeks after partial hepatectomy, permitting life-saving procedures such as split-liver living donor liver transplantation to be performed. The molecular mechanisms governing this phenomenon are starting to become clear, but sufficient detail to develop new therapeutics to stimulate liver regeneration or inhibit cancer

Correspondence to: dean.yimlamai@childrens.harvard.edu, fernando.camargo@childrens.harvard.edu.

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growth remain in its infancy. To this end, many scientists have used various model systems to define the molecular mechanisms that initiate and restrict tissue growth. The Hippo pathway has emerged as an important biochemical signaling pathway that tightly governs tissue growth. This review will focus on the known roles for Hippo signaling in the liver, how it affects its primary regulatory target, YAP, and promising avenues for future study.

Initially, the Hippo pathway was defined through genetic screens in *Drosophila* for regulators of growth (Figure 1A). The pathway is aptly named for the serine/threonine kinase *hippo* (*hpo*), which when lost, results in tissue overgrowth due to excessive proliferation and decreased apoptosis [1]. Two other *Drosophila* mutants had been discovered several years prior to this publication, the *warts* (*wts*) kinase [2] and the scaffolding molecule, *salvador* (*sav*) [3]. The primary activity of the pathway is to restrict the cytoplasmic localization of *yorkie* (*yki*) through critical phosphorylation sites [4–6]. *yki* is a transcriptional co-activator that integrates Hippo signaling, acting as a “gatekeeper” regulating proliferation and growth. The *hpo*, *sav* and *wts* mutants exhibit a similar overgrowth phenotype as overexpression of *yki*, suggesting that these proteins interact to form a growth regulatory gene program. Through a number of biochemical and genetic epistasis experiments, these molecules form the core of the canonical Hippo pathway. Hippo signaling is commonly referred to as a tumor suppressor, as its “baseline” activity is to restrict *yki* to the cytoplasm. Hippo signaling loss results in accumulation of *yki*, its translocation into the nucleus to trigger a downstream gene program. Subsequent work has greatly expanded our biochemical understanding of the pathway (Figure 1A), but a major challenge is developing a clear understanding of the contexts within which Hippo regulates *yki*.

Remarkably, many of the components of this pathway that were initially described in *Drosophila*, have vertebrate homologs, which share functional and biochemical activity (Figure 1B). For example, YAP, the vertebrate homolog of *yorkie*, when expressed in *Drosophila* can rapidly expand its tissues [7]. There are two homologs of *yorkie* in vertebrates, YAP and TAZ. Although, these homologs have different sizes, both molecules have strong co-transcriptional activity, have critical phosphorylation sites that control their nuclear localization which regulates their ability to activate downstream genes and contain WW motifs that modulate protein-protein binding [8–10]. A large body of work supports the Hippo pathway regulating YAP’s activity in the liver leading to stem cell renewal, regeneration and oncogenesis. Because of the high degree of evolutionary conservation, invertebrate members of the Hippo pathway serve as a resource to interrogate their potential roles in the liver.

With regards to TAZ, it may have similar or overlapping roles in the liver to YAP, but this has not been as extensively explored. Future studies will have to address what roles, if any TAZ may play in liver homeostasis and regeneration.

The Consequence of Manipulating Hippo/YAP Activity in the Liver

Two groups independently provided the first direct evidence that high levels of YAP in the liver rapidly leads to hepatomegaly and eventually hepatocellular carcinoma in the mouse

[7, 11]. Within a week, the liver has doubled in size and by two weeks is typically 20% of the mouse's body weight (Figure 2A, normal 3–5%). Persistent elevation of YAP levels results in tumor development (Figure 2A, 5 month). Restoring endogenous YAP levels after a period of overexpression leads to rapid reversal of the hepatomegaly, and normalization of the parenchymal architecture; strongly suggesting that Hippo signaling may act as an important regulator of overall liver size.

When Hippo signaling is active, YAP is phosphorylated and restricted to the cytoskeleton through its binding to 14-3-3 [12]. Loss of phosphorylation whether by decreased kinase activity [13, 14] or through increased phosphatase activity is associated with nuclear localization of YAP [15] and the subsequent activation of downstream proliferative and anti-apoptotic gene programs. Several mouse liver knockouts of Hippo pathway regulation have been reported including MST1/2 [16–18], NF2 [19, 20], and WW45 [18, 21]. These models universally display increased levels of YAP, decreased YAP phosphorylation resulting in its nuclear relocalization. Each model developed liver overgrowth due to excessive cellular proliferation, but the primary cells composing these enlargements varied with the targeted gene. MST1/2 deletion affects hepatocyte proliferation, NF2 deletion leads to biliary hyperplasia and WW45 mutants demonstrated an abundance of hepatic progenitor cells. These results suggest that loss of various components of the Hippo pathway have differential effect on YAP activity, or that there is heterogeneity in how liver epithelial cells respond to changes in Hippo signaling. Accumulating evidence suggests that both of these hypotheses regarding Hippo signaling in the liver may be true.

Hippo Signaling Fine-tunes YAP Activity and Its Resulting Output

For many years, it had been presumed that a linear relationship between the core Hippo pathway components existed (Figure 2B, Linear). This model predicts that loss of one or more core members of the pathway lead to comparable levels of YAP activation and phenotypes that would be highly similar. As noted previously, this is not the case in the liver.

Duojia Pan and his group has refined this view; supporting a model that an actin-associated MST1/2-WW45 complex phosphorylates a plasma membrane-associated NF2/LATS1/2 complex (Figure 2B, Parallel). The parallel model predicts that these core components intimately cooperate to regulate YAP activity. Loss of a single regulator would yield a mild phenotype, while loss of multiple components leads to high levels of YAP activity and profound liver overgrowth. In the liver, loss of either NF2 or WW45 leads to a mild proliferative biliary phenotype with minimal liver hypertrophy. Concomitant loss of both molecules results in potent biliary hyperplasia, near complete loss of YAP phosphorylation—a sign of high YAP activity, and a profound enlargement of the liver [22].

Similarly, we have found that varying the level of YAP expression in the liver leads to different cellular phenotypes. Several years ago, using a hepatocyte specific driver to control a tetracycline-inducible form of YAP, we described that YAP's primary function at high levels was to drive cell proliferation [11]. Although some progenitor markers began to appear in this context, the cells that drove YAP expression did not completely lose their

phenotypic and biochemical hepatocytic markers. More recently, using a different system, we documented that high levels of YAP can drive hepatocytes to completely develop the properties of hepatic progenitor cells. These cells are easily expanded in tissue culture, can be transplanted to other animals for therapy, and revert to hepatocytes or assumed the characteristics of cholangiocytes when endogenous levels of YAP were restored [23]. The difference in driving a hepatocyte to proliferate versus dedifferentiating under the influence of YAP is likely accounted for by the level and persistence of YAP expression in these contexts. In our earlier publication, YAP expression was driven by a liver specific promoter, which limited YAP expression to those cells in the “hepatocyte-specific” context. The more recent publication utilized a driver of expression that is less affected by cell type, resulting in consistently high YAP expression that uncovered the dedifferentiation phenotype. Our results as well as those of the Pan group, suggest that varying levels of YAP activity may act as a rheostat for guiding cell behavior. Modest YAP levels may cause proliferation with higher levels being associated with changes in cell fate (Figure 3). Levels of TAZ activity have previously been suggested to guide cell fate choices as well [24], making it likely that tight regulation of the Hippo pathway transcriptional co-activators is a common mean by which cells guide these choices. Additional cellular choices involving varying degrees of Hippo activity will likely be uncovered, as we understand how cells and tissues tightly regulate the activity of these proteins.

The hepatocyte response to increased YAP activity throughout the liver is not uniform. Our recent studies have shown that there is a clear preferential expression of progenitor markers and proliferation that occurs in a periportal to central venous pattern (Figure 2C). These results suggest that Hippo signaling synergizes with other biochemical signaling pathways that predominate in the periportal areas. Preexisting factors such as hepatocyte growth factor, fibroblast growth factor, bone morphogenic factor 4, angiopoietin-2, transforming growth factor β 1, jagged1 and other ligands are found in these areas [25, 26]. Modulating these ligands or their cognate receptors in the context of Hippo signaling will provide useful insight on how we might improve our ability to generate liver stem cells.

The Requirement for YAP Activity During Liver Development, Homeostasis and Regeneration

Of the liver epithelial cells, biliary cells have the highest levels of YAP protein and activity [23, 27, 28]. YAP activity is critical for normal biliary development as liver-specific YAP knockout mice are born with hypoplastic biliary ducts, which are progressively lost as they age. YAP knockout hepatocytes are more sensitive to injury, due to a loss of survival factors. These mice gradually developed hepatitis and fibrosis, likely due to cholestatic liver injury from the immature biliary system and hepatocyte hypersensitivity to injury [19]. In the adult, YAP appears to be inconsequential, as liver YAP knockouts do not acutely develop biliary duct loss or hepatocyte necrosis. In contrast, YAP is required for recovery from liver injury as extensive hepatic necrosis and high mortality occurs after bile duct ligation [23, 29].

During chronic liver injury, ductal reactions often develop around the portal areas. They are likely responsible for pathologic liver remodeling and serve as a nidus for cancer stem cells

[30]. Several recent reports support hepatocyte dedifferentiation as a significant contributor to the ductal reaction [31–33]. Since YAP activity is important to biliary development/survival [19] and induced YAP activity in the liver can generate a ductal reaction [23], human biliary diseases should also display evidence of Hippo Signaling loss. In the ductal reactions associated with human cases of primary sclerosing cholangitis, primary biliary cirrhosis and biliary atresia, high concentrations and evidence of nuclear YAP is present. This suggests that dysregulated Hippo signaling leads to the aberrant formation of a dysfunctional biliary system [29, 34]. In principle, inhibitors of YAP activity could ameliorate some of the pathology associated with these diseases.

Connecting Hippo/YAP Signaling to Organ Size

The liver is renowned for its ability to rapidly respond to partial hepatectomy by synchronously activating proliferation in the remaining hepatocytes. After hepatectomy, YAP protein levels rise, its phosphorylation status decreases and Hippo target genes are upregulated [35–37]. In particular, YAP localizes in the nucleus shortly (<4 hr) after partial hepatectomy suggesting that Hippo target genes are involved in liver regeneration [35]. What kinds of signals could facilitate these changes, particularly ones that may be rapidly transmitted throughout the liver?

One possible mechanism where partial hepatectomy could activate hepatocyte proliferation would be if cells could detect changes in shear stress from increased blood flow [38]. YAP is intimately associated with the actin cytoskeleton with the majority of it being cytoplasmic. It is clear that this association is to not simply act as a YAP reservoir, but directly facilitates its ability to sense overall changes in cell tension. Cytoskeletal changes can affect YAP localization in both a phosphorylation-dependent and -independent manner. The most direct evidence for cytoskeletal changes affecting YAP localization comes from work in fibroblasts where increased extracellular matrix stiffness leads to nuclear YAP localization in a Hippo pathway kinase independent manner [39, 40]. Mutations in YAP that uncouple its ability to sense cell tension lead to disorganized tissue and organ development, thereby providing a paradigm by which YAP activity can orchestrate liver size [41].

In addition, a number of cell surface molecules have been identified as YAP interactors, potentially transmitting changes in cell tension into the liver Hippo pathway. In the skin, the adherens junction (AJ) complex is an important sensor of tissue tension with loss of critical components resulting in hyperproliferation and cancer. α -catenin, an intracellular member of the AJ complex associates with YAP, anchoring it to the actin cytoskeleton with its loss being associated with increased YAP activity [42]. A similar situation exists in the adult liver, where α -catenin knockdown livers have disorganized liver sinusoids and are 60% larger in size after partial hepatectomy than controls. This disorganization is associated with elevated serum bile acids, presumably due to improper development of bile canaliculi. [43].

Another cell surface signaling complex that coordinates with YAP are the microtubule affinity-regulating kinase (MARK) family. A targeted siRNA screen of the known human kinome found several members of the MARK family to affect YAP phosphorylation and activity [44]. YAP is tightly associated with the MARK family as well as Scribble and

LKB1, which have been previously described to regulate cell growth and proliferation [45, 46]. LKB1 knockdown *in vitro* affects YAP activity [47] and LKB1 liver knockouts show a 40% increase in liver to body weight that is attenuated by YAP knockout [44].

Finally, several groups have identified the cytoskeletal protein angiomin 1 (AMOT) as a strong binding partner of YAP [48–50]. While it had been predicted that loss of AMOT would result in excessive proliferation, genetic deletion of AMOT is indistinguishable from controls. Upon liver injury, AMOT knockout mice display less biliary proliferation than expected, suggesting that rather than restricting YAP's activity, it potentiates downstream YAP gene activation by acting as a nuclear co-factor [51].

Of the three cytoskeletal proteins/complexes cited above, AMOT, α -catenin, and LKB1, only loss of LKB1 in the adult liver causes changes in organ size. Genetic loss of the other noted proteins requires some form of injury to reveal the process it modulates. This suggests there may be some sort of hierarchy to liver Hippo pathway regulation, or that these cell surface complexes detect fundamentally different forms of input. An understanding of how these complexes cooperate to modulate YAP activity will significantly contribute to our understanding of liver disease.

Alternatively, partial hepatectomy increases blood flow to the remaining lobes, effectively upregulating the concentration of metabolic products and signaling molecules that may activate hepatocyte proliferation [52]. G protein coupled receptors (GPCRs) are an important link into the Hippo pathway that may sense increases in metabolites associated with hepatectomy. GPCRs were identified through small molecule screening for extracellular ligands that can modulate Hippo signaling. Small molecules such as epinephrine, estrogen, lysophosphatidic acid, sphingosine 1-phosphate and thrombin have been identified as GPCR ligands [53–55]. Moreover, this work described that the Hippo pathway could be either positively regulated through the $G\alpha$ subset or negatively impacted by the G12/13 subset. The GPCRs and their ligands should be considered potential endogenous sensors and signals of liver size.

The mechanisms above preferentially affect hepatocytes, but biliary cells display high YAP activity. Could these cells be in direct contact with molecules that inactivate Hippo signaling? A primary physiologic activity of biliary cells is to efflux of bile salts from the liver and obstructive cholestasis often results in biliary proliferation. Bile salts can act as signaling molecules to stimulate regeneration [56] or as detergents, disrupting the cell membrane. Directly increasing bile salt concentration without mechanical obstruction increases YAP levels in the liver, resulting in liver hypertrophy and the development of hepatocellular carcinoma [57]. These results suggest bile acids may upon biliary cells through an unidentified receptor, or by directly changing the properties of the plasma membrane to inactivate the Hippo pathway.

Accumulating evidence indicates that the Hippo/YAP pathway integrates phosphorylation dependent and independent inputs (likely through changes in cytoskeletal tension) to make decisions to activate downstream transcription. In the liver and even at the cellular level, it is unlikely that all of these mechanisms, which are described above are simultaneously present

and active in the same cell. Which cellular subsets utilize these mechanisms and can we identify the extracellular inputs that regulate downstream Hippo gene programs in the liver?

Transcriptional Partners (and Antagonists) of YAP and Signaling Cross Talk

As a transcriptional coactivator, YAP cannot bind to DNA, but it interacts with transcription factors to activate gene expression. The primary binding partners of YAP are the TEAD family of transcription factors [58–62]. They are highly associated with anti-apoptotic, proliferative and “stemness” gene programs [23, 62–64]. Vertebrates have four TEAD family members that are expressed in a tissue-restricted pattern, but seem to have similar affinity for YAP. VGLL4 antagonizes the effects of nuclear YAP by binding to TEAD and interfering with its ability to activate downstream target genes [65, 66].

Prior to the identification of the TEAD family of proteins as major effectors of the Hippo transactivators, several other transcription factors were identified as binding partners of YAP including p73 [67], PEBP2 α [9] and ErbB4 [68]. There is accumulating evidence that although, proliferative/anti-apoptotic/“stemness” programs may be transmitted through the TEAD family, YAP has critical interactions with other transcription factors. Some phenotypes include intestinal cell fate changes through Klf4 [69], enable pancreatic adenocarcinoma relapse utilizing E2F and TEAD2 [70], specifying the mouse trophectoderm in conjunction with RBP-J [71] as well as facilitating epithelial to mesenchymal transition in lung cancer through co-binding with KRAS/FOS [72]. While YAP-TEAD interactions appear to be the most prevalent, other potential YAP binding partners as described above, may facilitate critical activities or cell fate choices in the liver.

Notch signaling is well known to be important in the normal development and maintenance of the liver [73–75]. Subsequent work has shown that Notch and Hippo signaling synergize to potentiate liver cell growth and remodeling. The Notch pathway components, Jagged-1 and Notch2 are downstream targets of Hippo signaling leading to the dedifferentiation of hepatocytes into hepatic progenitors [23, 76]. Loss of Notch signaling in the context of increased YAP activity leads to poor cellular growth and blunting hepatocyte dedifferentiation, supporting the importance of Notch targets and its synergy with the Hippo pathway to support efficient clonal growth.

The WNT pathway interacts with Hippo signaling on multiple levels to modulate cellular output. WNT signaling is well known for its potent roles in stem cell maintenance, cell fate determination, cell migration, neural patterning and oncogenesis [77]. Our group has shown an antagonist relationship regarding YAP and WNT signaling occurs in the cytoplasm through DISHEVELLED inhibition [78]. In other contexts, YAP and β -catenin directly cooperate to activate gene targets [79] and the transcription factor TBX5 can direct the YAP/ β -catenin complex to target genes [80]. More recently, another group has proposed that YAP and β -CATENIN may be primed in a quiescent cytoplasmic complex with AXIN. Upon WNT stimulation, there is a concomitant release of YAP and β -CATENIN, whereupon these molecules translocate into the nucleus and separately activate their respective pathways [81].

From studies in our laboratory as well as others, there is clear heterogeneity in the signaling complexes that contain YAP. Defining the composition and contexts that these YAP complexes operate in the liver is the next challenge to develop a clear understanding of how Hippo/YAP signaling contribute to liver disease.

Loss of Hippo signaling is an oncogenic driver of Liver Cancer

A role for Hippo signaling in cancer began to emerge approximately 10 years ago, when it was discovered that the chromosome region containing YAP is amplified in breast and liver cancer [82, 83]. Embryonic hepatoblasts with a p53 null, c-myc overexpressing background were used in these early screens, making it unclear how characteristic these tumors might compare to HCCs that arise in the adult. Subsequently, it was shown that Yap overexpression in the adult mouse liver, even for as little as 2 months results in hepatocellular carcinoma [7]. Of the HCC models that exist, Yap overexpression results in the most rapid means of generating HCC, which could bode well for *in vivo* chemical drug screening. YAP expression has since been demonstrated to be elevated in a number of human cancer samples including including colon [78], breast [84], lung [85, 86], ovarian [87, 88], medulloblastoma [89], cholangiocarcinoma (CC) and hepatocellular carcinoma [16, 27, 90–93]. It is estimated that 5–10% of human HCCs have genomic amplification of the genomic locus containing YAP [83].

Chemically-induced injury models of HCC suggest that increased YAP activity may be an early oncogenic event. Nuclear YAP was found in preneoplastic hepatic foci within 4 weeks of injury with the number and size of tumors being dramatically reduced when YAP inhibitors were administered [94]. In contrast, late induction of YAP expression can also be a common mechanism that tumors use to overcome targeted blocks to their growth. For example, in hepatocytes, loss of the Retinoblastoma (RB) protein is initially accompanied by rapid cellular proliferation, which slows due to cellular senescence. Genes initially activated by RB loss become silenced during this process. YAP expression restores hepatocyte proliferation partially by reactivating some of these silenced genes [95].

What other mutations may lead to Hippo inactivation and liver cancer? In a small study of HCC patients, more than half had evidence of reduced MST1/2 activity, which leads to increased YAP activity. The mouse model of MST1/2 loss consistently developed HCC supporting its mechanistic role [16]. Hepatitis B infection is commonly associated with HCC. One of the viral products, Hepatitis B Virus X protein directly interacts with the cyclic AMP responsive element binding protein to promote YAP transcription and increase its activity [90].

Mouse knockouts of WW45 [18, 21] and NF2 [19, 20] knockouts lead to the development of HCC and CC. It is interesting to note, that while loss of NF2 clearly leads to liver cancer in the mouse, there are no clinical reports of NF2-associated liver cancers in humans. Neurofibromatosis 2 patients are often reported to have spontaneous schwannomas and meningiomas. In addition, mutations in NF2 have been commonly documented in glioblastoma [96], mesothelioma [97], renal [98] and breast cancer [99] suggesting that in humans, the cells of the liver that lead to HCC or CC may not be susceptible to NF2

mutation or loss. Other unidentified genetic modifiers may make these kinds of cancer particularly rare due to monoallelic loss of NF2.

WNT signaling is commonly activated in a large number of cancers including HCC and hepatoblastoma [100, 101]. An important target of WNT signaling in liver cancer is TRIB2. This pseudokinase stabilizes YAP levels and downregulates C/EBP α expression, which antagonizes YAP-TEAD interactions [102]. *In vivo*, activating mutations of β -catenin, the WNT pathway co-activator does not lead to oncogenesis. Using similar techniques, *in vivo* hepatocyte transfection of YAP also does not lead to cancer, but co-transfection of activated YAP and β -catenin consistently led to the development of HB [103]. These two reports suggest that liver cancer, particularly HB develops in the context of simultaneous WNT and Hippo pathway activation.

In a similar fashion, transducing the biliary epithelium with activated forms of YAP and AKT results in the development of cholangiocarcinoma in mice. These tumors share a transcriptional profile similar to CC arising in human patients, suggesting that the PI3K and Hippo pathway interact in this oncogenic context [104]. Several prior reports suggested that increased YAP activity alone is sufficient for HCC or CC to develop [16, 18–21, 102, 105], although these models were not well characterized for mutations in other signaling pathways. It is likely that cells with Hippo pathway mutations rapidly proliferate and acquire additional mutations that lead to cancer.

The immunohistochemical localization of YAP has long been interpreted to be a sign of high YAP activity, but this may neglect modest levels of YAP activity that also have clinical significance. Uniform classification of cancer by molecular markers, such as by high throughput RNA sequencing is becoming commonplace [106, 107]. This should be done more uniformly for patients as well as our mouse models so that accurate comparisons particularly across studies can be made. This can then be used to help predict potentially effective treatments for a group of tumors that currently have limited available therapy.

Mutations in the Hippo pathway leading to elevated YAP levels/activity commonly lead to liver cancer in mice and humans (Figure 4). Targeting the inactivation of the Hippo/YAP pathway to hepatocytes versus cholangiocytes can partially explain how HCC, HB or CC arises. Why do MST1/2 mutants only develop HCC while NF2 and WW45 mutants can develop both HCC and CC remains a mystery. This suggests that there are subtle differences in the construction of the Hippo pathway in cells that generate HCC versus CC. The studies of how Hippo Signaling interacts with other important signaling programs is adding to our understanding of cancer evolution and should allow us to specifically targeted cancerous cells over normal cells.

Therapeutic avenues for Modulating Hippo Signaling

The mechanisms through which Hippo signaling can be therapeutically inhibited are so far limited, but it is a particularly active area of research. As noted previously, NF2 loss results in the liver cancer; interestingly, this can be rescued by hemizygous loss of YAP [19]. Dominant-negative TEAD2 (which binds to YAP, but not DNA) can also ameliorate the liver tumors that develop in the NF2 knockout model [108]. It is also reassuring that loss of

YAP in quiescent adult hepatocytes does not immediately lead to their widespread loss [23]. These data suggest that partial inhibition of YAP activity is sufficient to suppress the development of oncogenesis. YAP siRNA lipid nanoparticles have been shown to effectively treat a mouse model of advanced HCC. Reduction in YAP using lipid-encapsulated siRNA converted highly aggressive HCC to undergo hepatocyte differentiation [109]. These data support the idea that reduced YAP activity can serve as the basis of treatment for HCC (and likely other forms of YAP-dependent cancers) with limited effect upon normal tissues.

Since much of the transcriptional activity of the Hippo pathway is directed through the TEAD family of transcription factors, a number of groups have focused on disrupting the YAP-TEAD interaction. Screening chemical libraries of commercially available compounds identified verteporfin, a porphyrin molecule currently in use as a photosensitizer for macular degeneration. Verteporfin binds to YAP interfering with its ability to bind TEAD. Importantly, verteporfin shows efficacy *in vivo* to limit YAP driven liver cancer [108]. Currently, the affinity of this drug is poor for clinical use, but serves as an important lead compound for future development.

Many of the proteins identified in the Hippo pathway are adaptor proteins. It is conceivable that development of a competitive inhibitor at critical junctions will have important therapeutic benefit. For several years, vestigial-like4 (VGLL4) has been reported to be an important competitive inhibitor of TEAD activation acting to partially mask the YAP-TEAD binding site [110, 111]. By identifying the critical peptides that mediate VGLL4-TEAD binding, this group generated a cell permeable peptide that out competes YAP for TEAD4 binding. It appears a promising candidate for the treatment of gastric cancer [65] and could be a useful adjunctive therapy for liver cancer particularly since TEAD4 is the highest expressed member of this family in the liver.

Elevating YAP activity has been proposed to be a potentially effective way of expanding the liver in the transplantation setting, particularly where there may be some graft to host size mismatch and accelerating graft growth may prove advantageous. Utilizing RNAi technology to reduce expression of regulatory kinases of the Hippo pathway, small molecule activation of the Gα GPCR or even transient expression of YAP are possible means of momentarily accelerating cell proliferation. Recent reports evaluating hepatocyte ploidy in the context of YAP overexpression raises some concern over the long-term stability of cells after YAP induction or if induced YAP expression may accelerate the accumulation of potential oncogenic mutations. Typically mature hepatocytes are tetraploid. In the presence of YAP, the vast majority of hepatocyte ploidy shifts to 8N and higher [112]. Presumably, normalization of YAP levels results in either loss of the 8N hepatocytes, or subsequent cell division reducing the ploidy of these hepatocytes to the normal 4N number. As YAP expression is used to overcome a number of physiologic checkpoints during expansion, what will be the quality of these expanded cells and can techniques or strategies be used to minimize the potential deleterious effects? Additional work to understand cell health and quality after induced YAP expression is important for potential application in the transplantation setting.

Conclusions

The Hippo/YAP pathway has been recognized as important in regulating overall tissue proliferation and growth with loss of proper control associated with oncogenesis. But, these ideas are likely an oversimplification due to the limitations of our current models. Roles for Hippo/YAP in cell fate determination, metabolism and epithelial to mesenchymal transition are beginning to emerge and it is likely that there are many important nuances left to be appreciated.

Many inputs into Hippo signaling have been identified, but the composition of the pathway is presumed to be context dependent. A number of Hippo Pathway members have been identified to be active in the liver (Table 1), but an even larger number are known in other contexts. Some of these mechanisms will apply to Hippo/YAP in the liver as well, such as in the case of α -catenin. The future challenge is in identifying the biologically and clinically relevant mechanisms to liver processes in order to develop new therapeutic paradigms.

Acknowledgments

We appreciate the members of the Camargo laboratory for stimulating and insightful discussions. DY is a Gilead Sciences Scholar in Liver Disease and is supported by a Boston Children's Hospital Career Development Award. This work was supported by awards from the NIH K08 DK105351 (DY), NIH R01 AR064036 (FDC) and NIH R01 DK099559-01 (FDC).

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KEY POINTS

- Hippo signaling is a recently described tumor suppressor pathway which tightly regulates overall liver size by controlling pro-proliferative and anti-apoptotic gene programs. Hippo pathway mutants commonly lead to liver overgrowth phenotypes and the development of cancer.
- The transcriptional co-activator YAP is the primary regulatory target of Hippo signaling. Hippo signaling sequesters YAP to the cytoplasm through phospho-specific and cell-tension based mechanisms.
- YAP primarily binds to the TEAD family of transcription factors to regulate genes associated with Hippo signaling. Small molecule and peptide-based inhibitors of the YAP-TEAD interaction can attenuate phenotypes associated with inactivation of Hippo signaling.
- There is emerging evidence for Hippo/YAP signaling as important for stem cell maintenance/renewal, cell fate choice, overriding senescence, regulating cell metabolism and conferring epithelial to mesenchymal transition.
- Increased YAP expression and activity is associated with the development of multiple types of liver cancer including hepatoblastoma, hepatocellular carcinoma and cholangiocarcinoma. Experimental models support YAP activity to be an early event and an important oncogenic driver of liver cancer development.

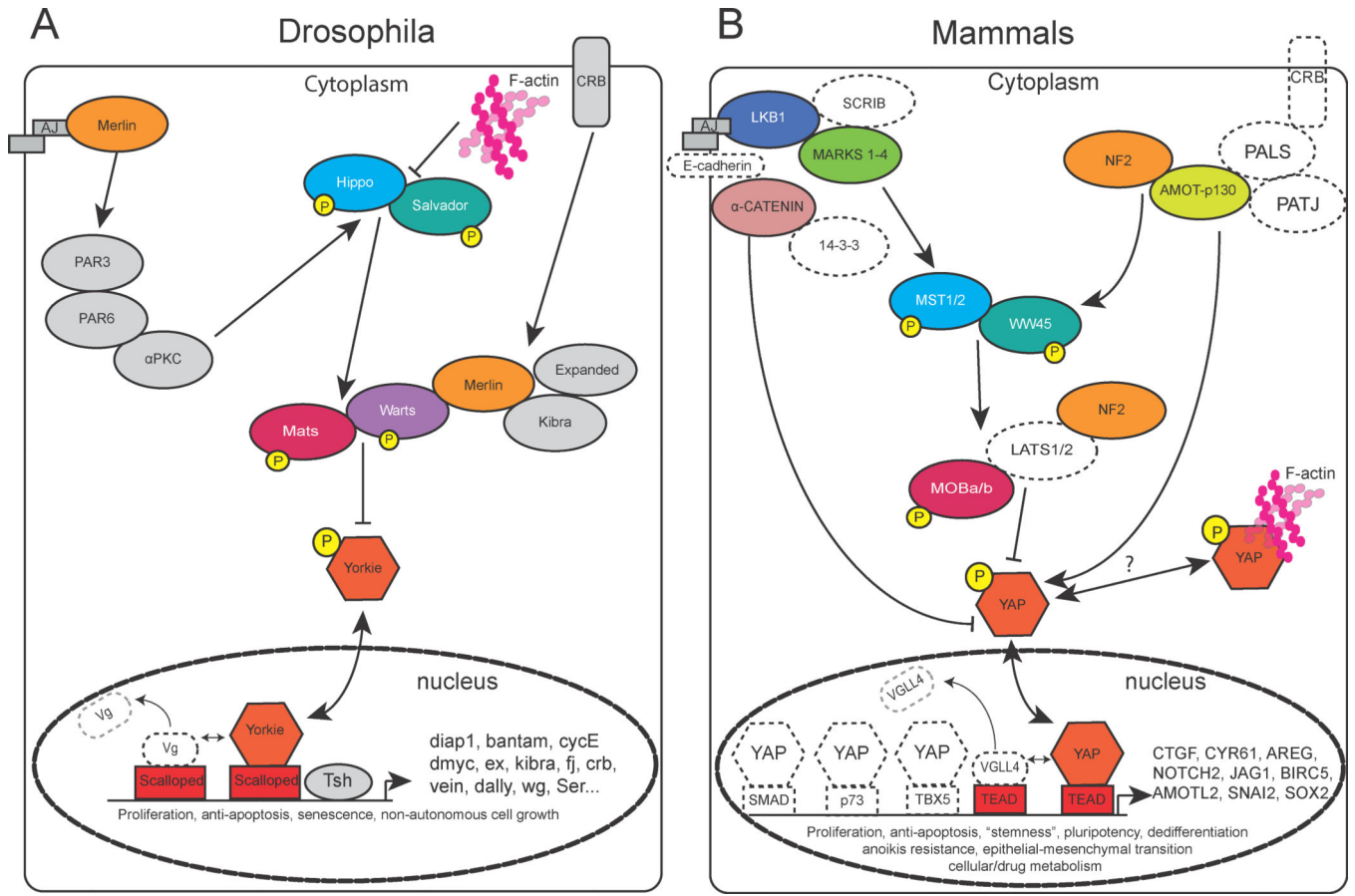


Figure 1. The Hippo pathway in *Drosophila* and Mammals

Schematic of a subset of the Hippo pathway with known roles in the liver. The primary activity of the Hippo pathway is to restrict *yorkie* (YAP in mammals) to the cytoplasm. Reduced activity of the Hippo pathway results in translocation of *yorkie* to the nucleus where it binds to the TEAD family of transcription factors to upregulate gene expression. Known target genes of the pathway in *Drosophila* and Mammals are indicated after the arrowhead in the nucleus. Known roles for the pathway are indicated below the diagram of the transcriptional element.

A. The Hippo pathway in *Drosophila*. Colored shapes represent known effectors of the Hippo pathway. Selected effectors have homologous partners in mammals that are associated with liver proliferation/cancer. These are color/shape-matched for clarity.

B. The Hippo pathway in Mammals. Colored shapes represent known effectors of liver proliferation/cancer in the mammals. Open/dotted shapes represent potential/expected partners of the Hippo pathway that have yet to be functionally demonstrated in the liver, but have evidence from other tissues for their interaction.

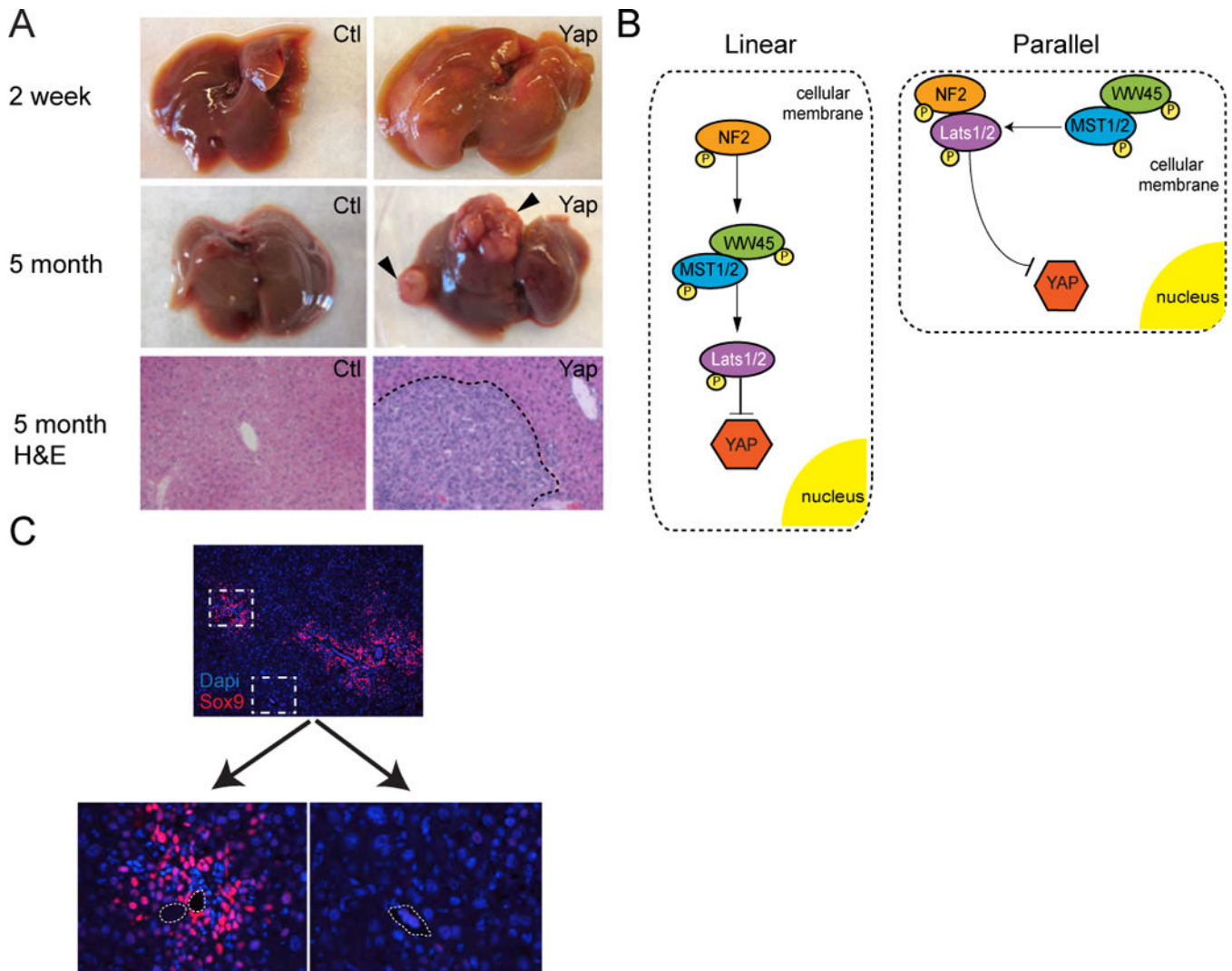


Figure 2. The Effect of Liver YAP Expression

A. 2 weeks – Gross morphology of littermate control and YAP S127A overexpressing livers 2 weeks after high yield YAP induction in the adult. 5 month – Gross morphology of littermate control and a YAP S127A overexpressing liver with tumors (arrowheads) 5 months after low yield induction in the adult. H&E slides show histology of a highly undifferentiated HCC in the YAP overexpressing liver (dotted line).

B. Linear versus Parallel models of YAP modulation. Colored shapes represent known effectors of the Hippo Pathway that directly modulate YAP expression. They are arranged in the two proposed models of YAP regulation, linear and parallel [22].

C. Sox9 staining demonstrates differential hepatocyte response to YAP S127A expression in periportal (PP) as compared to central venous (CV) areas 2 weeks after induction. Dashed boxes are enlarged in pictures noted below. Dashed lines in the PP/CV pictures indicate the borders of the blood vessel [23].

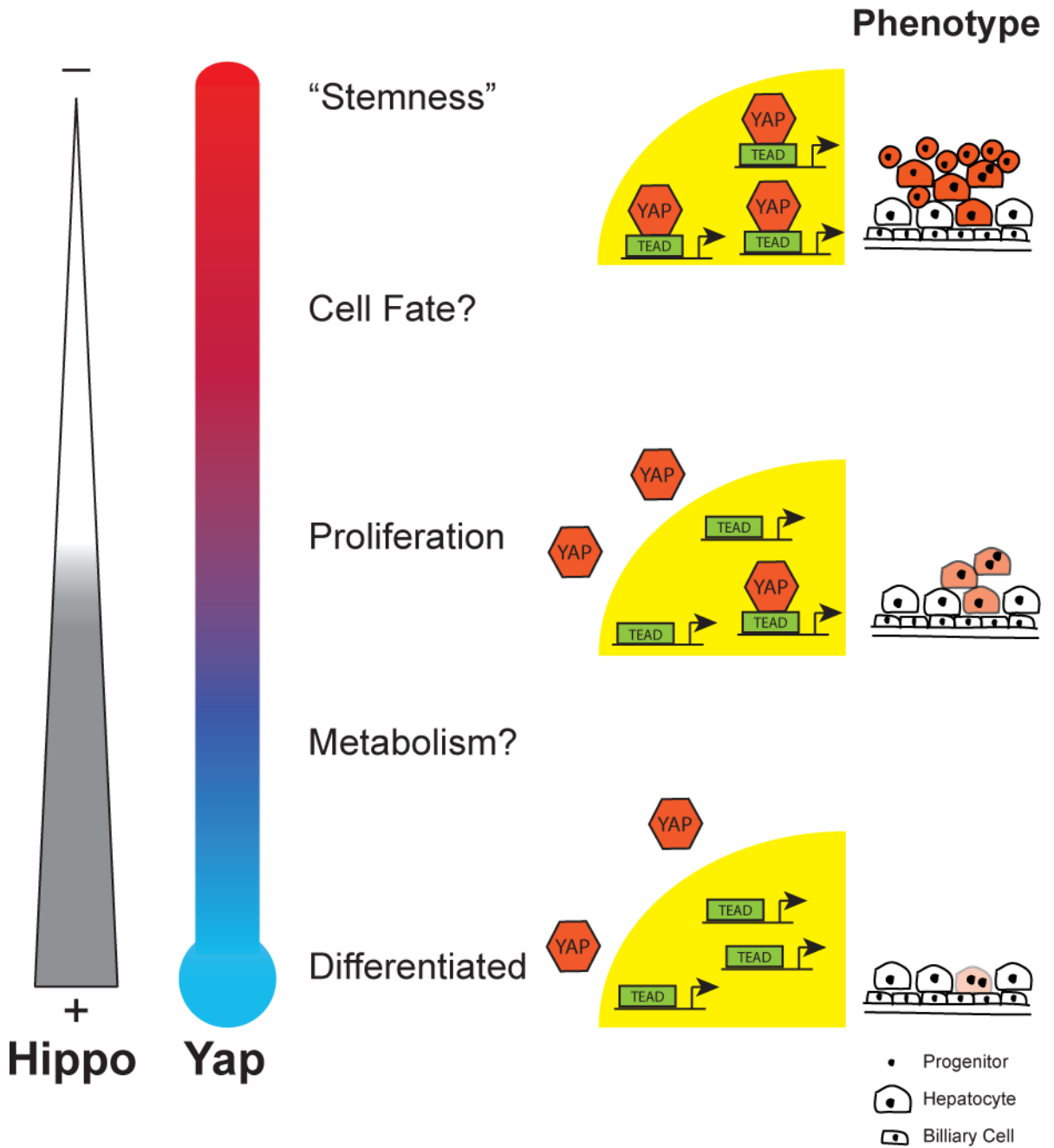


Figure 3. Hippo/YAP Levels Mediate Cellular Behavior

Hippo signaling and YAP activity is inversely related and determines particular cellular processes at various levels. High Hippo signaling activity is correlated with low levels of YAP protein and cytoplasmic YAP. Low Hippo activity is associated with high levels of YAP and a propensity for it to be localized to the nucleus. Proposed activities for varying levels of Hippo/YAP are depicted next to the YAP “thermometer”. At the far right, animations represent the proliferation and dedifferentiation of cells associated with the various levels of YAP expression. Darker shading suggests increased YAP expression.

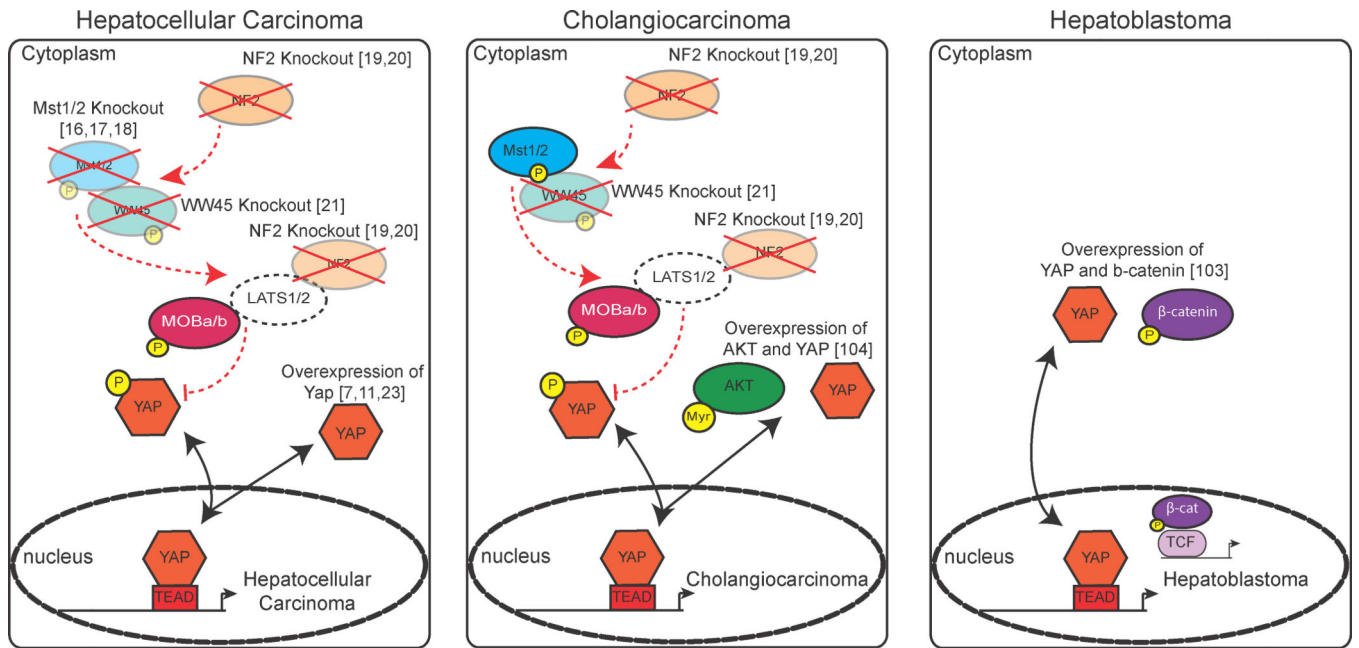


Figure 4. Known Mutations in Hippo/YAP signaling in Liver Cancer

Colored shapes represent the known effectors of the Hippo pathway that, when knocked out or overexpressed, are associated with the development of the indicated form of cancer.

References to the papers are indicated.

Table 1

Hippo pathway mutations cause liver specific phenotypic changes.

Genetic Mutation	Mutation induction	Liver Phenotype	Reference
α -catenin knockdown	siRNA-LNP	Increased hepatocyte proliferation and liver size after hepatectomy	Herr, K et. al. 2014
Amot-p130 knockout	Amot ^{flox/flox} /Alb-Cre	Reduced biliary hyperplasia after bile duct ligation	Yi, C et. al. 2013
LKB1 knockout	Lkb1 ^{flox/flox} /Ad-Cre	Increased hepatocyte proliferation and liver size	Mohseni, M et. al. 2014
Mob1a/1b double deletion	Mob1a ^{-/-} 1b ^{tr/+}	19% of mice develop HCC	Nishio, Miki et. al 2012
MST1/MST2 knockout	Mst1 ^{-/-} Mst ^{flox/-} /Ad-Cre Mst1 ^{-/-} Mst ^{flox/-} /Alb-Cre Mst1 ^{-/-} Mst ^{flox/flox} /Alb-Cre	Development of HCC	Zhou, D et. al. 2009; Song, H et. al. 2010; Lu, L et. al 2010
Nf2 knockout	Nf2 ^{flox/flox} /Alb-Cre Nf2 ^{flox/flox} /Ad-Cre	Increased/disorganized biliary cell proliferation, biliary hyperplasia and HCC, rare cholangiocarcinoma	Zhang, N et al. 2010; Benhamouche, S et. al. 2010
Nf2/Salvador double knockout	Nf2 ^{flox/flox} Sav1 ^{flox/flox} /Alb-Cre	Biliary epithelial cell hyperplasia	Feng, Y et. al. 2013
Salvador knockout	Sav1 ^{flox/flox} /Alb-Cre	Increased proliferation and expansion of hepatic progenitor cells leading to HCC and CC phenotypes	Lee, K.P. et. al. 2010;
Yap knockout	Yap ^{flox/flox} ; AAV-TBG-Cre Yap ^{flox/flox} ; Alb-Cre	Biliary hypoplasia, hepatocyte sensitivity to stress	Yimlamai, D et al. 2014; Zhang et. al. 2001
Yap Overexpression	TetOYap-S127A-R26-rtTa LAP1/rtTA-Yap(S127A) ApoE/rtTa-Yap	Hepatocyte dedifferentiation, liver overgrowth, HCC development	Yimlamai, D et. al. 2014; Camargo, F et. al. 2007 Dong, J et. a.; 2007

Liver-specific gene mutations within the Hippo pathway leading to survival and proliferative changes of epithelial cells in the mouse liver. Adeno-associated thyroid binding globin virus Cre (AAV-TBG-Cre); Adenovirus Cre (Ad-Cre); albumin promoter Cre (Alb-Cre); villin-Cre); siRNA packaged in lipid nanoparticles (LNP).

Table 2

Description of Hippo pathway Components

<i>Vertebrates</i>		
Name	Abbreviation	Role
14-3-3	YWHA	Regulatory binding proteins
α -catenin	CTNNA	Adherens junction structural proteins
Adherens junction	AJAP	Protein complex found in cell-cell junctions in epithelium and endothelium
Angiomotin	AMOT	Adaptor protein
E-cadherin	CDH	Transmembrane receptor
Large tumor suppressor homolog 1/2	LATS1/2	Serine/threonine kinase
Liver kinase B1	LKB1	Serine/threonine kinase
Microtubule affinity-regulating kinase	MARK	Serine/threonine kinase
Mob kinase activator 1a/1b	MOB1A/B	Co-factor
Mammalian STE20-like protein kinase	MST1/2	Serine/threonine kinase
Neurofibromin 2	NF2	Adaptor protein
Salvador homolog 1	SAV1	Adaptor Protein
Scribble	SCRIB	Adaptor protein
Transcriptional co-activator with PDZ-binding motif	TAZ	Transcription co-activator
TEA domain-containing sequence-specific transcription factor	TEAD	Transcription factor
Vestigial-like protein 4	VGL-4	Co-factor
Yes-associated protein	YAP	Transcriptional Co-activator

<i>Drosophila</i>		
Name	Abbreviation	Role
Crumbs	crb	Transmembrane receptor
Expanded	ex	Adaptor protein
Kibra	kibra	Adaptor protein
Mob as tumor suppressor	mats	Co-factor
Merlin	mer	Adaptor protein
Salvador	sav	Transcription factor
Scalloped	sd	Transcription factor
Hippo	hpo	Serine/threonine kinase
Warts	wts	Serine/threonine kinase
Yorkie	yki	Transcriptional co-activator