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REVIEW

The Hippo–YAP pathway in organ size control and tumorigenesis: an updated version

Bin Zhao,[1] Li Li,[1] Qunying Lei,[2] and Kun-Liang Guan[1,3]

[1]Department of Pharmacology and Moores Cancer Center, University of California at San Diego, La Jolla, California 92093, USA; [2]Department of Biological Chemistry, School of Medicine, and Molecular and Cell Biology Laboratory, Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China

The Hippo signaling pathway is gaining recognition as an important player in both organ size control and tumorigenesis, which are physiological and pathological processes that share common cellular signaling mechanisms. Upon activation by stimuli such as high cell density in cell culture, the Hippo pathway kinase cascade phosphorylates and inhibits the Yes-associated protein (YAP)/TAZ transcription coactivators representing the major signaling output of the pathway. Altered gene expression resulting from YAP/TAZ inhibition affects cell number by repressing cell proliferation and promoting apoptosis, thereby limiting organ size. Recent studies have provided new insights into the Hippo signaling pathway, elucidating novel phosphorylation-dependent and independent mechanisms of YAP/Yki inhibition by the Hippo pathway, new Hippo pathway components, novel YAP target transcription factors and target genes, and the three-dimensional structure of the YAP–TEAD complex, and providing further evidence for the involvement of YAP and the Hippo pathway in tumorigenesis.

Precise control of cell number is an essential function of multicellular organisms under physiological conditions such as development and organ regeneration. Impairment of this function leads to human diseases such as cancer. Signaling pathways communicating extra- and intracellular cues to gene transcription are at the center stage of cell number regulation. In the past decade, a new signaling pathway, the Hippo pathway, has been shown to have a critical role in controlling organ size by regulating both cell proliferation and apoptosis (for review, see Zhao et al. 2008a; Kango-Singh and Singh 2009). Many components of the Hippo pathway were discovered initially by *Drosophila* mosaic genetic screens, due to a strong overgrowth phenotype shared by these mutants (Justice et al. 1995; Xu et al. 1995; Kango-Singh et al. 2002; Tapon et al. 2002; Harvey et al. 2003; Pantalacci et al. 2003; Wu et al. 2003).

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[3]**Corresponding author.**
E-MAIL kuguan@ucsd.edu; FAX (858) 534-7628.
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The Hippo pathway was named after the *Drosophila* Hippo kinase that was discovered using this approach. Components of the Hippo pathway are highly conserved in mammals (Fig. 1). Later genetic and biochemical studies gradually shaped the current working model, in which the mammalian Mst1/2 kinase (Hippo homolog), complexed with a scaffold protein, Sav1, phosphorylates and activates the Lats1/2 kinase. Lats1/2 is also activated by another scaffold protein, Mob1 (Fig. 2). These four proteins are often referred to as the core components of the Hippo pathway. At the upstream, several components have been implicated by *Drosophila* genetic studies, including Merlin (Mer), Expanded (Ex), and Fat (Bennett and Harvey 2006; Cho et al. 2006; Hamaratoglu et al. 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007). Lats1/2 kinase directly phosphorylates and inactivates a transcription coactivator Yes-associated protein (YAP) (Zhao et al. 2007; Hao et al. 2008) and the YAP paralog transcriptional coactivator with PDZ-binding motif (TAZ) (Lei et al. 2008). Functions of YAP in organ size regulation and tumorigenesis have been confirmed in mammals, using transgenic mouse models (Camargo et al. 2007; Dong et al. 2007). In this review, we briefly summarize the overall picture of the Hippo pathway in *Drosophila* and mammals, highlighting important new discoveries in the last 2 years regarding the regulation and function of the Hippo pathway and YAP/TAZ.

The *Drosophila* Hippo pathway

The *Drosophila* genetic mosaic screen is a powerful tool in discovering tumor suppressors, such as the first Hippo pathway component, *wts* (Justice et al. 1995; Xu et al. 1995). *wts* encodes a nuclear Dbf-2-related (NDR) family protein kinase. Mutation of *wts* leads to robust tissue overgrowth without affecting cell fate determination. *sav* (*shar-pei*) encoding a WW domain-containing protein and *hippo* (*hpo*) encoding a STE20 family protein kinase are two other Hippo pathway components identified in a similar fashion to that of *wts* (Kango-Singh et al. 2002; Tapon et al. 2002; Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003).

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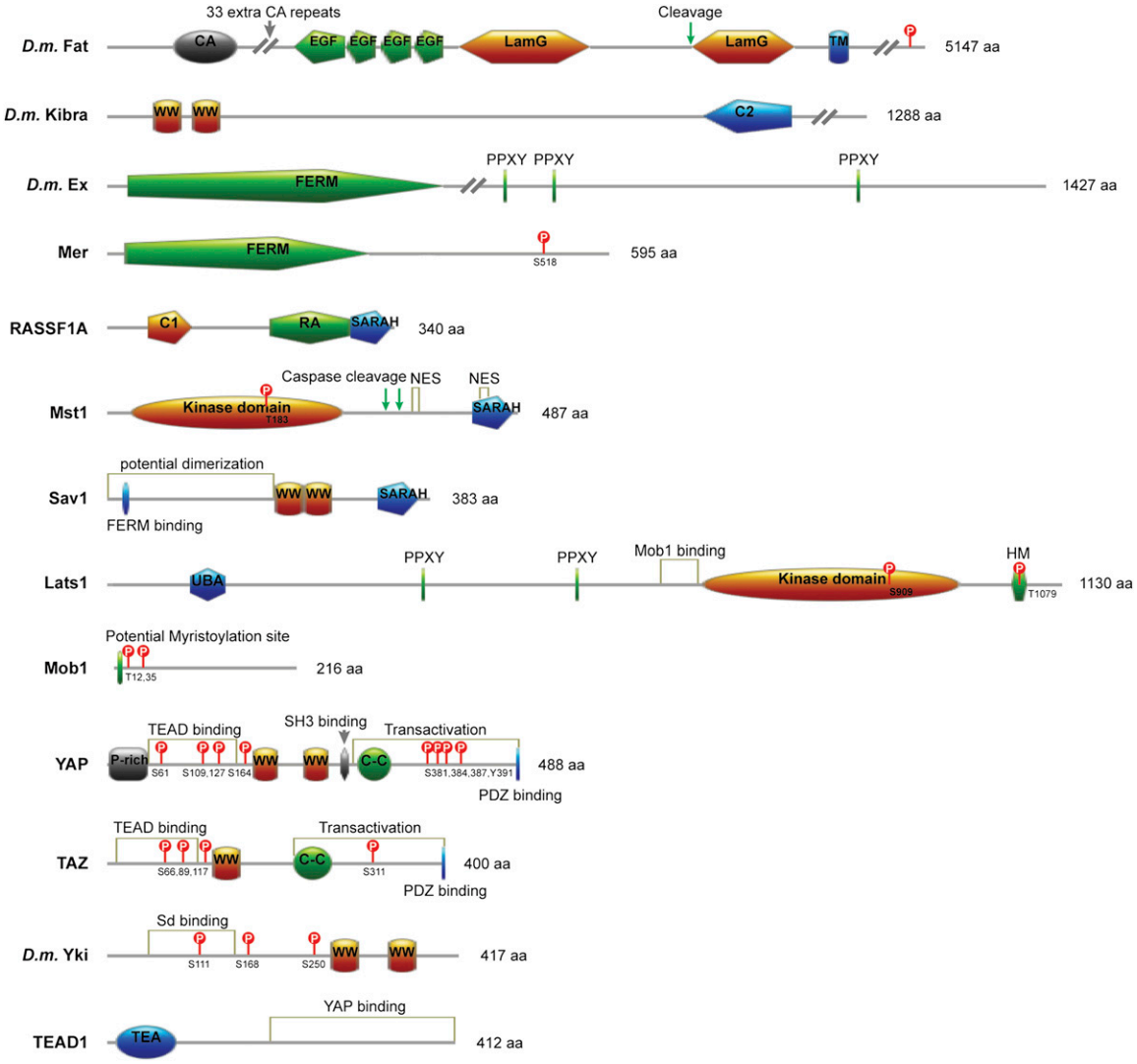

Figure 1. Domain organization and key modifications of the Hippo pathway components. The illustrations are drawn in scale unless indicated otherwise. Human sequences are drawn unless indicated by *D.m.*, which stands for *Drosophila* sequences. (CA) Cadeherin repeats; (EGF) EGF-like domain; (LamG) Laminin G domain; (TM) transmembrane region; (WW) WW domain; (C2) C2 domain; (FERM) FERM domain; (C1) C1 domain; (RA) Ras association domain; (SARAH) SARAH domain; (UBA) ubiquitin-associated domain; (PPXY) PPXY motif; (HM) hydrophobic motif; (P-rich) proline-rich domain; (C-C) coiled-coil domain; (TEA) TEA DNA-binding domain. *Drosophila* Fat is processed into two fragments (Feng and Irvine 2009; Sopko et al. 2009). The approximate cleavage site is indicated. Fat cytoplasmic domain is phosphorylated by Dco (Feng and Irvine 2009; Sopko et al. 2009). Mer is phosphorylated by PAK1/2 on S518, which affects Mer conformation and inactivates Mer as a tumor suppressor (Rong et al. 2004). Mst1 activation loop autophosphorylation (T183) is essential for its kinase activity. Caspase cleavage, as indicated, activates Mst1 (Graves et al. 1998). Lats1 is activated by autophosphorylation on the activation loop (S909), and phosphorylation by Mst1/2 on the hydrophobic motif (T1079) (Chan et al. 2005). Mob1 is phosphorylated by Mst1/2 on T12 and T35, and this phosphorylation stimulates its interaction with Lats1/2 (Praskova et al. 2008). Sav1 is also phosphorylated by Mst1/2 on an unidentified site (Callus et al. 2006). YAP/TAZ/Yki is phosphorylated by Lats1/2 on S61, S109, S127, S164, and S381 (TAZ S66, S89, S117, S311, Yki S111, S168, and S250) in the HXRXXS motifs (Zhao et al. 2007; Lei et al. 2008; Oh and Irvine 2008). S127 phosphorylation induces 14–3–3 binding and cytoplasmic retention (Zhao et al. 2007). S381 phosphorylation primes CK1δ/ε phosphorylation of S384, and S387 finally leads to $SCF^{\beta\text{-}TRCP}$-mediated ubiquitination and degradation (Zhao et al. 2010). YAP is also phosphorylated by c-Abl on Y391 (Levy et al. 2008).

Hpo interacts directly with Sav and promotes Sav and Wts phosphorylation (Wu et al. 2003). Subsequently, *mats* mutations were shown to phenocopy other Hippo pathway component mutations. The Mats protein interacts physically with Wts as an activating subunit (Lai et al. 2005; Wei et al. 2007; Shimizu et al. 2008). Mats is also phosphorylated by Hpo, resulting in increased interaction with Wts. These observations have established the core components of the *Drosophila* Hippo pathway, showing the Hpo kinase—in association with Sav—phosphorylating and activating the Wts kinase–Mats complex (Fig. 2).

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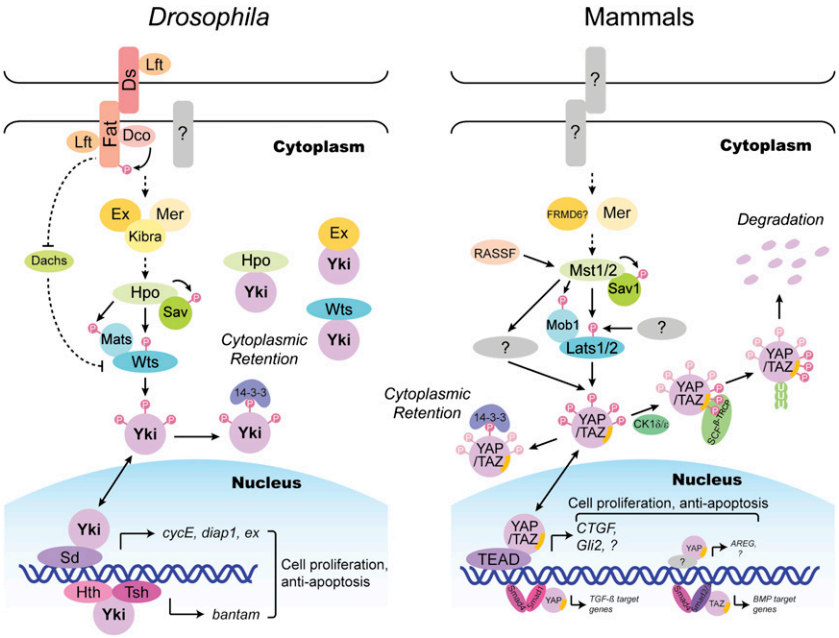

Figure 2. Models of the Hippo pathway in *Drosophila* and mammals. In *Drosophila*, Fat protocadherin may initiate the Hippo pathway signal in response to Ds binding, and is modulated by binding of Lft and phosphorylation by Dco (Feng and Irvine 2009; Mao et al. 2009; Sopko et al. 2009). Fat may inhibit a nonconventional myosin Dachs, which represses Wts protein levels (Cho et al. 2006). Fat may also activate Ex with an unknown mechanism (Bennett and Harvey 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007). Mer and Ex also activate the Hippo pathway (Hamaratoglu et al. 2006). They may form a complex with Hpo and Sav (Yu et al. 2010). Kibra interacts with both Mer and Ex, and may also be in the complex (Yu et al. 2010). Hpo kinase interacts with and phosphorylates a scaffold protein, Sav (Wu et al. 2003). Together, they phosphorylate and activate Wts kinase and its associated protein, Mats (Lai et al. 2005). Wts phosphorylates a transcription coactivator, Yki, on three sites (Oh and Irvine 2009). Phosphorylation of Yki S168 induces 14–3–3 binding and cytoplasmic retention (Dong et al. 2007). Yki may also be retained in the cytoplasm by physical interaction with Ex, Wts, and Hpo (Badouel et al. 2009; H Oh et al. 2009). When Yki is relieved from inhibition and gets into the nucleus, it binds and activates a transcription factor, Sd, to induce *cycE*, *diap1*, and *ex* expression (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008). Yki induces *bantam* microRNA through Hth and Tsh (Peng et al. 2009). In mammals, functional significance of Fat and Ex homologs are not clear. However, Mer may still activate the Hippo pathway (Yokoyama et al. 2008). RASSF, a subgroup of Ras effector proteins, may also activate Mst1/2 (Hpo homolog) (Oh et al. 2006). Relationships between Hpo, Sav, Wts, and Mats are basically conserved in mammalian Mst1/2, Sav1 (Sav homolog), Lats1/2 (Wts homolog), and Mob (Mats homolog). Lats1/2 phosphorylates YAP on five conserved HXRXXS motifs (four on TAZ) (Zhao et al. 2007). Dependent on cell context, there may exist another YAP kinase in response to Mst1/2 and another Lats1/2 kinase (Zhou et al. 2009). S127 (S89 in TAZ) phosphorylation-dependent 14–3–3 binding and cytoplasmic retention are conserved in YAP/TAZ (Zhao et al. 2007; Lei et al. 2008). YAP is also inhibited by S381 phosphorylation, which primes CK1δ/ε phosphorylation of S384, and S387 finally leads to $SCF^{\beta\text{-}TRCP}$-mediated ubiquitination and degradation (Zhao et al. 2010). Sd homologs, TEADs, are major YAP target transcription factors. They mediate expression of CTGF, Gli2, and many other target genes (Zhao et al. 2008b). AREG is induced by YAP through an unidentified transcription factor (J Zhang et al. 2009). YAP and TAZ also bind Smad1 and Smad2/3 to activate expression of TGF-β and BMP target genes, respectively, to maintain stem cell pluripotency (Varelas et al. 2008; Alarcon et al. 2009).

These pioneering studies converge on one special feature of the Hippo pathway: It not only functions to inhibit cell proliferation, but also to promote apoptosis (Edgar 2006). This function is achieved at least in part by transcriptional activation of *cycE*, *diap1* (for review, see Edgar 2006), and *bantam* microRNA (Nolo et al. 2006; Thompson and Cohen 2006). Therefore, like many other signaling pathways, the Hippo pathway regulates a transcription program. The missing transcriptional link was identified to be Yki (YAP homolog) transcription coactivator using Wts as bait in yeast two-hybrid (Huang et al. 2005). Yki regulates transcription of the Hippo pathway target genes, and its overexpression phenocopies the loss of Hippo pathway components. A biochemical study showed that Wts directly phosphorylates Yki and leads to Yki cytoplasmic retention and inactivation (Dong et al. 2007).

The Yki transcription coactivator possesses no DNA-binding activity. Therefore, a key question was the identification of target transcription factors that mediate Yki activity. Clues from mammalian YAP-interacting TEAD family transcription factors and reported Yki yeast two-hybrid data led to the identification of Scalloped (Sd), a critical regulator of proliferation and survival of wing imaginal disc cells and the *Drosophila* TEAD homolog, as a direct Yki target transcription factor mediating Yki-induced gene expression and overgrowth phenotype (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008; Zhao et al. 2008b). Therefore, Sd is the first DNA-binding factor identified to mediate the Hippo pathway effects in *Drosophila*.

A search for mutations with similar phenotypes to Hippo pathway defects yielded the discoveries of Mer and Ex, two FERM domain-containing cytoskeleton-related proteins that act upstream of the Hippo pathway core components (Fig. 2; Hamaratoglu et al. 2006). While the double mutant of *mer* and *ex* mimics mutation of other Hippo pathway components, the *mer* or *ex* single mutation had only a weak effect on inducing extra interommatidial cells, a common phenotype in Hippo pathway mutants. It was shown later that Mer and Ex may have different contributions to the phenotypes observed, where *mer* mutant clones showed defects in apoptosis and *ex* mutant clones showed impaired cell cycle exit (Pellock et al. 2007). However, the biochemical mechanisms of Hippo pathway regulation by Mer and Ex remain unclear, possibly including an indirect effect on receptor

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endocytosis (Maitra et al. 2006). Interestingly, a recent study identified a FERM domain-binding consensus motif in Sav, which likely mediates direct interaction with the FERM domain of Mer (Yu et al. 2010). Moreover, Ex has also been shown to coimmunoprecipitate with both Hpo and Sav (Yu et al. 2010). Thus, Mer/Ex may activate Hpo/Sav via direct interaction.

Kibra, a protein known to interact with Mer and Ex, was identified as a new component of the Hippo pathway (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010). *kibra* mutations share similar phenotypes to mutated components of the Hippo pathway in both oocyte polarity and notch signaling defects in posterior follicle cells. More importantly, *kibra* mutations generate extra interommatidial cells, a signature phenotype of Hippo pathway mutations. This phenotype is weak, analogous to that of *mer* or *ex* mutations. However, when a *kibra* mutation was combined with either a *mer* or *ex* mutation, a strong overgrowth phenotype similar to that of a *mer/ex* double mutant was observed. Epistatic analysis shows that *kibra* is upstream of *hpo* and *sav*, and its overexpression increases Hpo, Wts, and Yki phosphorylation. In mammalian cells, overexpression of the *kibra* homolog also potently induces Lats1/2 phosphorylation. These data suggest a role for *kibra* acting upstream of the hippo pathway. It has not been determined whether Kibra indeed functions by mediating or increasing Mer/Ex interaction with Hpo/Sav. Another remaining question is whether Kibra relays some upstream signals to the hippo pathway.

Several studies have identified a regulatory role for Fat protocadherin, a member of the hyperplastic group of *Drosophila* tumor suppressors, upstream of the Hippo pathway (Bennett and Harvey 2006; Cho et al. 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007). Fat is a cell surface molecule with multiple cadherin repeats in an enormous extracellular domain that may serve as a receptor for the Hippo pathway (Figs. 1, 2). *fat* mutants resemble *ex* mutants, both of which have a mild overgrowth phenotype. Notably, removing one copy of *yki* dramatically suppresses the *fat* mutant overgrowth phenotype. Two mechanisms have been suggested for Fat in activation of the Hippo pathway. Fat may regulate Ex protein level and localization (Bennett and Harvey 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007), or control the abundance of Wts through Dachs (Fig. 2; Cho et al. 2006; Feng and Irvine 2007). Interestingly, it has also been reported that Fat activation by Dachsous (Ds), another protocadherin, might be responsible for the effect of Dpp gradient on cell proliferation (Rogulja et al. 2008). Furthermore, Fat and Four-jointed, a transmembrane kinase, form a complementary gradient that might be important for the regulation of the Hippo pathway (Willecke et al. 2008). Moreover, Lowfat (Lft), a known Fat- and Ds-interacting cytosolic protein, has been shown to regulate Fat and Ds protein levels and genetically interact with Fat to control wing development (Mao et al. 2009). Simultaneous knockdown of Ds and Lft cause a wing disc overgrowth, although mutation of *lft* itself exerts only weak phenotype in the wing. Whether *lft* modulates Hippo pathway signaling remains to be determined.

The mammalian Hippo pathway

Components of the Hippo pathway are highly conserved from *Drosophila* to mammals, including Mst1/2 (Hpo homolog); Sav1 (Sav homolog); Lats1/2 (Wts homolog); Mob1 (Mats homolog); YAP and its paralog, TAZ (also called WWTR1, both are Yki homologs); Mer (also called NF2, Mer homolog); and, to a lesser degree, FRMD6 (Ex homolog) and Fat4 (Fat homolog) (Fig. 1). More strikingly, human YAP, Lats1, Mst2, and Mob1 can functionally rescue the corresponding *Drosophila* mutants in vivo, suggesting a functional conservation of these genes (for review, see Edgar 2006).

Most studies of Mst1/2 have been done in the context of apoptosis. It has been shown to be activated by caspase-dependent cleavage (Graves et al. 1998), dimerization, autophosphorylation (Lee and Yonehara 2002), and RASSF (Khokhlatchev et al. 2002; Praskova et al. 2004; Oh et al. 2006), and inhibited by the proto-oncogene product Raf-1 (O'Neill et al. 2004). Recently, Mst1/2 was reported to partially colocalize with actin cytoskeleton, disruption of which leads to mild activation of the kinase (Densham et al. 2009). Mst1 and Mst2 double knockout in liver largely abolished YAP phosphorylation, and induced an enlarged liver phenotype strikingly similar to YAP overexpression (Zhou et al. 2009; Lu et al. 2010; Song et al. 2010). However, the effect of Mst1 and Mst2 ablation on organ size is more dramatic in some organs, such as liver and stomach, than in others, such as kidney and limb, which do not show an increase in size (Song et al. 2010). This is possibly due to an organ-specific contribution of impaired cell differentiation versus cell number on the overall size of the organ. Furthermore, the function of Mst1/2 in the Hippo pathway is likely to be cell context-dependent, since Mst1/2 are not required for Lats1/2 phosphorylation and cell density-induced YAP nuclear–cytoplasmic translocation in mouse embryonic fibroblast (MEF) cells (Zhou et al. 2009), or YAP phosphorylation in early embryos (S Oh et al. 2009).

Sav1 is an adaptor protein that interacts with Mst1/2 through the SARAH domains in both proteins. Sav1 can also be phosphorylated by Mst1/2 (Callus et al. 2006). Recently, Sav1 was shown to be required for Mst1 activation and translocation to the nucleus upon keratinocyte differentiation (Lee et al. 2008). Sav1 homozygous deletion is embryonic-lethal, exhibiting hyperplasia and immature differentiation in epithelium, while heterozygous mice are prone to tumorigenesis (Lee et al. 2008). Conditional ablation of Sav1 in liver leads to liver size enlargement and tumor formation (Lu et al. 2010). Surprisingly, Lats1/2 and YAP phosphorylation are not affected, suggesting that Sav1 is not absolutely required for Lats1/2 activation and may limit liver growth by other mechanisms.

Current data suggest that the hydrophobic motif of Lats1/2 is phosphorylated by Mst1/2, and the activation loop is autophosphorylated (Fig. 2; Chan et al. 2005).

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Lats1-deficient mice develop soft tissue sarcoma and ovarian tumors (St John et al. 1999), and Lats2-null mice are embryonic-lethal (McPherson et al. 2004; Yabuta et al. 2007). Lats1/2 has been shown to phosphorylate YAP/TAZ in vitro and in cell culture (Zhao et al. 2007; Hao et al. 2008; Lei et al. 2008). However, some studies suggest that the function of Lats1/2 in YAP inhibition is cell type-dependent. In MCF10A cells, overexpression of Lats1 but not Lats2 was shown to inhibit YAP-induced epithelial–mesenchymal transition (EMT), migration, and anchorage-independent growth (J Zhang et al. 2008). However, in Lats1-null MEF cells, YAP phosphorylation is not significantly affected (B Zhao and K-L Guan, unpubl.). In HeLa cells, simultaneous knockdown of Lats1 and Lats2 is required for an efficient inhibition of YAP phosphorylation. Controversial results have been reported on the requirement of Mst1/2 in liver for Lats1/2 phosphorylation, although YAP phosphorylation is reduced in both cases (Zhou et al. 2009; Lu et al. 2010). The possibility of YAP phosphorylation by a Mst1/2-regulated kinase distinct from Lats1/2 is supported by fractionation experiments showing that a Lats1/2-independent kinase phosphorylates YAP (Zhou et al. 2009). The possibility that the other NDR family members, NDR1/2, act as candidate kinases for YAP is dampened by the fact that NDR1/2 do not interact with YAP in vitro and do not seem to induce YAP phosphorylation in cell culture when overexpressed (Hao et al. 2008). Therefore, Lats1/2 are likely the major kinases responsible for YAP phosphorylation and inhibition; however, other kinases may also phosphorylate YAP on S127. Lats2 itself is also regulated by oncogenic microRNA miR-372 and miR-373 (Voorhoeve et al. 2006; Cho et al. 2009; Lee et al. 2009). It will be interesting to test if YAP/TAZ actually mediate the oncogenic activity of these microRNAs.

MOBKL1A/MOBKL1B (highly homologous and collectively referred to as Mob1 below) are the only human Mob proteins that interact strongly with Lats1/2 (Chow et al. 2010). Mob1 is phosphorylated by Mst1/2 (Fig. 2), which enhances the interaction between Mob1 and Lats1/2 (Praskova et al. 2008). However, the mechanism of Mob1 inducing Lats1/2 activation is not fully understood. It has been shown that artificial targeting of Mob1 to plasma membrane induces Lats1 membrane localization and activation (Hergovich et al. 2006). Consistently, *Drosophila* Mats has been reported to localize to plasma membrane, and the constitutively membrane-localized form of Mats, but not the wild-type protein, inhibits tissue overgrowth when overexpressed in vivo (Ho et al. 2009). However, membrane localization of endogenous mammalian Mob1 has not been confirmed, and it is not clear if Mob1/Mats membrane localization is regulated. Mob1 can activate NDR2 in vitro in the presence of Mst3 without affecting NDR2 hydrophobic motif phosphorylation (Stegert et al. 2005), suggesting an additional mechanism of NDR activation by Mob, possibly by relieving an autoinhibitory binding. It is not clear whether such a mechanism also applies to Lats1/2 activation.

Mer, acting upstream of the Hippo pathway, has been shown to regulate YAP localization and inhibit YAP activity in cell culture (Zhao et al. 2007). In NF2-deficient glioma and mesothelioma cells, Mer re-expression induces Mst1/2 and Lats1/2 phosphorylation as well as YAP phosphorylation, further supporting a role of Mer in activating the Hippo pathway (Lau et al. 2008; Yokoyama et al. 2008). The mechanism of how Mer regulates this pathway remains unresolved. Functional conservation of other upstream components in mammalian cells, such as FRMD6 and Fat4, is less clear. In zebrafish, knockdown of a cell junction protein, Scribble, was shown to intensify the cyst-promoting phenotype of Fat1 depletion (Skouloudaki et al. 2009). Scribble was also shown to interact physically with Fat1 and inhibit YAP nuclear localization and activity. However, whether scribble affects hippo pathway activity requires further investigation.

YAP/TAZ are the major downstream effectors of the Hippo pathway. YAP and TAZ mRNA is expressed in a wide range of tissues, except peripheral blood leukocytes (Kanai et al. 2000; Komuro et al. 2003). YAP-null mice die at embryonic day 8.5 (E8.5), with defects in yolk sac vasculogenesis, chorioallantonic fusion, and body axis elongation (Morin-Kensicki et al. 2006). TAZ knockout mice are viable and are predisposed to glomerulocystic kidney disease and pulmonary disease (Hossain et al. 2007; Tian et al. 2007; Makita et al. 2008). YAP has an N-terminal proline-rich domain, a TEAD-binding region, two WW domains (or one in the YAP1 splicing variant), an SH3-binding motif, a coiled-coil domain, a transcription activation domain, and a C-terminal PDZ-binding motif (Fig. 1). TAZ has a similar domain organization, although it lacks the proline-rich domain, the second WW domain, and the SH3-binding motif. Without a DNA-binding domain, YAP/TAZ have to work through target transcription factors. Under physiological conditions, the TEAD family proteins serve as key transcription factor targets of YAP/TAZ and mediate their functions (Zhao et al. 2008b; H Zhang et al. 2009).

The *cycE*, *diap1*, and *bantam* genes are directly induced by Yki and contribute to tissue overgrowth. However, the situation in mammalian cells is not identical. CycE is not induced by YAP in mammalian cells, while Bric5 and Birc2, two Diap1 homologs, are induced depending on cellular context (Dong et al. 2007; Zhao et al. 2007; Hao et al. 2008). Bantam, which encodes a microRNA, is not conserved in the human genome, and a functional counterpart has not been identified yet. However, other proteins—including many cytokines such as CTGF, BDNF, and FGF1—are up-regulated by YAP (Zhao et al. 2007; Hao et al. 2008). CTGF has been shown to be a direct target gene induced by YAP–TEAD, and to play a role in YAP-induced proliferation and anchorage-independent growth. In a recent report, amphiregulin (AREG), an EGF family member, was also identified as a target gene of YAP (J Zhang et al. 2009). The induction of AREG by YAP is only observed under EGF starvation conditions. However, the AREG promoter does not contain a TEAD-binding element, and the transcription factor mediating AREG induction remains elusive (Fig. 2). Interestingly, AREG was shown to be the factor in medium conditioned by active YAP-overexpressing cells mediating a

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non-cell-autonomous growth-promoting effect on MCF10A cells. However, the main function of the Hippo pathway and YAP seems to be cell-autonomous in vivo.

Phosphorylation-dependent inhibition of YAP by the Hippo pathway

YAP is directly phosphorylated by Lats1/2 on five HXRXXS consensus motifs (Zhao et al. 2010). Three of the sites are conserved in Yki and are shown to be phosphorylated (Oh and Irvine 2009). Phosphorylation of YAP S127 and the corresponding site in Yki or TAZ generates a 14–3–3-binding site that leads to YAP/TAZ/Yki cytoplasmic retention through 14–3–3 binding, and their spatial separation from nuclear target transcription factors and target gene promoters (Zhao et al. 2007; Lei et al. 2008; Oh and Irvine 2008). The involvement of 14–3–3 in *Drosophila* Yki inhibition was confirmed recently by RNAi and genetic mutation, which enhanced Yki nuclear localization and activity in vivo (Ren et al. 2009). This Hippo pathway-dependent nuclear–cytoplasmic translocation of YAP, originally observed in cell culture, has also been suggested to be responsible for the cytoplasmic translocation of YAP in inner cell mass during mouse embryo development (Nishioka et al. 2009). Mutation of the S127 residue activates YAP. However, further mutations of the other four serine residues in the HXRXXS motifs boost YAP activity even more (Zhao et al. 2007). Furthermore, the YAP-5SA mutant can potently transform NIH-3T3 cells, whereas YAP-S127A cannot (Zhao et al. 2009). These observations support the idea that the other Lats1/2 phosphorylation sites in YAP are also important for its regulation.

Indeed, later experiments showed that the presence of either S127 or S381 is sufficient to suppress the oncogenic potential of YAP-5SA mutant (Zhao et al. 2010). Moreover, double mutation of S127A and S381A is sufficient to turn on transforming activity of YAP. These results demonstrate that Lats1/2 phosphorylation on S127 and S381 are key events for YAP inhibition. We reported recently that phosphorylation on YAP S381 primes subsequent phosphorylation by another kinase, possibly casein kinase 1 (CK1δ/ε), activating a phosphodegron and causing the recruitment of β-TRCP E3 ubiquitin ligase leading to YAP polyubiquitination and degradation (Zhao et al. 2010). This mechanism of YAP destabilization provides a possibility of long-term YAP inactivation and is implicated in YAP inhibition upon cell contact inhibition. Increased YAP phosphorylation at high cell density is accompanied by YAP destabilization, which can be blocked by mutation of both S127 and S381. Interestingly, YAP S381A mutation is sufficient to block ubiquitination. Therefore, the requirement of both S127 and S381 mutation for YAP stabilization also suggests that there might be a ubiquitination-independent but S127-dependent mechanism of YAP degradation. The Hippo pathway-dependent destabilization of YAP is supported by the recent observation of increased YAP protein levels accompanying decreased YAP phosphorylation in Mst1/2 double-knockout mice liver (Song et al. 2010). The phosphodegron is also conserved in TAZ, and modulates TAZ stability in a manner similar to YAP (Q Lei and K-L Guan, unpubl.).

However, a similar mechanism may not be applicable to Yki because the phosphodegron is not conserved. Notably, *discs overgrown* (*dco*), the *Drosophila* homolog of CK1δ/ε, has been shown by genetic epistatic analysis to be involved in the Hippo pathway upstream of Dachs (Cho et al. 2006). Recently, Dco has also been shown to phosphorylate Fat, although the effect of this phosphorylation on the Hippo pathway activity has not been clarified (Feng and Irvine 2009; Sopko et al. 2009). However, the effect of CK1δ/ε on YAP phosphorylation appears to be direct, supporting CK1δ/ε as a new player in the Hippo–YAP pathway. YAP Y391 residue is in close proximity to the phosphodegron and is phosphorylated by c-Abl in response to DNA damage, resulting in YAP stabilization (Levy et al. 2008). It is not clear if Y391 phosphorylation stabilizes YAP through modulation of S381 phosphorylation-mediated YAP degradation. The proposed mechanism also raises an interesting analogy between YAP and another oncogenic transcription coactivator, β-catenin, as β-catenin is similarly degraded by a phosphodegron and β-TRCP (Liu et al. 2002).

Yki is phosphorylated by Wts on S168 (YAP S127 counterpart), as well as S111 and S250. Similar to YAP, S168 phosphorylation plays a major role in the cytoplasmic localization and inhibition of Yki (Oh and Irvine 2008). Phosphorylation of S111 and S250 also inhibits Yki activity, and the Yki S111/250A mutant promotes *Drosophila* eye overgrowth more potently than wild-type Yki (Ren et al. 2009). Yki S111/250A appears to be more nuclear-localized than the wild-type Yki, although the effect is less dramatic than Yki S168A (Ren et al. 2009). Therefore, S111/250 phosphorylation may also regulate Yki subcellular localization through an elusive mechanism. 14–3–3 binding and a direct sequester by Ex are apparently not involved (Ren et al. 2009). It is clear that the Hippo pathway-dependent phosphorylation inhibits YAP/TAZ/Yki through multiple mechanisms.

Inhibition of Yki by Hippo pathway components via direct physical interaction

An interesting phenomenon in the Hippo pathway is the abundance of protein–protein interactions, such as those mediated by WW domains that bind PPXY motifs (Fig. 1). The functions of WW domains in YAP regulation are controversial. They have been shown to positively mediate YAP function, and mutation of the WW domains attenuated YAP activity in promoting cell proliferation and oncogenic transformation in NIH-3T3 cell culture and diminished the ability of YAP/Yki to promote tissue overgrowth in *Drosophila* in vivo (X Zhang et al. 2009; Zhao et al. 2009). However, in other cells, such as MCF10A, WW domains were shown to promote cell proliferation or inhibit cell transformation (X Zhang et al. 2009; Zhao et al. 2009). This discrepancy might be due to the interaction of WW domains with multiple binding partners, such as transcription factors in nucleus or inhibitory proteins in cytoplasm.

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The WW domains in YAP have long been suggested to interact with Lats1/2 (Hao et al. 2008; Oka et al. 2008). However, the WW domain mutant YAP can still be inhibited by Lats2, indicating they are not required for YAP phosphorylation by Lats2 (Zhao et al. 2009). The WW domain in Yki has been shown recently to interact with the PPXY motifs in Ex, Wts, and Hpo. Such interactions may inhibit Yki by sequestering it in cytoplasm (Badouel et al. 2009; H Oh et al. 2009), adding another layer of complexity to Yki regulation. However, one concern from these studies is that most experiments were performed with overexpressed proteins, which may create nonphysiological interactions. Even if the WW domain indeed inhibits Yki by interacting with PPXY-containing proteins, the Wts-induced phosphorylation-dependent inhibition would play a more prominent role in Yki inhibition. This is supported by the finding that, in 14–3–3 knockdown *Drosophila* wing discs, Hippo pathway components are unable to hold Yki in the cytoplasm by physical interaction (Ren et al. 2009). This suggests that 14–3–3-dependent translocation of phosphorylated Yki is responsible for sequestering a large fraction of Yki in the cytoplasm.

YAP-–TEAD structure

As a transcription coactivator, YAP is brought to gene promoters by target transcription factors. Recent biochemical and genetic studies established a key role of the TEAD family transcription factors in mediating the biological activity of YAP/TAZ (Zhao et al. 2008b; H Zhang et al. 2009), and their *Drosophila* homolog Sd in mediating the functions of Yki (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008). In mammalian cells, it has been reported that as high as 75% of purified TEAD2 is in association with YAP (Vassilev et al. 2001). A screen of a human transcription factor library also identified TEADs as the targets that are most potently activated by YAP (Zhao et al. 2008b). Knockdown of TEADs or disruption of YAP–TEAD interaction blunts the regulation of the majority of YAP-dependent genes and largely diminishes the activity of YAP in promoting cell proliferation, oncogenic transformation, and EMT (Zhao et al. 2008b). Conversely, when TEAD was fused with VP16 transactivation domain, it produced a gene expression profile similar to that activated by YAP (Ota and Sasaki 2008). In addition, the phenotype of TEAD1/TEAD2 double-knockout mice resembles YAP knockout mice with decreased proliferation and increased apoptosis (Sawada et al. 2008). These discoveries were supported by studies indicating that *sd* interacts genetically with *yki* and is required for *yki*-induced target gene expression in vivo (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008).

The three-dimensional structures of human YAP–TEAD1 complex and mouse YAP–TEAD4 complex have been solved recently (Chen et al. 2010; Li et al. 2010). Both structures contain only the TEAD-binding domain of YAP and the YAP-binding domain of TEAD. Nevertheless, the complex structures provide important insights into the molecular basis of YAP–TEAD interaction. The TEAD C-terminal domain forms a globular structure with a β-sandwich fold surrounded by four α-helices on one side. The YAP N-terminal domain wraps around TEAD, forming extensive interactions.

Of particular interest are the two residues that have been implicated previously in YAP–TEAD binding based on biochemical studies. Mutation of S94 in YAP or mutation of Y406 in TEAD1 disrupts the YAP–TEAD interaction and abolishes YAP-induced gene expression (Zhao et al. 2008b). TEAD1 Y406H mutation is associated with a human genetic disease, Sveinsson's chorioretinal atrophy (Fossdal et al. 2004). The three-dimensional structure shows clearly that S94 directly forms a hydrogen bond with TEAD1 Y406 (Chen et al. 2010; Li et al. 2010). The YAP–TEAD complex structure provides a beautiful biochemical explanation of the disrupted interaction between YAP and TEAD1 by YAP S94 or TEAD1 Y406 mutations, and reveals the molecular basis for TEAD1 mutation in Sveinsson's chorioretinal atrophy. Given that a small region of YAP makes a critical contribution to the YAP–TEAD complex formation, it will be interesting to test if small peptides could be designed to target the conserved interfaces and to inhibit YAP–TEAD function in vivo. Such inhibitors would have potential therapeutic application for cancers with high YAP activity.

New transcription factor targets of YAP/Yki

In most tissues, there is at least one TEAD expressed (Kaneko et al. 1997), supporting a ubiquitous role of YAP–TEAD transcription complex in cell proliferation and survival. Other YAP target transcription factor partners—including RUNX, ErbB4 cytoplasmic domain, and p73 (Yagi et al. 1999; Strano et al. 2001; Komuro et al. 2003; Omerovic et al. 2004)—interact mainly with YAP via their PPXY motifs, although none of them has been shown to mediate the growth-promoting function of YAP. A recent report identified Smad1 in the BMP signaling pathway as a new transcription factor interacting with the YAP WW domains (Alarcon et al. 2009). This interaction is also conserved in *Drosophila* Yki and the Smad1 homolog Mad. Importantly, phosphorylation of the linker region of Smad1 following BMP stimulation significantly increases the interaction with YAP, and mutation of the phosphorylation sites decreases their interaction. YAP has also been shown to mediate BMP target gene expression in mouse embryonic stem (ES) cells, which relies on BMP signal for pluripotency maintenance (Alarcon et al. 2009). Therefore, this regulated interaction of Smad1 and YAP could possibly mediate the cross-talk between the BMP and the Hippo signaling pathways.

The above work is reminiscent of a previous report indicating the requirement of TAZ in maintaining Smad2/3 nuclear localization and target gene expression in response to TGF-β signaling (Varelas et al. 2008). Knockdown of TAZ not only impairs TGF-β-induced gene expression, but also induces human stem cell differentiation, which relies on TGF-β signaling for maintenance of pluripotency. It is noteworthy that TAZ binds to Smad2/3 through the coiled-coil region instead of the

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WW domain. Thus, the regulations of YAP and TAZ interaction with Smads are different. Intriguingly, the effect of YAP or TAZ knockdown is not compensated by the presence of the other, indicating the physiological differences between YAP and TAZ. One possible explanation is that YAP and TAZ have different expression patterns in human and mouse stem cells. Alternatively, YAP and TAZ are functionally distinct, but respond specifically to BMP and TGF-β, respectively. However, both the WW domains and the coiled-coil domain are conserved in YAP and TAZ. It is therefore unclear how they achieve specific binding with certain Smads.

In *Drosophila*, Yki shows a broader expression pattern than Sd (Campbell et al. 1992), and *yki* mutant cells have more dramatic growth defects than *sd* mutant cells (Huang et al. 2005; Wu et al. 2008), suggesting other transcription factors also mediate Yki function. This speculation is supported by the observation that the Sd-binding-defective Yki mutant elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (Zhao et al. 2008b). In a recent report, Homothorax (Hth), a TALE homeodomain transcription factor, and Teashirt (Tsh), a zinc finger transcription factor, were shown to mediate part of Yki activity in promoting tissue overgrowth by inducing *bantam* microRNA (Peng et al. 2009). Hth and Tsh form a physical complex, and have been studied mostly in the context of patterning and morphogenesis. Hth and Tsh are required for cell survival and proliferation in the progenitor domain of the eye imaginal disc anterior to the morphogenetic furrow (MF). However, the growth of Hth and Tsh overexpression clones in the anterior disc was largely inhibited by Yki mutation. On the other hand, mutation of Hth also abolished Wts mutation or Yki overexpression-induced clone expansion specifically in the anterior compartment (Peng et al. 2009). Therefore, there is a codependent relationship between the Hippo pathway and Hth/Tsh in inducing proliferation in the *Drosophila* eye progenitor domain. Hth and Tsh stimulate the promoter activity of *bantam* microRNA. *bantam* expression partially rescues the phenotype of *hth* mutation, while *bantam* mutation represses Hth and Tsh overexpression-induced overgrowth. Finally, Hth and Yki are shown to interact with each other and bind to a short DNA sequence 14 kb upstream of the *bantam* gene, suggesting that they may directly activate bantam expression.

The relationship between Hth/Tsh and Sd or possibly other Yki target transcription factors remains an interesting question. In the eye disc, Hth/Tsh and Sd expression is restricted to anterior and posterior to the MF, respectively, and may therefore function in different cells. Glass multimer reporter (GMR) specifically drives gene expression posterior to the MF in *Drosophila* eye discs. Intriguingly, when Yki overexpression was driven by GMR, mutation of *bantam* still attenuated the effect of Yki (Nolo et al. 2006; Thompson and Cohen 2006). Similarly, the effect of Hippo overexpression by GMR could also be rescued by simultaneous expression of *bantam* (Nolo et al. 2006; Thompson and Cohen 2006). These observations indicate that other transcription factors could regulate *bantam* expression posterior to the MF, where Hth/Tsh are not expressed. There are two mammalian homologs of Hth (Meis1 and Meis2), and three Tsh homologs (Tsh1/2/3). Although mouse Tsh1/2/3 share low sequence homology with the *Drosophila* Tsh, they have been shown to compensate for developmental defects due to Tsh loss in *Drosophila* (Manfroid et al. 2004). More studies are needed to examine the function of Hth and Tsh mammalian homologs in mediating YAP/TAZ function.

YAP and the Hippo pathway in cancer

The Hippo pathway was initially defined as a tumor suppressor pathway in *Drosophila*. The best-characterized human tumor suppressor gene in the Hippo pathway is the neurofibromatosis tumor suppressor *NF2* (*Mer*). An *NF2* mutation is present in one out of 25,000 individuals, half of which is due to de novo mutation and the other half to germline inheritance. Patients with *NF2* mutations develop lesions in the nervous system, eyes, and skin, with a close to 100% penetrance by the age of 60 (Asthagiri et al. 2009). Although mutations of other Hippo pathway components in human cancers are understudied, compelling evidence supports a role of this pathway in human tumorigenesis. Mutations of Sav1 and Mob1 have been observed in a human renal carcinoma cell line (ACHN) and melanomas, respectively (Tapon et al. 2002; Lai et al. 2005). Attenuated expression of Mob1 has been observed in colorectal cancer tissue compared with matched normal samples (Kosaka et al. 2007). In a retroviral tumorigenesis study in mice, Mob1 has also been shown to be one of the genes with multiple insertions, indicating a potential tumor suppressor role (Uren et al. 2008). Down-regulation of Lats1/2 has been reported in human sarcomas, ovarian carcinomas, aggressive breast cancers, astrocytomas, retinoblastomas, and acute lymphoblastic leukemia (Hisaoka et al. 2002; Jimenez-Velasco et al. 2005; Chakraborty et al. 2007). In some cases, this attenuated Lats1/2 expression was attributed to promoter hypermethylation (Takahashi et al. 2005; Jiang et al. 2006). Decreased Mst1/2 expression has been observed in human soft tissue sarcomas and colorectal cancers (Minoo et al. 2007; Seidel et al. 2007). Importantly, genetic studies in mice have unequivocally demonstrated Mst1/2 as tumor suppressors. The germline $Mst1^{-/-}Mst2^{+/-}$ mice mainly developed hepatocellular carcinoma (HCC) due to *Mst2* loss of heterozygosity (Zhou et al. 2009). Moreover, tissue-specific ablation of both *Mst1* and *Mst2* in liver leads to massive HCC (Zhou et al. 2009; Lu et al. 2010; Song et al. 2010). Knockdown of YAP reversed the transformed phenotype of HCC-derived cells from these mice (Zhou et al. 2009). Strikingly, 70% of human HCC samples examined show markedly reduced Mst1/2 activity, as determined by Mob phosphorylation, and most are also confirmed by loss of the cleaved, presumably active form of Mst1 (Zhou et al. 2009). It is worth noting that in all but three of those samples with attenuated Mst1/2 activity, YAP phosphorylation is also decreased.

As the major downstream effector of the Hippo pathway, it is not surprising that YAP functions as an oncogene. The

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YAP gene locus is known to be amplified in human cancers, including intracranial ependymomas, oral squamous cell carcinomas, and medulloblastomas (Baldwin et al. 2005; Snijders et al. 2005; Modena et al. 2006; Fernandez et al. 2009). More interestingly, two reports identified YAP as a driving oncogene in human HCC and breast cancer 11q22 amplicons (Overholtzer et al. 2006; Zender et al. 2006). Consistently, elevated YAP expression and nuclear localization have been observed in multiple types of human cancers, including liver cancers, colon cancers, ovarian cancers, lung cancers, and prostate cancers. (Zender et al. 2006; Dong et al. 2007; Zhao et al. 2007; Steinhardt et al. 2008). Recently, by analyzing 177 pairs of HCC and matched normal samples with complete clinical records, YAP was determined to be an independent prognostic marker for overall survival and disease-free survival for HCC patients (Xu et al. 2009). At this moment, the data are still insufficient to delineate the tissue specificity and frequency of Hippo pathway components and YAP mutations/alterations in human cancers. However, current data indicate that dysregulation of the Hippo pathway and YAP plays a role in tumorigenesis, at least in HCC. This might be due to tissue-specific function of the Hippo pathway, or simply because more studies of this pathway have been performed in liver tissues. YAP is also reported to have proapoptotic activity by coactivation of p73 (Strano et al. 2005; Matallanas et al. 2007; Oka et al. 2008), and was proposed to be a breast tumor suppressor (Yuan et al. 2008). This might be cell context-specific, and needs to be supported further by in vivo genetic study. In addition, TAZ has been shown to be overexpressed in ~20% of breast cancer samples (Chan et al. 2008).

A recent study also identified YAP and TEAD1 overexpression in Sonic hedgehog (Shh) and Wnt-dependent medulloblastomas, which were believed to arise from cerebellar granule neuron precursors (CGNPs) (Fernandez et al. 2009). Treatment of CGNPs with Shh was shown to induce YAP transcription, stabilization, and nuclear localization. YAP, together with TEAD1, may also directly induce expression of Gli2, a downstream effector of Shh signaling. In medulloblastoma mouse models with abnormal Shh signaling, YAP and TEAD1 levels were consistently elevated in tumors. Also, YAP expression was strikingly high in perivascular cells, which are believed to have cancer stem cell properties (Fernandez et al. 2009). These high-YAP-expressing cells also express CD15, a marker of medulloblastoma tumor-propagating cells. Considering the function of YAP in preventing mouse ES cell differentiation, it is tempting to hypothesize that YAP may also function in maintaining cancer stem cells in certain tumors. In support of this hypothesis, Mst1 and Mst2 ablation in liver is also shown to induce abundant accumulation of adult liver stem cells, termed oval cells, in periductal regions (Lu et al. 2010).

Future perspectives

The rapid progress of the Hippo pathway research in the last several years has built a road map of this pathway and its role in physiological organ size control and pathological tumorigenesis. However, the picture is still not complete, and many key questions remain to be addressed. At the top of the list are: Which upstream signals trigger the Hippo pathway, and which receptors detect these signals? Related to this is the question of how the Hippo pathway senses organ size. Other important questions include the biochemical mechanism of Mst1/2/Sav1 activation by upstream components. The extent to which the Hippo pathway varies in terms of cell context and the new components involved have yet to be determined. In addition, other transcription factors and target genes mediating YAP/TAZ/Yki function remain to be identified. Furthermore, it has not been fully elucidated how the Hippo pathway and YAP/TAZ are deregulated in human cancer. Finally, there awaits the challenge of developing an inhibitor of YAP/TAZ for potential therapeutic use against cancer.

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