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Minireview

# The Emerging Role of the Hippo Pathway in Cell Contact Inhibition, Organ Size Control, and Cancer Development in Mammals

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**The Hippo pathway defined originally in *Drosophila melanogaster* is conserved in mammals. The fly core components Hippo, Sav, Wts, and Mats are conserved in mammals as Mst1/2, WW45, LATS1/2, and Mob1. The pathway impinges on transcriptional coactivator Yorkie in fly and YAP in mammals to coordinate cell proliferation and apoptosis. Several recent publications establish that the pathway is one major conserved mechanism governing cell contact inhibition, organ size control, and cancer development. This advance opens new vistas in exploring fundamental mechanisms in cell and developmental biology and offers potential targets to interfere with cancer development.**

During normal development, each organ is patterned not only to a specific architecture but also to a defined final size (Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007). Organ size control is also evident during regeneration after tissue injury or experimental procedures. For example, after partial hepatectomy, the hepatocytes in the liver are mobilized to divide rapidly to increase the size of the regenerating liver. Upon reaching the original size, the cells cease dividing to ensure that the regenerating liver is not over-grown (Fausto et al., 2006).

At the cellular level, epithelial cells in vivo and cultured in vitro exhibit contact inhibition arising from cell-cell and cell-substrata interactions (with basement membrane in vivo and cultureware in vitro) (Eagle and Levine, 1967). Contact inhibition ensures that epithelial cells will stop proliferation once they have reached confluence. Most human cancer cells are refractory to contact inhibition. As a consequence, they are able to continue proliferation in spite of interactions with neighboring cells and substrata. In more aggressive stages, the dividing cancer cells can invade the surrounding tissue to achieve unlimited growth and can eventually metastasize to secondary sites. Many established cancer cell lines also exhibit growth in vitro that is impervious to contact inhibition, and often display anchorage-independent growth in soft agar. The loss of contact inhibition and the gain of anchorage-independent growth are hallmarks of cancer cells in vitro (Hanahan and Weinberg, 2000), suggesting that many oncogenic alterations either uncouple cell proliferation from the mechanism that subjects it to contact inhibition, or alter the contact inhibition mechanism itself.

The Hippo pathway was initially identified in the fly to control organ size, and its core components are evolutionally conserved in mammals. The discoveries made in the fly have provided the paradigm for follow-on studies of mammalian components (Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007). Several recent studies have clearly established a role for

**Table 1. Summary of the Core Components of the Hippo Pathway and the Downstream Target YAP in *Drosophila melanogaster* and in Human**

| *Drosophila* | Accession No. | Size (aa) | Human | Accession No. | Size (aa) |
|---|---|---|---|---|---|
| Hippo | Q8T0S6, AAF57543 | 669 | Mst1/STK4 | NP_006273 | 487 |
| | | | Mst2/STK3 | NP_006272 | 491 |
| Salvador | Q9VCR6 | 608 | WW45/Sav1 | NP_068590 | 383 |
| Warts/LATS | AAA70336, AAA73959 | 1099 | LATS1 | NP_004681 | 1130 |
| | | | LATS2 | NP_055387 | 1088 |
| Mats | Q95RA8 | 219 | Mob1 | NP_060691 | 216 |
| Yorkie | AAZ42161, NP_001036568 | 418 | YAP | NP_006097 | 454 |
| | | | TAZ/WWTR1 | NP_056287 | 400 |

The name, accession number (at http://www.ncbi.nlm.nih.gov/), and length (number of amino acid [aa] residues) of each protein are indicated. Mammalian TAZ/WWTR1 is a candidate target of the Hippo pathway.

the Hippo pathway in regulating cell contact inhibition, organ size control, and cancer development in mammals (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007).

## Core Machinery of the Hippo Pathway

Genetic screens in the 90s on *Drosophila melanogaster* for mutants exhibiting tissue overgrowth identified Warts (Wts) as the first component of the Hippo pathway (Justice et al., 1995; Xu et al., 1995). Yet, only in 2002 was the second component of the pathway, Salvador (Sav), identified. Functional loss of Sav results in overexpression of the cell-cycle regulator cyclin E and the antiapoptotic dIAP1 (Tapon et al., 2002). Subsequent studies uncovered the Hippo (Hpo) component and placed Hippo upstream of Wts (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) and established that Sav potentiates the activity of Hippo (Wu et al., 2003). Mats was identified 3 years ago as the fourth component that interacts with and enhances the activity of Wts (Lai et al., 2005). Hippo, Sav, Wts, and Mats are core components of the fly Hippo pathway and are homologous to mammalian Mst1/2, WW45, LATS1/2, and Mob1, respectively (Table 1). Functional conservation is evident, since LATS1, Mst2, and Mob1 can rescue corresponding fly mutants in vivo (Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007).

Hippo, Mst1, and Mst2 belong to the STE20 (Sterile20) family of protein kinases. Their N-terminal region contains a Ser/Thr protein kinase domain (Figure 1), while coiled-coil domains are predicted in the central and C-terminal regions. The C-terminal coiled-coil region has been referred to as the SARAH (*Sav*/*Ras*sf/*H*po) domain (Scheel and Hofmann, 2003). Sav and WW45 contain two WW domains, and the C-terminal region contains a SARAH domain (Figure 1). Type I WW domains are known to recognize PPXY motifs. The C-terminal SARAH domain of Mst1/2 interacts with the corresponding SARAH domain of WW45 (Callus et al., 2006). Wts, LATS1, and LATS2 belong to the NDR (Nuclear Dbf-2-related) protein kinase family with the Ser/Thr protein kinase domain in the C-terminal region (Figure 1). Further downstream of the kinase domain is the S_TK_X region, while the N-terminal region contains a UBA (ubiquitin-associated) motif that is implicated in ubiquitin-mediated processes. Several PPXY motifs are present in both Wts and LATS1/2 and are expected to interact with WW domain proteins. Knockout of the LATS1 gene in mice causes ovarian tumors and soft-tissue sarcoma (St John et al., 1999), while LATS2-deficient mouse embryonic fibroblasts overcome contact inhibition (McPherson et al., 2004). Mats and Mob1 are small proteins consisting essentially of a Mob1 domain implicated in protein-protein interaction (Figure 1). The four Hippo core components form two protein kinase complexes (each with a regulatory subunit) acting in a cascade such that Hippo/Mst1/2 interacts with Sav/WW45 to phosphorylate and activate the complex formed by Wts/LATS1/2 and Mats/Mob1 (Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007) (Figure 2).

## Yorkie as the Major Target of the Hippo Pathway

Given that Wts/LATS1/2-Mats/Mob1 is a protein kinase complex, its downstream substrates would define the mechanics of the Hippo pathway. To this end, protein interaction screens

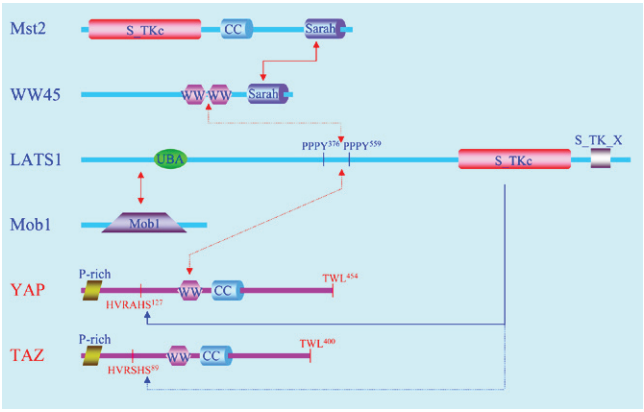


**Figure 1. Schematic Illustration of Domain Organization and Key Structural Features of Human Hippo Core Components and YAP**

Mst1 and LATS2, structurally similar to Mst2 and LATS1, respectively, are not shown. The indicated domains are S_TKc for catalytic Ser/Thr protein kinase, CC for coiled coil, Sarah for Sav/Rassf/Hpo domain, WW for domain with two conserved Trp (W) residues, UBA for ubiquitin associated domain, S_TK_X for extension to Ser/Thr-type protein kinases, Mob1 for Mob1/phocein, and P-rich for Pro-rich. The two PPXP motifs in LATS1 are indicated. The HXRXXS motif with S127 targeted by LATS1 and the C-terminal PZD-binding motif of YAP are also indicated. The interaction of Mst2 with WW45 (mediated by the C-terminal Sarah domains) and the interaction of LATS1 and Mob1 are indicated by double-arrow-ended solid red lines, while the potential interactions of the PPXY motifs on LATS1 with the WW domain of WW45 and YAP are indicated by double-arrow-ended dotted red lines. The link between the catalytic domain of LATS1 and S127 phosphorylation site of YAP is indicated by an arrowed blue line. The general organization and the corresponding S89-containing HXRXXS motif of TAZ/WWTR1 are also included, as TAZ is a candidate target of the Hippo pathway.

using Wts identified the transcriptional coactivator Yorkie as a major downstream target of the Wts-Mats complex in the fly (Huang et al., 2005). Mechanistically, Yorkie is phosphorylated and inactivated by Wts, whereas overexpression of Yorkie yields a tissue overgrowth phenotype similar to Wts mutation. Conversely, inactivation of Yorkie through mutations leads to tissue atrophy. Thus, the Hippo core components can negatively regulate Yorkie through direct phosphorylation (Figure 2). Analogous to overexpression of Yorkie itself, mutations of the Hippo core components are thus expected to increase the activity of Yorkie, resulting in enhanced transcription of its target genes such as cyclin E and dIAP1 that promote cell proliferation and/or suppress cell death.

YAP is the mammalian homolog of Yorkie and can functionally rescue Yorkie mutation in the fly, which suggests that YAP is a functional counterpart of Yorkie and is likely to promote proliferation. However YAP may also exhibit proapoptotic activity under certain conditions, such as DNA damage (Matallanas et al., 2007). YAP contains a WW domain for interaction with PPXY motifs, a predicted coiled-coil region, and a C-terminal TWL motif for interaction with PDZ domains (Figure 1), while its N-terminal region is rich in Pro.

Recent studies support that YAP is an oncogene in mammalian cells (Overholtzer et al., 2006; Zender et al., 2006). First, genome-wide analysis of mouse liver tumors revealed recurrent chromosome amplification at 9qA1, syntenic to human chromosome region 11q22. Amplification of 11q22 has been observed in several human cancers. Two candidate oncogenes

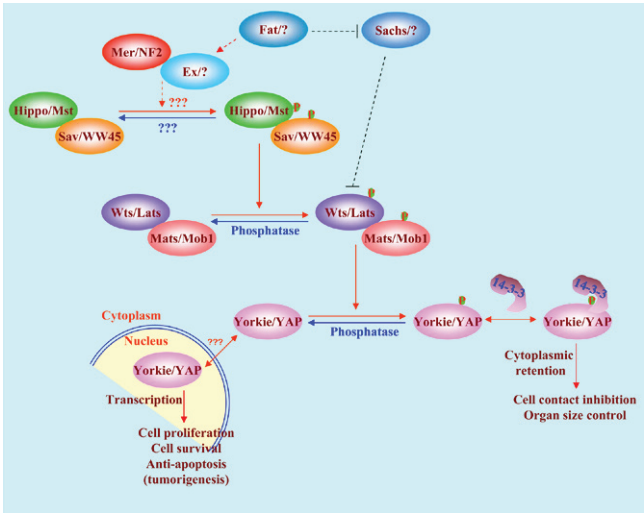


**Figure 2. Schematic Illustration of the Signaling Cascade of the Hippo Pathway**

Hippo/Mst, Sav/WW45, Wts/Lats, and Mats/Mob1 are the core components of the Hippo pathway. Upstream signals transmitted from proteins such as Fat, Mer, and Ex in the fly (or NF2 and other yet to be established human counterparts) upon reaching cell confluence and/or defined organ size cause activation of the Hippo/Mst-Sav/WW45 complex via an unknown mechanism, although protein phosphorylation of Hippo/Mst is a likely possibility. In addition to regulating Ex, Fat may act in parallel to Ex/Hippo to directly activate LATS through relieving its inhibition by the atypical myosin Dachs. Wts/Lats-Mats/Mob1 is activated by Hippo/Mst-mediated phosphorylation. Yorkie/YAP is normally translocated into the nucleus, where it acts as a transcriptional coactivator to coordinate proproliferating and antiapoptotic programs and is phosphorylated (primarily at S168/S127) by the activated Wts/Lats-Mats/Mob1 complex, causing cytoplasmic sequestration of phosphorylated Yorkie/YAP via interactions with 14-3-3 proteins. Cytoplasmic sequestration of Yorkie/YAP suppresses the proproliferating and antiapoptotic program, leading to the arrest of cell proliferation and organ growth. Mutations of the Hippo core components or overexpression of Yorkie/YAP increase the levels of functional Yorkie/YAP in the nucleus to sustain the proliferating and antiapoptotic transcriptional programs and overcome normal contact inhibition and organ size control to promote cancer development. TAZ/WWTR1 is a candidate downstream target of the Hippo pathway in mammalian cells and is likely to be regulated similar to YAP.

in the amplified region are YAP and cIAP1, the latter being a dIAP1-related protein. YAP and cIAP1 are individually oncogenic, but they can cooperate to yield accelerated tumor growth (Zender et al., 2006). In a separate screen, an amplification of a smaller chromosomal region within 9qA1 was identified in mouse mammary tumors, and YAP is the only gene within this narrower region. Overexpression of YAP in nontransformed human MCF10A mammary epithelial cells causes epithelial-mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation, and anchorage-independent growth in soft agar (Overholtzer et al., 2006). Consistent with the proproliferating and antiapoptotic functions of YAP, knockout of the YAP gene in mice leads to early embryonic lethality (Morin-Kensicki et al., 2006).

## A Conserved Mechanism Governing Cell Contact Inhibition, Organ Size Control, and Cancer Development from Fly to Mammals

Three recent reports have elucidated the molecular mechanism through which Yorkie/YAP is regulated by the Hippo pathway to govern cell contact inhibition, organ size control, and cancer development (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007). Guan's laboratory noted that the subcellular location of YAP is dependent on cell density. YAP is primarily present within the nucleus in sparsely growing cells, where it functions as a transcriptional coactivator. Upon confluence, when contact inhibition comes into play, YAP accumulates in the cytoplasm, thereby rendering it unable to function as a transcriptional coactivator. This cytoplasmic sequestration of YAP in response to cell density correlates with its increased phosphorylation. Mst2 and LATS2 act coordinately to phosphorylate YAP at HXRXXS motifs, with the S127-containing motif being the major site. Yorkie has a corresponding motif with S168 as the major site targeted by Wts. In sparsely cultured cells, overexpression of LATS2 leads to S127 phosphorylation of YAP and its cytoplasmic sequestration, but mutation of the S127 into Ala abrogates this cytoplasmic shift. Circumventing the regulation of YAP and Yorkie by LATS2 and Wts through the S127A/S168A mutation also results in enhanced growth-promoting activity. The cytoplasmic sequestration of S127-phosphoryalted YAP results from its enhanced interactions with 14-3-3 proteins (Zhao et al., 2007), which are known to sequester phosphorylated YAP in the cytosol (Basu et al., 2003). This study suggests that when cultured cells reach confluence, cell-cell interactions trigger a cascade of signaling events that activate the Hippo pathway. The activated LATS-Mob1 complex phosphorylates YAP (preferentially at S127), leading to enhanced interactions with 14-3-3 proteins and cytoplasmic sequestration. This results in reduced transcription of YAP target genes, manifesting a growth cessation that is referred to as cell contact inhibition.

Working with *Drosophila*, Pan's laboratory showed that the Hippo pathway leads to cytoplasmic sequestration of Yorkie and that S168 in the HXRXXS motif is the principle site phosphorylated by Wts. Wts-mediated phosphorylation of Yorkie S168 is the mechanistic basis for cell growth suppression by the Hippo pathway. In addition, loss of Hippo signaling due to mutation of Hippo or Wts results in nuclear accumulation of Yorkie, reflecting the role of the Hippo pathway in phosphorylating S168 of Yorkie to mediate its cytoplasmic sequestration. Pan's team also established the biochemical and functional conservation of the Hippo pathway in mammals, its growth-suppressing effects, and the S127 phosphorylation of YAP by LATS1/2 as the substantive mechanism. More significant was the demonstration that variations of YAP levels can overcome organ size control; a regulated increase of YAP expression in the liver of transgenic mice led to a striking enlargement of the liver due largely to increased cell numbers. Sustained overexpression of YAP can expand liver mass from 5% of bodyweight to about 25%, yet this effect is reversible, as the enlarged liver reverts to almost normal size when overexpression of YAP is restrained for a sufficient period of time. This dramatic and reversible manipulation of liver size through YAP changes alone positions the YAP-regulating Hippo pathway as the major mechanism controlling organ size in mammals. Presumably, when an organ reaches its programmed size, the Hippo pathway is triggered to inactivate YAP through phosphorylation and sequestration in the cytoplasm via interactions with 14-3-3 proteins. Many growth-promoting or anti-

apoptotic genes are upregulated by YAP, including Ki67, c-Myc, Sox4, H19, AFP, BIRC5/survivin, and BIRC2/cIAP1. Indeed, the enhanced expression of BITC5/survivin is necessary for YAP to induce anchorage-independent growth. Also, the upregulation of cIAP1 levels by YAP would effectively coordinate cell proliferation by YAP and the suppression of apoptosis by cIAP1 and would explain the coamplification of BIRC2/cIAP1 and YAP genes observed in mouse and human hepatocellular carcinoma. Finally, sustained high-level expression of YAP in the liver of transgenic mice leads eventually to tumorigenesis characteristic of hepatocellular carcinoma (Dong et al., 2007).

A third study from Brummelkamp's laboratory independently demonstrated that YAP is sufficient for inducible and reversible liver enlargement in transgenic mice. Significantly, this study also linked YAP expression to stem/progenitor cells in the intestine, since YAP is primarily expressed in the crypt compartment where these cells reside. Regulated YAP overexpression in the intestine of transgenic mice correlates with elevated levels of cyclin D and BclXL and causes dysplasia due to proliferation of the crypt stem/progenitor cells. Interestingly, this parallels the correlation between YAP expression levels and enhanced levels of cyclin D and BclXL in human colon cancers. This study thus implicates YAP as a critical link between stem/progenitor cells and colon cancer cells (Camargo et al., 2007). Collectively, these three studies suggest that overexpression of YAP or its overactivation due to intrinsic Hippo pathway mutations is able to abrogate cell contact inhibition and organ size control to promote cancer development.

## Future Challenges

Despite this major advance in our understanding of the Hippo pathway, many questions remain to be answered. For example, how does the cell-cell interaction contingent upon confluence or the acquisition of normal organ size activate the Hippo pathway? Although surface proteins such as Fat and submembrane scaffold proteins such as Merlin and Expanded (Ex) are known to be functionally associated with the Hippo pathway (Bennett and Harvey, 2006; Cho et al., 2006; Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007; Silva et al., 2006; Willecke et al., 2006), the precise mechanism of these linkages with the Hippo core components has not been established. In addition to regulating Ex, Fat may act on an atypical myosin Dachs to prevent it inhibiting Wts so that Wts can be activated without engaging Hippo (Figure 2) (Cho et al., 2006; Feng and Irvine, 2007). While the mammalian homolog of Merlin is clearly the tumor suppressor neurofibromin 2 (NF2) (Hamaratoglu et al., 2006), the definitive mammalian Fat (speculated to be FatJ/Fat4) and Ex (speculated to be FRMD6) remain uncertain, as there are many mammalian proteins that are homologous to Fat or Ex. Although Merlin and Ex are likely to transmit signals from surface proteins to the Hippo core machinery (Hamaratoglu et al., 2006), we know little about the molecular detail.

The biochemical mechanism for activating the Mst1/-WW45 complex has yet to be defined, although the regulated phosphorylation of Hippo/Mst1/2 is a prime candidate. If that is indeed the case, then identification of the regulatory kinases and phosphatases will be key to understanding the integration of signals via the Hippo pathway. Although the sites on Wts/LATS1/2 phosphorylated by Hippo/Mst1/2 have been defined (Chan et al., 2005) and Hippo also phosphorylates Mats (Wei et al., 2007), we know little about the phosphatases that dephosphorylate Wts/LATS and/or Yorkie/YAP. Since these phosphatases are likely to enhance the level of unphosphorylated Yorkie/YAP, leading to its nuclear accumulation, they will likely be growth-promoting. The systematic examination of all known phosphatases is one approach to identify them.

The mechanism of nuclear accumulation of Yorkie/YAP has not been explored. Also not established are the partner(s) of nuclear YAP in executing the proproliferating and antiapoptotic transcriptional program. The Bantam microRNA is a downstream target of Yorkie in the fly (Nolo et al., 2006; Thompson and Cohen, 2006) and it will be interesting to examine whether YAP also regulates similar microRNA species in mammalian cells.

In addition to its major target, YAP, the Hippo core machinery may target other substrates yet to be identified. Mammalian TAZ is homologous to YAP and Yorkie and contains several HXRXXS motifs with S89 corresponding to S127 of YAP (Kanai et al., 2000). A recent report established that TAZ is indeed a substrate of the Hippo pathway in human cells (Lei et al., 2008). It would be interesting to explore polymorphisms of Hippo core components and/or YAP as a potential basis for the differing size and height of individuals.

Finally, unraveling the interconnections between the Hippo pathway and other signaling cascades that coordinate the cellular programs in cell growth, proliferation, and apoptosis will be important. A known tumor suppressor, RASSF1, has recently been shown to regulate the Hippo pathway via interaction with Hippo/Mst1/2 (Guo et al., 2007; Matallanas et al., 2007). It is tempting to speculate that RASSF1 functions as the entry point for Ras and other signaling pathways to modulate the Hippo pathway. Mst1/2 has recently been shown to connect with the Akt kinase pathway (Cinar et al., 2007; Jang et al., 2007). With recent advances establishing the foundation, and exploiting the sophisticated genetic, genomic, and proteomics technologies at hand, we can expect rapid progress in addressing these questions. These answers will facilitate the development of assays to screen for molecules that interfere with the functionality of the Hippo pathway and YAP as candidates for anticancer drugs. Cellular components or small chemical compounds that are able to regulate YAP activity in a reversible manner offer potential handles to transiently regulate the organ size or even the size of an organism itself.

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