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INSIGHT INTO HIPPOSIGNLING PATHWAY AND CLINICAL IMPLICATIONS

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ABSTRACT

The pace of discovery in the Hpo signaling field over the past few years has been remarkable, and because of its many advantages for gene discovery and characterization, research in Drosophila has continued to play a leading role. Substantial progress in identifying pathway components has been made recently, but we still lack a mechanistic understanding of crucial steps in the pathway, such as how the activity of the Hpo kinase is controlled by upstream regulators, or how the levels of Wts protein are controlled by Dachsand Zyx. Attaining these mechanistic insights will require substantial investments in both biochemical approaches (eg to reconstitute key steps in signal transduction in vitro), and better reagents for imaging steps in signal transduction. Reagents that would enable discrete identification and localization of active versus inactive isoforms of pathway components would be especially valuable. In short, in a field that has been dominated by geneticists, opportunities for talented biochemists and cell biologists abound. One of the remarkable features of the pathway is the number of different inputs into upstream regulation. A key challenge for the future is to understand how all of the different inputs into the pathway are related to each other. A further challenge will be to understand how diverse inputs are integrated to achieve appropriate levels of Yki activity. Another continuing challenge is to understand how the Hpo pathway is integrated with other growth control pathways. To date most research has focused on a limited set of partners and downstream target genes, and the full extent and cell type diversity of transcriptional responses to Yki activity remains an open question.

KEYWORDS: Hippo, signaling, Drosophila, disease, cancer.

INTRODUCTION

In developmental biology control of organ size is a long-standing puzzle. Classic embryological studies suggest that many organs possess intrinsic information about their final size. For example, when two-thirds of a mouse liver is surgically removed, the remaining one-third regenerates its original mass within 7-10 days and then ceases growth. Similarly, when imaginal discs from newly hatched larvae are transplanted into adult flies, they grow to a final size characteristic of that seen in situ. The molecular mechanisms that stop organ growth at the appropriate point during development or regeneration remain poorly understood today.

Discovery of the Hippo signaling pathway provides an important entry point to addressing many standing questions. The first four components of the Hippo pathway, including the NDR family protein kinase Warts (Wts), the WW domain containing protein Salvador (Sav), the Ste20 like protein kinase Hippo (Hpo) and the adaptor protein Mob as tumor suppressor (Mats) were discovered in genetic screens in *Drosophila* for tumor suppressor genes. Loss of function mutant clones for any of these four genes lead to a strong tissue overgrowth phenotype characterized by increased proliferation and diminished cell death. Biochemically, these four tumor suppressors form a kinase cascade in which the Hpo-Sav kinase complex phosphorylates and activates the Wts-Mats kinase complex.[1] The prime target of this kinase cascade in growth regulation is transcriptional co-activator Yorkie (Yki), which was isolated as a Hippo pathway component in a yeast two-hybrid screen for Wts-binding proteins. Yki functions as an oncogene and its overexpression phenocopies loss of Hippo signaling. Genetic analysis placed *yki* downstream of *hpo*, *sav*or *wts*, and biochemical studies. demonstrated that Wts directly phosphorylates and inactivates Yki in Hpo regulated manner.[2] Thus, from these pioneering studies, a kinase cascade leading from Hpo to Yki phosphorylation emerged.

The elucidation of the Hippo kinase cascade in *Drosophila* has stimulated intense research into the molecular mechanism and the physiological function of this emerging pathway in both flies and vertebrates. While still a relatively young field, research on Hippo signaling is escalating rapidly.[1]

THE HIPPO SIGNALING NETWORK IN DROSOPHILA

The discovery of the four tumor suppressors that constitute the core kinase cassette, candidate gene based approaches and forward genetic screens have implicated at least seven additional tumor suppressors whose activities converge on Hpo and/or Wts, include the FERM domain proteins Merlin (Mer) and Expanded (Ex), the proto cadherins Fat (Ft) and Dachsous (Ds), the CK1 family kinase Disc overgrown(Dco), the WW and C2 domain containing protein Kibra, and the apical transmembrane protein Crumbs (Crb) (Table 1).[1]

Table 1 Hippo Pathway Components in *Drosophila* and Human

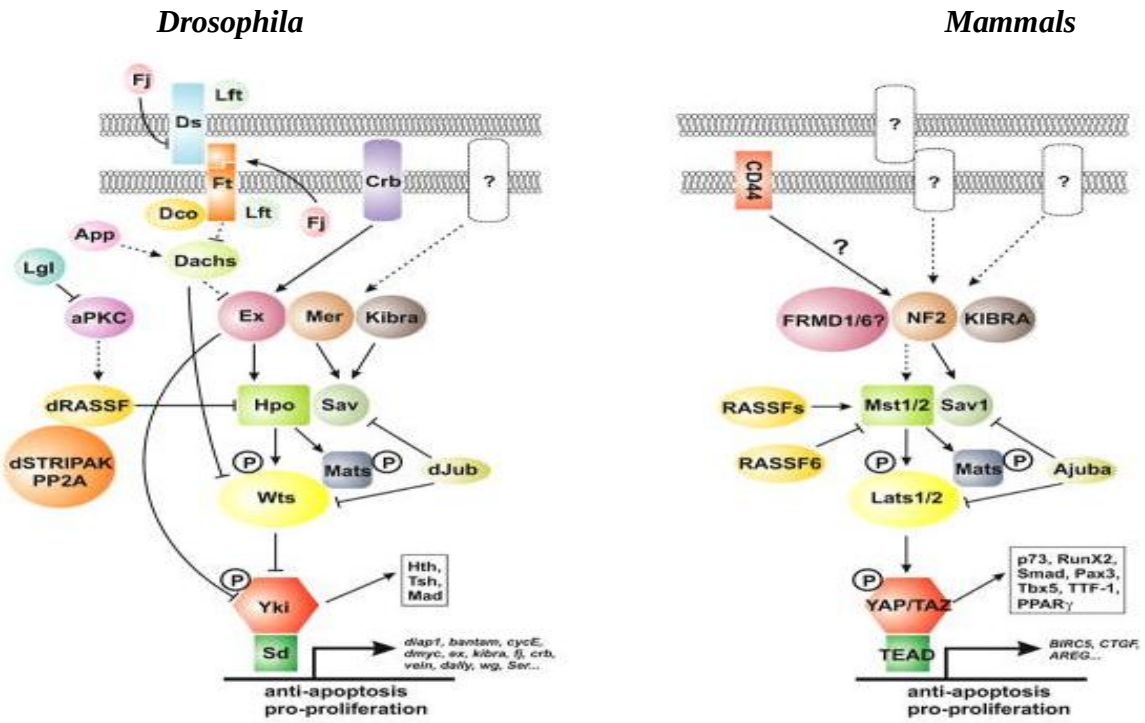
Drosophila	**Human**	**Conserved Domains and Motifs**
The Core Pathway		
Hippo	Mst1-2	Ste20 Ser/Thr kinase and SARAH domains
Salvador	Sav1/WW45	WW and SARAH domain
Warts	Lats1-2	NDR Ser/Thr kinase domain, PPXY motif
Mats	MOBKL1A-B	Mob1/phocein domain
Yorkie	YAP, TAZ	WW and TEAD-binding domains
Scalloped	TEAD1-4	TEA/ATTS and Yki/YAP- binding domains
Upstream Apical Complex		
Kibra	KIBRA/WWC1,WWC2	WW and C2 domains
Expanded	FRMD6/Ex1,FRMD1/Ex2	FERM domain
Merlin	NF2/Merlin	FERM domain
Fat Effectors and Regulators		
Fat	Fat4/Fat-j	EGF-like, Laminin G and Cadherin repeat domains
Dachsous	Dchs1-2	Cadherin repeat domain
Four-jointed	Fjx1	Golgi Ser/Thr kinase
Dachs	?	Myosin motor domain
Approximated	ZDHHC14	DHHC zinc finger domain
Discs overgrown	CK1δ, CK1ε	Ser/Thr kinase domain
Low fat	Lix1, Lix1L	unknown conserved domain
Apical-Basal Polarity		
Crumbs	Crb1-3	EGF-like and Laminin G domains, PDZ- and FERM-binding motifs
Lgl	Lgl1-2	LLGL2 domain
aPKC	aPKCλ, aPKCζ	PKC kinase, PB1 and C1 domains
Lgl	Lgl1-2	LLGL2 domain
Other Modulators		
dRASSF	RASSF1-6	Ras association and SARAH domains

Drosophila	Human	Conserved Domains and Motifs
dJuba	Ajuba, LIMD1, WTIP	LIM domain
dSTRIPAK PP2A	STRIPAK PP2A	PP2A Ser/Thr phosphatase complex

While the mechanisms by which these upstream regulators converge on the Hippo kinase cascade are complex and in some instances unresolved, a notable feature is that mutations in each of these genes lead to a relatively mild over growth phenotype, suggesting that these upstream components function in a combinatorial or additive manner to regulate the Hippokinase cassette.[1]

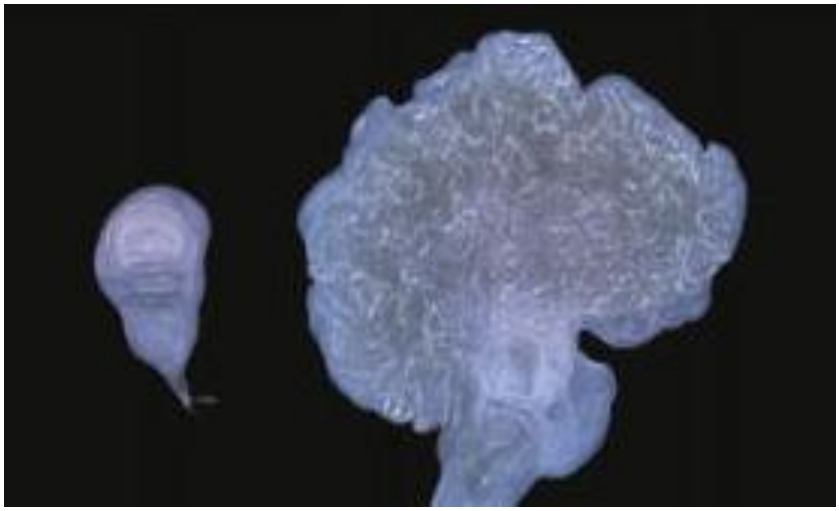
HIPPO, A CONSERVED PATHWAY FOR GROWTH CONTROL

At its most basic, the Hippo pathway is, like many other path-ways, a signal transduction pathway that conveys signals perceived at the plasma membrane to a transcriptional response in the nucleus (Fig. 1) (1).

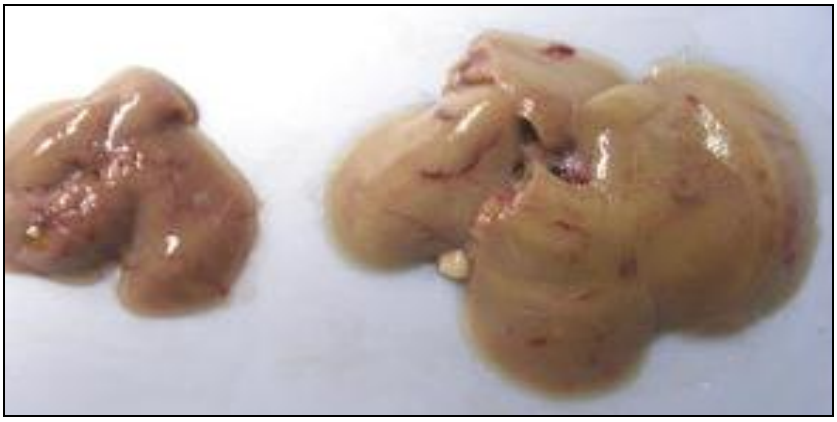


(1) Signaling diagram. Corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. Direct biochemical interactions are indicated by solid lines or drawn as proteins in direct contact with each other. Dashed lines indicate genetic interactions for which no direct protein-protein interactions have been reported. Arrowed or blunted ends indicate activation or inhibition, respectively. Also shown are selected target genes. Yki- or

YAP/TAZ-interacting transcription factors other than Sd (*Drosophila*) or TEAD (mammals) are collectively listed in a box.



(2) A normal (left) and yki-overexpressing (right) *Drosophila* wing imaginal disc.



(3) A normal (left) and a YAP-overexpressing (right) mouse liver. The dramatic increase in organ size induced by Yki/YAP overexpression illustrates the potent growth-regulatory activity of Hippo signaling in *Drosophila* and mammals.

In many cases, the transcriptional output of the pathway appears to be directed toward the regulation of organ growth, and consequently Hippo signaling has major roles in developmental growth control, and its dysregulation has been linked to a number of cancers. Some individual components of Hippo signaling were first discovered years (e.g. Yap, Warts), or even decades (e.g. Fat, Dachs) before the term Hippo signaling was coined, but it is only within the last decade that the interconnectedness of these components into a conserved intercellular signaling pathway has become appreciated.[5] At the core of both the discovery of the pathway and its signal transduction mechanism is a conserved kinase

cascade, in which the protein kinase Hippo (Mst1,2 in vertebrates) promotes activation of the protein kinase Warts (Lats1,2 in vertebrates). This is achieved by Hippo directly phosphorylating Warts, and two additional components of the core kinase cassette: Mob as tumor suppressor (Mats, Mobkl1a,b in vertebrates) and Salvador (Sav, WW45 in vertebrates). Warts kinase can then negatively regulate a transcriptional co-activator protein, Yorkie (Yki, Yap and Taz in vertebrates)[2], mainly by phosphorylating it to promote its cytoplasmic retention through 14-3-3 binding. Upstream and downstream of this conserved kinase cassette, the pathway becomes more complex, as there is a considerable diversity of upstream regulatory inputs. In Drosophila, three trans-membrane receptor proteins have so far been identified as Hippo pathway receptors: Fat, Crumbs, and Echinoid. Other upstream inputs whose regulation is less well understood have also been described in Drosophila, including regulation dependent upon Merlin, Lethal giant larvae, and Jun kinase (Jnk). There is also a surprising diversity amongst the upstream regulatory inputs into the pathway between phyla. Merlin is a conserved regulator between Drosophila and vertebrates, which at least in vertebrates plays a crucial role in contact dependent inhibition of cell proliferation through affects on Hippo signaling. How-ever, in vertebrates components of the Ecadherin/alphacatenin complex are Hippo pathway regulators[6,7], and an equivalent role in Drosophila has not yet been described. Conversely, components of the Fat branch of the Hippo pathway have been identified in vertebrates, but do not appear to have significant effects on the Hippo pathway in these species, at least in most tissues. Nonetheless, a common theme has emerged in which most regulators of Hippo signaling are associated with cell-cell junctions, and thus well positioned to inform cells about cell polarity and cell density, which thus have major influences on Hippo activity.[5]

One feature of Hippo signaling that provides significant potential for signal integration concerns the nature of the transcriptional output of the pathway. This is provided by a transcriptional co-activator protein (Yorkie in Drosophila, Yap and Taz in vertebrates) rather than a DNA binding protein.[2] Thus, interaction with other proteins is intrinsically required for regulation of down-stream transcriptional targets. Moreover, Yki/Yap/Taz co-activators have been found to be able to associate with multiple, distinct DNA-binding partners. The major partner for Yki/Yap/Taz appears to be Scalloped in Drosophila, and its homologues the Tead/TEF proteins in vertebrates. However, a number of additional DNA binding partners have also been identified, including some that are regulated by other signaling pathways, such as the Smad proteins, which are transcription factors of TGF-α related pathway.[5]

REGULATION OF OTHER PATHWAYS BY HIPPO SIGNALING

While integration between signaling pathways can occur at almost any level, conceptually one of the simplest forms is when one pathway regulates the amount of signal perceived by another, for example, by regulating the production of a ligand for that pathway. In most contexts, the main targets of Yki/Yap/Taz activity appear to be promoters of organ growth. These include a number of direct, autonomous promoters of cellular growth. However, a significant part of the growth regulating activity of Hippo appears to derive from regulation of secreted growth factors that activate other pathways. An early example of this was the regulation of the Drosophila Wnt ligand Wingless (Wg) in the proximal part of the developing wing.[8,9] Wg acts as a mitogen here, promoting proximal wing growth[10], and this regulation of Wg contributes significantly to upregulation of growth associated with certain Hippo pathway mutants.[9] Another example from Drosophila is the upregulation of Serrate, a ligand for the Notch pathway, which pro-motes leg growth.[11,12] Drosophila Hippo signaling has also been linked to regulation of glypicans, which play a prominent role in modulating signaling by several classes of secreted growth factors.[13] Finally, a striking recent example of growth promotion by Drosophila Hippo signaling through regulation of other pathways has come from studies of the adult intestine. The Drosophila intestine is maintained by intestinal stem cells (ISCs) and has a simple architecture comprising four basic cell types: ISCs, undifferentiated progenitors, and two types of differentiated cells.[14,15] The differentiated cells are continuously lost at a low rate, but are replaced from the ISCs, which are the only cells that proliferate. In response to infection or injury, ISC proliferation is increased to facilitate repair. This increased proliferation is mediated through the Hippo pathway, but the effect of Hippo signaling is at least mostly non-autonomous: infection or damage of the non-proliferating differentiated cells leads to activation of Yki, which then promotes the expression of cytokines that are secreted from these cells, and which stimulate the proliferation of nearby ISCs.[5] These cytokines include multiple ligands for the Jak-Stat pathway (Unpaired proteins), and also ligands for the EGFR pathway. The characterization of the central role of non-autonomous effects of Hippo signaling in controlling ISC proliferation emphasizes the importance of regulation of other pathways to growth control by Hippo signaling. Activation of secreted ligands for other pathways has also been linked to growth regulation by vertebrate Hippo pathways. One prominent example is the regulation of amphiregulin, a mammalian EGFR ligand, by Yap within breast epithelial and adenocarcinoma cells.[16,17] This upregulation of amphiregulin is essential to the ability of Yap to transform these cells. Another major growth factor target of Hippo signaling in

mammalian cells is Connective Tissue Growth Factor (CTGF, also known as CCN2).[18,19] Regulation of CTGF by Yap/Taz appears to be widespread amongst many different cell types, and consequently its expression is often now used as a marker of Hippo pathway activity. CTGF has diverse roles, including links to tissue repair and carcinogenesis, which could contribute to Hippo pathway influences on growth in vertebrates.[20]

INTEGRATION OF HIPPO SIGNALING WITH OTHER PATHWAYS

In addition to the upstream downstream connections between pathways described above, some pathways are even more closely integrated with Hippo signaling. TGF-β signaling pathways are intimately connected to Hippo pathways through direct binding of their transcription factors. TGF-β signaling influences transcription by regulating the sub-cellular localization of DNA-binding transcription factors of the Smad protein family.[21] Smad proteins can be partners for Yki/Yap/Taz proteins, and, in some cases, such as in the Drosophila wing, contribute to growth regulation by Hippo signaling.[22] The interaction between Yap/Taz proteins and Smad proteins has also been implicated in TGF-β signaling functions in some contexts, including mammalian ES cell renewal.[23] Notably, these proteins interact not only in the nucleus, where they can co-regulate downstream genes, but also in the cytoplasm. This cytoplasmic interaction, which has so far only been characterized in mammalian cells, enables Hippo signaling to exert another layer of control on TGF-β signaling. When Taz/Yap are cytoplasmic, their interaction with Smads in the cytoplasm restrains TGF-β signaling. This results in a cell density-dependent influence on TGF-β signaling[24], as cell density influences Yap/Taz localization through the Hippo pathway. Hippo also exhibits multiple layers of interaction with Wnt signaling pathways beyond the transcriptional regulation described above. As in TGF-β pathways, crucial cytoplasmic interactions between pathway components have been identified. Yap or Taz can interact in the cytoplasm with the transcription factor of canonical Wnt pathways, β-catenin, and thereby retain it in the cytoplasm.[25] Taz has also been reported to inhibit Wnt signaling by inter-acting in the cytoplasm with Dvl, another key component of Wnt pathways.[26] Conversely, under conditions where the Hippo pathway is inactivated and Yap is able to translocate to the nucleus, Yap can interact with β-catenin, within the nucleus to promote the expression of Wnt target genes. In the developing heart, this led to elevated cardiomyocyte proliferation.[27] Yki and Wnt also synergize to co-regulate Vg in the Drosophila wing, but the mechanism by which they synergize here has not been determined.[5]

PHYSIOLOGICAL FUNCTION OF HIPPO SIGNALING IN ANIMAL DEVELOPMENT

Although the Hippo pathway was first discovered for its pivotal role in restricting imaginaldisc growth by promoting cell-cycle exit and apoptosis, more recent studies in *Drosophila* have expanded the function of this pathway into other developmental contexts, such as the mitotic-to-endocycle switch of the posterior follicle cells in adult egg chambers, neuroepithelial cell differentiation in larval optic lobe, photoreceptor R8 subtype specification in pupa retina and dendrite morphogenesis of larval sensory neurons. These studies emphasize two general physiological functions for Hippo signaling: coordinating a timely transition from cell proliferation to cellular quiescence and ensuring proper cellular differentiation. These two processes are intimately linked in many development contexts.

Studies of Hippo signaling in vertebrates have reinforced these general themes. For example, overexpression of YAP in murine intestine or chick neural tubes resulted in expansion of progenitor cells and concomitant loss of differentiated cells.[1] Likewise, loss of WW45 led to progenitor cell hyperplasia and defective terminal differentiation in skin, intestine, and lung epithelia. In cell culture models of myogenesis and keratinocyte differentiation, it was shown that Hippo signaling is activated at the onset of cell differentiation and that YAP hyper activation led to failure of cell-cycle exit and terminal differentiation. The loss of differentiated cell types upon YAP hyper activation may explain, at least in part, why ubiquitous inactivation of Hippo signaling resulted in increased organ size in only selected tissues. [28-30]. In some developmental contexts, however, the often coupled roles for Hippo signaling in promoting cellular quiescence and differentiation may be separated from each other. For example, Hippo signaling is required to maintain the terminally differentiated hepatocytes of mammalian livers in a quiescent state. Overexpression of YAP or loss of Mst1/2 leads to ectopic proliferation of the differentiated hepatocytes.[1] In this context, Hippo signaling regulates cell proliferation without an obvious effect on hepatocytes differentiation. There are also contexts in which the opposite is true. In blastocyst stage mouse embryos, differential Hippo signaling activity leads to cytoplasmic and nuclear YAP localization in the presumptive inner cell mass (ICM) (inside the blastocyst) and the presumptive trophectoderm lining the exterior of the blastocyst, respectively.[31] This position dependent Hippo signaling activity may derive in part from the difference in the degree of cell-cell contacts between the inside and the outside cells. In this context, cell-contact-mediated Hippo signaling regulates cell fate specification without inducing proliferation arrest. This dedicated role in cell differentiation is reminiscent of the requirement for Hippo

signaling in photoreceptor R8 subtype specification and dendrite morphogenesis in *Drosophila*, although the latter involve post mitotic rather than proliferating cells. Thus, Hippo signaling may regulate distinct cellular outcomes indifferent contexts.[1]

HIPPO SIGNALING IN HUMAN DISEASES

Consistent with the critical roles of Hippo signaling in mammalian physiology, mutations in Hippo pathway components have been linked to human diseases. Besides Mer/NF2 as the tumor suppressor underlying Neurofibromatosis 2, a heterozygous missense mutation inTEAD1, Y421H, was identified in two independent pedigrees as the cause of Sveinsson's chorioretinal atrophy (SCRA) (also known as helicoid peripapillary chorioretinal degeneration), a rare autosomal dominant disease characterized by progressive lesions radiating from the optic disc involving the retina and the choroid.[32] In the recently resolved three dimensional structure of the TEAD1-YAP or the TEAD4-YAPcomplex, Y421 was shown to be engaged in hydrogen bond and hydrophobic interaction with YAP. Accordingly, TEAD1/2/4 proteins carrying the disease-mimicking mutation disrupted TEAD1/2/4-YAP/TAZ binding [1]. It is unknown at present whether SCRA lesions result from loss of heterozygosity or haplo insufficiency of the dominant Y421G allele. Identifying the relevant target genes should shed light on the pathophysiological mechanism of SCRA. Other than NF2/Mer, DNA mutations in tumor suppressor components of the Hippo pathway are rare in human cancers. Deletion of WW45 was reported in two renal cancer cell lines[33], and mutations in Mob1 were found in two cDNAs derived from a human melanoma and a mouse mammary carcinoma, respectively.[34] In contrast, there is increasing evidence implicating epigenetic silencing as a prevalent mechanism of inactivating Hippo pathway tumor suppressor genes. Besides the frequent hypermethylation of RASSF family genes inhuman cancers[35], hypermethylation of Mst1/2 (in soft tissue sarcoma;[36] and Lats1/2 (in astrocytoma and breast cancers; has also been reported. More generally, decreased expression ofMst1/2 (in colorectal and prostate cancers; and Mob1 (in colorectal and lung cancers; may be functionally significant, irrespective of methylation status. miRNA-mediated silencing of Hippo pathway tumor suppressors in human cancers has also emerged, as exemplified by the suppression of Lats2 expression by miR372 and miR373, two related oncogenic miRNAs in testicular germ cell tumors.

The YAP/TAZ-TEAD transcription factor complex represents a common target of oncogenic transformation. Amplification of the *YAP* gene locus has been reported at varying in a wide spectrum of human and murine tumors, such as medulloblastomas, oral squamous-cell

carcinomas, and carcinomas of the lung, pancreas, esophagus, liver, and mammary gland Interestingly, in nearly all cases, the *YAP* amplicon also contains *cIAP2*, a mammalian homolog of *diap1*, suggesting a potential cooperation between YAP and cIAP2 in tumorigenesis.[1] Consistently, comprehensive survey of the most common solid cancer types revealed widespread and frequent YAP overexpression in lung, ovarian, pancreatic, colorectal, hepatocellular, and prostate carcinomas, and YAP was shown to be an independent prognostic marker for disease-free survival and overall survival of HCC patients.[37] In one study focusing on mammary tumors, TAZ overexpression was detected in 21% of primary breast cancers.[38] This further suggested that TAZ may govern the invasiveness of breast cancer cells. Consistent with these findings, overexpression of YAP or TAZ can induce anchorage in dependent growth and EMT of immortalized mammary and pancreatic epithelial cells invitro. Interestingly, the YAP/TAZ partner TEAD4 has also been reported to be amplified in various cancers and TEAD4 alone promoted anchorage-independent growth of MCF10A cells invitro. Whether other TEAD homologs are amplified or overexpressed inhuman cancers remains to be determined. It is worth noting that besides its role as a potent oncogene, YAP has also been implicated as a potential tumor suppressor by potentiating gp73-mediated apoptosis). Paradoxically, this proapoptotic activity of YAP was reported to be activated or inhibited by Lats1-mediated phosphorylation. Future studies are required to determine whether the seemingly opposing function of YAP as an oncoprotein versus a tumor suppressor is dictated by cell contexts.[1]

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. 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 co-activator. Upon confluence, when contact inhibition comes into play, YAP accumulates in the cytoplasm, thereby rendering it unable to function as a transcriptional co-activator. This cytoplasmic sequestration of YAP in response to cell density correlates with its increased phosphorylation. Mst2 and LATS2 act coordinately to phosphorylated YAP at HXRXXS motifs, with the S127-containingmotif 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.[39] 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 ofS127-phosphoryalted YAP results from its enhanced interactions with 14-3-3 proteins[40], which are known to sequester phosphorylated YAP in the cytosol.[41] 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 LATSMob1complex 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 phosphorylatingS168 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 byLATS1/2 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 body weight 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 co amplification 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.[42] 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 Bcl-xL 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 Bcl-xL in human colon cancers. This study thus implicates YAP as a critical link between stem/ progenitor cells and colon cancer cells. Collectively, these three studies suggest that overexpression of YAP or its over activation due to intrinsic Hippo pathway mutations is able to abrogate cell contact inhibition and organ size control to promote cancer development.[39]

THE HIPPO PATHWAY AND CANCER

Accumulating evidence indicates that the Hippo pathway also plays important roles in cancer development. Mer is a well established human tumor suppressor gene, the mutation of which causes lesions in the nervous system, eyes and skin. Mutation of Mob1and Sav1, two other Hippo pathway core components, has also been observed in a human renal cancer cell line and in skin melanoma, respectively.

Other Hippo pathway components have also been reported to show abnormal expression levels in cancer samples. For example, RASSF1A is one of the most commonly silenced genes in human cancers, owing to hypermethylation of its promoter and the expression of Mst and Lats is down regulated in several human cancers. By contrast, TAZ was found to be upregulated in breast cancers, especially in invasive ductal carcinomas. Furthermore, YAP gene amplification was found in various cancers, including hepatocellular carcinoma (HCC). Consistent with these findings, increased protein levels of YAP and nuclear accumulation was also observed in several cancers. Recently, a clinical study identified YAP as a predicting factor for HCC-specific disease free survival and overall survival through assessing its protein level and nuclear localization.[43]

THE ROLE OF THE HIPPO PATHWAY IN STEM CELLS

Recent studies demonstrated previously unknown functions of YAP and TAZ in the renewal and proliferation of ESCs and organ specific progenitor cells. For instance, YAP is inactivated during ESC differentiation and activated in induced pluripotent stem (iPS) cells.

In addition, knock down of YAP in murine D3 ESCs leads to loss of pluripotency. By contrast, ectopic expression of YAP prevents ESC differentiation, but the exact underlying mechanism is not clear. Intriguingly, knockdown of TAZ also leads to differentiation of human CA1 stem cells, but it is not clear how these functions of YAP and TAZ are coordinated to maintain 'stemness'. YAP also plays a role in tissue specific progenitor cells. For instance, in mouse intestines, expression of endogenous YAP is restricted to the progenitor cell compartment, which expands when YAP is overexpressed. Consistent with this observation, activation of YAP and TEAD also causes marked expansion of neural progenitor cells in a chicken neural tube model. In the past few years, it has been suggested that cancer could arise from a small population of cancer cells that have stem-cell-like properties, the so called cancer stem cells. A function of YAP and thus of the Hippo pathway in cancer stem cells is also emerging. In a subtype of medulloblastomas, YAP expression was found to be strikingly high in the perivascular cancer stem cell compartment, suggesting a role for YAP in maintaining cancer stem cells. In addition, double knockout of Mst1 and Mst2, knockout ofSav1 or knockout of Mer in mice liver also induces abundant accumulation of oval cells, the adult liver stem cells, which possibly are the cell type of origin for subsequent tumor formation. Further investigations are necessary to address how regulation of cell proliferation; apoptosis and stem cell self-renewal contributes to the functions of the Hippo pathway in organ size control and cancer development.[43]

HIPPO SIGNALING IN MAMMALS: GROWTH CONTROL OR CELL CYCLE EXIT?

Overexpression of Yap and targeted deletion of Mst1/2 in the mouse liver provide evidence that Hippo signaling has a pivotal role in regulating organ size, suggesting that Hippo signaling is a universal growth regulatory mechanism. However, other studies question whether Hippo signaling regulates growth in all mammalian tissues. Clearly, some tissues do not require Hippo signaling to regulate organ size. For example, the targeted deletion of Mst1/2 (Stk3/4) in mouse limb bud tissues has a relatively mild effect on the growth plate, but does not result in enlarged limbs. Furthermore, overexpression of activated Yap in the mouse small intestine leads to Notch dependent hyperplasia and loss of terminally differentiated cell types, but does not appreciably increase the overall size of the organ. Likewise, targeted deletion of the adaptor protein Sav1 (Salvador homolog 1) in various mouse epithelial tissues leads to hyperplasia and the loss of terminal differentiated phenotypes, including in the small intestine and skin, but does not result in markedly

enhanced organ size. These studies suggest that an important function of Hippo signaling in mammals might be to modulate cell cycle exit and terminal differentiation, as has been suggested in Drosophila neural development. How this is achieved at a mechanistic level is poorly understood. Additional studies in which the conditional deletion of core Hippo signaling components is carried out in a range of tissues will be required to resolve this issue.[44]

The Hippo pathway and organ size Control In Drosophila, mutation of the Hippo pathway results in increased organ size; the ectopic expression of its components leads to an opposite phenotype of reduced organ size, similar to what has been observed for Ykimutations These alterations of organ size are attributed to a combinatory effect of changes in both cell proliferation and apoptosis. A function of the Hippo pathway in controlling organ size was also confirmed in mammals. For example, liver-specific expression of YAP in transgenic mice leads to an up to four fold increase in liver size and to the development of liver tumors at a later stage. Similar phenotypes have been observed in liver specific knockouts of Mst1 and Mst2, Sav1 or Mer. Taken together; these genetic studies present convincing evidence that the Hippo pathway is indeed a key regulator of organ size.[39]

CONCLUSIONS AND FUTURE PERSPECTIVES

Research in the past several years has greatly advanced our understanding of the molecular mechanism and the physiological function of the Hippo signaling pathway. These studies have firmly established the Hippo signaling pathway as a central mechanism that regulates organ size and tissue homeostasis in species spanning from Drosophila to mammals. The fundamental importance of this pathway is further solidified by the realization that dysregulation of Hippo signaling underlies various human diseases including cancer. Despite recent progress, our knowledge about this important growth regulatory pathway remains incomplete. First and foremost, while a conserved Hippo kinase cascade has been established, potentially important variations on this cascade await characterization in mammals, and many of the upstream inputs remain to be defined. A major challenge in the future is to elucidate the molecular nature of these upstream signals, the physiological contexts of their action, and the molecular mechanism by which they regulate the Kibra-Ex-Mer complex and/or the core kinase cassette. Conversely, the outputs of the pathway remain incompletely defined, especially in intact mammalian tissues. Another challenge is to understand how the growth-regulatory Hippo pathway is integrated with other developmental pathways involved in

pattern formation, cell growth, survival, and differentiation to coordinately define the characteristic size, shape, and cellular composition of a given organ during animal development. Likewise, investigation of how dysregulation of Hippo and other pathways cooperate to drive tumorigenesis and other forms of human disease is likely to be an active and exciting topic. Finally, small molecule modulators of Hippo signaling may be exploited for tissue engineering and regenerative medicine, as well as therapeutic intervention of relevant human cancers.

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