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Hippo signaling pathway and organ size control

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Abstract

Initially discovered in *Drosophila*, the Hippo (Hpo) pathway has been recognized as a conserved signaling pathway that controls organ size during development by restricting cell growth and proliferation and by promoting apoptosis. In addition, abnormal activities of several Hpo pathway components have been implicated in human cancer. Here, we review the current understanding of the molecular and cellular basis of Hpo signaling in development and tumorigenesis, and discuss how the Hpo pathway integrates spatial and temporal signals to control tissue growth and organ size.

Different organs exhibit characteristic size, which is determined by the number and size of their constituent cells.[1] How the organ size is controlled during animal development has been a fascinating problem in modern biology. The control of organ size depends on a delicate balance of cell proliferation and cell death, which are properly coordinated in response to both global and local stimuli. Although tissue growth is influenced by environmental factors such as hormonal signals and nutrients, organ-intrinsic mechanisms also play important roles. By genetic screen and characterization of mutants that cause tissue overgrowth in *Drosophila*, several signaling pathways, including the Hpo pathway, have been unraveled as organ intrinsic mechanisms that control organ size.[2]

Finding Hippo---an emerging size control pathway

The imaginal discs of *Drosophila*, which give rise to adult structures such as wings, legs, and eyes, provide an attractive system to study size control.[3] Imaginal discs are specified during embryonic development but growth occurs at larval stages during which the number of cells of each disc increases exponentially. For example, a wing disc has less than 50 cells at the beginning of first instar; however, it contains over 50,000 cells at the end of third instar. Imaginal discs appear to possess intrinsic mechanisms to determine their final size and defects in these mechanisms result in overgrowth in a disc autonomous fashion.[4, 5] In the past, tumor suppressor mutants were identified by genetic screens for mutations that either result in enlarged imaginal discs in homozygous late third instar larvae or cause overgrowth of imaginal disc derivatives in mosaic flies that carry clones of homozygous tissues in

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otherwise heterozygous background (Fig. 1).[2, 6] In particular, genetic mosaic screens have led to the identification of a number of tumor suppressor genes, including *warts/large tumor suppressor* (*wts/lats*),[7, 8] *salvador* (*sav*),[9] *hpo/dMST*,[10–14] that fall into an emerging tumor suppressor pathway, the so-called Hpo pathway (Fig. 2).

Central to the Hpo pathway is a kinase cascade consisting of four proteins including Hpo, Sav, Wts, and Mats (Fig.2). Hpo is the *Drosophila* homolog of mammalian Ste20 family kinases MST1 and MST2, and forms a complex with the WW-repeat scaffolding protein Sav to phosphorylate and activate the downstream kinase Wts, a member of the Nuclear Dbf-2-related (NDR) kinase family.[7–14] Wts acts in association with a small regulatory protein called Mats (Mobs as tumor suppressor) to restrict cell growth and proliferation and promote cell death.[15] Like Wts, Mats is phosphorylated by Hpo, which increases its association with Wts and its ability to upregulate Wts kinase activity.[16, 17]

Yki/Sd transcriptional complex mediates Hpo signaling

Hpo signaling pathway restricts cell growth and proliferation and promotes apoptosis mainly through transcriptional regulation of genes involved in these processes. Several transcriptional targets of the Hpo pathway have been identified, including *cycE*, *diap1*, and *bantam*, as well as two upstream Hpo pathway components: *merlin* (*mer*) and *expanded* (*ex*).[9, 10, 13–15, 18–20] Hpo signaling influences gene expression by regulating Yorkie (Yki), the *Drosophila* homolog of mammalian transcriptional coactivator YAP, which binds to and is phosphorylated by Wts.[21] Overexpression of Yki phenocopies loss of Hpo signaling activity, suggesting that Hpo signaling restricts cell growth and promotes cell death by inhibiting Yki-mediated gene expression.[21] Indeed, Yki regulates all the known target genes of the Hpo pathway.[19, 21]

What is the DNA-binding transcription factor that associates with Yki to regulate Hpo pathway target genes? Three recent studies provided an answer by showing that the TEAD/TEF family transcription factor Scalloped (Sd) acts in a complex with Yki to promote the expression of Hpo pathway responsive genes.[22–24] Overexpression of Sd enhances Hop target gene expression and tissue overgrowth caused by excessive Yki or tumor suppressor mutations in the Hpo pathway. Conversely, inactivation of Sd suppresses these effects. Moreover, a constitutively active form of Sd can promote tissue overgrowth as well as Hpo target gene expression.[23] Characterization of *diap1* enhancer elements suggests that Sd directly binds *diap1* regulatory elements.[22, 23] Sd promotes Yki nuclear localization[23, 24] and recruits Yki to the *diap1* promoter.[23] On the other hand, phosphorylation of Yki at S_{168} by Wts restricts Yki nuclear localization.[23, 25, 26] Thus, the Yki/Sd complex serves as a Hpo pathway transcriptional effector that is negatively regulated by Hpo signaling via phosphorylation and cytoplasmic retention of Yki (Fig.2).

Of note, loss of Sd has less severe phenotypes than loss of Yki.[22, 23] For example, loss of Sd does not affect basal levels of *diap1* expression but loss of Yki does. One possibility is that Yki can hook up with another transcription factor to regulate the basal expression of *diap1*. Alternatively, Sd may function as a default transcriptional repressor in the absence of Yki, as

are the cases for the transcription factors of many signaling pathways.[27] A prediction of the latter model is that removal of *sd* in *yki* mutant cells should restore *diap1* expression.

Exploring upstream regulators of the Hpo pathway

While the regulatory events downstream of the Hpo kinase cascade have been relatively well-defined, the upstream regulatory mechanisms remain much less understood. Several studies suggested that the protocadherin Fat may function as a receptor for the Hpo pathway.[28–31] *fat* was originally identified as a tumor suppressor gene whose mutations caused tumorous overgrowth of imaginal discs.[32] *fat* mutant clones deregulated many Hpo target genes including *cycE*, *diap1*, and *wg*, and genetic epistasis study suggested that *fat* acts upstream of *hpo*, *wts*, and *yki*.[28–31] Consistent with Fat acting upstream of Hpo signaling, overexpression of a truncated form of Fat (FatΔECD), which lacks the extra cellular domain and can suppress cell growth *in vivo*, induced Wts phosphorylation in cultured cells.[31, 33] In addition, Wts levels diminished in *fat* mutant discs,[29] and overexpression of Wts can rescue *fat* mutants to viability.[34] Taken together, these observations suggest that Fat acts as a Hpo pathway receptor that regulates both Wts phosphorylation and turnover.

How Fat is linked to the Hpo kinase cascade is not clear but several proteins have been implicated as components acting downstream of Fat and upstream of Hpo. Two FERM domain containing proteins, Ex and Mer, were identified as partially redundant activators of Hpo.[35] The mammalian orthologue of Mer is the product of tumor suppressor gene *Neurofibromatosis type-2* (*NF2*), whose loss of function leads to the development of tumors in the central nervous system.[36] In *Drosophila*, *mer ex* double mutant cells upregulate Hpo pathway target genes and deregulate both proliferation and apoptosis in a manner similar to *hpo* mutant cells, whereas *mer* or *ex* single mutant cells exhibit less severe phenotypes.[35] Genetic and biochemical studies place Mer and Ex upstream of Hpo--overexpression of Hpo suppresses tissue overgrowth in *mer ex* double mutants and overexpression of Mer and Ex in S2 cells induces Warts phosphorylation and downregulates Yki activity. Interestingly, Mer and Ex are both transcriptional targets of the Hpo pathway and act in a negative feedback loop to regulate Hpo pathway activity.[35]

Ex and Fat colocalize at the adherens junctions and loss of Fat leads to reduced membrane localization of Ex, suggesting that Fat may regulate Ex activity by controlling its subcellular localization.[30, 31] In the eye, the phenotype associated with overexpression of Ex is dominant over the phenotype caused by loss of Fat, consistent with Ex acting downstream of Fat.[30, 31] In the wing, however, overexpression of Ex is not sufficient to suppress *fat* mutant phenotype even though high levels of Ex accumulate at normal subapical position.[34] It is possible that Fat may not only regulate the subcellular localization but also control the activation of Ex so that Ex cannot function in the absence of Fat even when it localizes properly. Alternatively, or in addition to the mechanism stated above, Fat may act through a different pathway to regulate downstream signaling events. In support for the latter possibility is the observation that *fat* and *ex* mutations have additive effects on imaginal disc growth and development.[34] Indeed, a previous study suggested that Fat acts through an unconventional myosin encoded by *dachs*.[37] *dachs* mutations suppress tissue overgrowth as well as altered gene expression caused by fat mutations. Consistent with *dachs* acting

downstream of *fat*, *dachs* protein levels at the membrane are negatively regulated by Fat. In addition, the normal subcellular localization and activity of Dachs require Approximated (App), a member of DHHC family of palmitoyltransferase.[38] Dachs physically associates with Warts in cultured cells.[29] As Fat signaling acts at least in part by stabilizing Wts,[29] it would be interesting to determine whether Dachs mediates this aspect of Fat output.

Another classic tumor suppressor gene is *discs overgrown* (*dco*), which encodes a casein kinase 1 (CK1) family member, CK1ε.[39] *dco* mutant cells exhibit deregulated expression of Fat/Hpo target genes, and epistasis analysis places *dco* between *fat* and *dachs*.[29] However, the relevant Dco/CK1 substrate(s) in the Fat/Hpo pathway remains to be determined.

How does the Hpo pathway read the spatial and temporal signals?

The protocadherin Dachsous (Ds) functions as a ligand for Fat in the planer cell polarity (PCP) pathway.[40] Several lines of evidence suggest that Ds functions as a candidate ligand for Fat in the Hpo pathway. *ds* mutations result in tissue overgrowth, albeit less severe than that caused by *fat* mutations.[32, 41, 42] Ds and Fat participate in heterophilic cell adhesion in cultured cells, and stabilize each other at the cell surface in imaginal discs.[33, 42] The expression of Fat and Hpo target genes is influenced by Ds in a nonautonomous manner.[29, 43] Ds and another protein, Four-jointed (Fj), a Golgi protein that phosphorylates Fat and Ds to influence their interaction,[44] are distributed in a graded fashion in developing imaginal discs.[41, 45] Interestingly, juxtaposition of cells expressing different levels of Ds or Fj stimulates the expression of Hpo target genes and cell proliferation in a manner depending on Fat signaling,[46, 47] suggesting that Fat signaling activity is modulated by discontinuities of Ds/Fj. The model implies that the steepness of Ds/Fj gradient drives disc growth by modulating the Fat/Hpo signaling activity. For example, at early stages during larval development when the discs are small, the Ds/Fj gradient is steep and disc growth is promoted. At later stages, the Ds/Fj gradient is flattened due to increased disc size; as a consequence, tissue growth is retarded. However, there is no direct evidence that Hpo pathway activity is modulated in space or over time in a manner correlating with cell proliferation during normal development. Thus, it remains possible that Hpo pathway activity could be maintained at a constant level throughout larval development.

Dpp signaling regulates the expression of both Ds and Fj.[46] In addition, juxtaposition of cells transducing different levels of Dpp signaling also stimulates cell proliferation and Hpo target gene expression through Dachs.[46, 48] These and other observations led to the proposal that the Fat/Hpo pathway may couple cell growth and organ size control to morphogen gradients such as Dpp gradient.[46] However, a recent study provided evidence that normal growth can occur even in the absence of graded Dpp signaling.[49] In addition, measurement of Dpp gradient or its activity gradient (through p-Mad staining) during disc growth did not detect any change in the steepness of these gradients at different larval stages.[50] Thus, it is unclear whether the steepness of Dpp gradient is a driving force for tissue growth during normal development. It is possible that other morphogen gradients such Wg morphogen may promote disc growth in the absence of graded Dpp signaling. It is also possible that cell proliferation stimulated by Dpp signaling discontinuity may reflect a growth control

mechanism utilized during wound healing and regeneration when cells exposed to different levels of Dpp are juxtaposed after injury.

Hpo signaling in mammals

The core components of the *Drosophila* Hpo pathway are highly conserved in mammals (see Table1).[51–53] In fact, several components of the *Drosophila* Hpo pathway can be functionally replaced by their mammalian homologs.[11, 15, 21, 22, 54, 55] Accumulating evidence has suggested that the mammalian Hpo pathway regulates cell contact inhibition, organ size, and cancer development.[52, 55–60] Mice lacking Lats1, a vertebrate orthologue of *Drosophila* Wts, develop soft-tissue sarcomas, ovarian tumours, and pituitary dysfunction.[61] Embryos deficient for Lats2, another mammalian orthologue of Wts, showed overgrowth in several mesodermal tissues, and fibroblasts derived from these embryos (MEFs) have growth advantages, exhibit a defect in contact inhibition and cytokinesis, and display centrosome amplification and genome instability.[62] Complete knock-out of Lats2 resulted in an acceleration of exit from mitosis and mitotic defects including centrosome fragmentation and cytokinesis defects, followed by nuclear enlargement and multinucleation.[63] Human Mst2, an orthologue of Hpo, phosphorylates and activates both Lats1 and Lats2.[64] The human homolog of Sav, hWW45, is mutated in several cancer cell lines.[9] Mice lacking WW45 revealed a crucial role for WW45 in cell-cycle exit and epithelial terminal differentiation, and WW45 is required for Mst1 activation for proper epithelial tissue development in mammals.[65]

Yap, a human orthologue of *yki*, is a candidate oncogene amplified in several types of tumor.[57, 66] Recent studies revealed that Yap is the primary effector of the mammalian Hippo pathway.[25, 58, 59, 67] Yap overexpression in cultured cells is able to overcome cell contact inhibition and Yap inactivation can restore contact inhibition in a human cancer cell line bearing deletion of hWW45/Sav.[58] Yap nuclear localization is inhibited by Lats phosphorylation as well as by cell-cell contact.[25, 58] Yap overexpression in mouse liver caused excessive tissue growth and reversibly increased liver size,[25, 59] and long-term overexpression of Yap led to hepatocellular carcinoma (HCC).[25] Furthermore, Yap protein levels and/or nuclear localization are elevated in many human cancers including liver cancer, prostate cancer, lung cancer, colon cancer, ovarian cancer, and breast cancer.[25, 58, 60] Several recent studies provided evidence that TEAD family of transcription factors mediate the function of Yap to regulate cell proliferation and contact inhibition in mammals.[68–70]

Much less is known about the upstream signals regulating the mammalian Hpo pathway, except that Merlin has been extensively studied for its tumor suppressor function in mammalian nervous tissues.[36, 71–73] A recent study showed that loss of Merlin in meningioma cells resulted in loss of contact-dependent growth inhibition, enhanced anchorage independent cell growth, and increased cell proliferation due to accelerated S-phase entry. In addition, loss of Merlin in meningioma cell lines or primary tumors resulted in increased protein level and nuclear localization of Yap.[74] Fat4 is essential for vertebrate PCP, and loss of Fat4 disrupts oriented cell divisions and tubule elongation during kidney development, leading to cystic kidney disease.[75] Furthermore, Fat4 is a candidate tumor

suppressor whose expression is lost in a fraction of human breast tumor cell lines and primary tumors.[76]

Future perspective

The Hpo pathway has emerged as an evolutionarily conserved signaling pathway that regulates cell growth, proliferation, and cell death during normal development and its malfunction has been linked to several types of human cancer. The past several years have witnessed an explosion of information regarding various aspects of Hpo signaling cascade. However, many important questions regarding the signaling mechanism as well as the physiological and pathological roles of the Hpo pathway remain. In mammals, the upstream signal(s) that regulates Hpo signaling activity remains obscure. One intriguing observation is that cell-contact inhibition can modulate Yki activity but the molecular pathway that links the detection of cell density to Yki regulation has not been defined. It also remains to be determined whether mammalian Fat homologs participate in this process. Whether the Hpo pathway activity is modulated in space and over the time course of normal development remains a critical issue. More direct measurement of pathway activity, e.g., by measuring the phosphorylation states of Yki, Wts, or Hpo should be informative but could be challenging, not only because these reagents are difficult to develop but also because the change in Hpo pathway activity over time might be subtle. Hpo pathway reporter genes might also be useful to monitor spatial and temporal changes in Hpo pathway activity.

The Hpo signaling pathway is unlikely to be linear. There is evidence that another yet to be identified receptor may act in parallel with Fat.[35] Similarly, the Hpo pathway may branch out at other levels. For example, Hpo may directly regulate the turnover of Diap1 in addition to controlling its expression.[12] The biological effect of Hpo signaling is likely to be context dependent, and there is evidence that Yap can promote cell death by binding to a p53 family member, p73.[77, 78] In *Drosophila*, Hpo pathway components also play roles in other developmental processes including retina cell patterning,[79] dendrite morphogenesis,[80, 81] regulation of oocyte polarity,[82–84] and salivary gland degeneration.[85] Furthermore, Hpo signaling regulates salivary gland cell death in a PI3K-dependent, but Yki-independent, manner.[85] The specification of posterior follicle cell fate identify during oogenesis, another process that involves Hpo signaling, does not require the action of Fat.[81] These observations indicate that there can be deviations from the canonical pathway depending on developmental contexts. With respect to situations in which Hpo pathway goes awry, Yap is upregulated in many cancers but the underlying mechanisms and functional significance remain largely undetermined. It is possible that other growth control pathways can feed into the Hpo pathway at different levels. Uncovering pathway crosstalk should provide better insight into the signaling network underlying the control of tissue growth and organ size during normal development and how cancer cells hijack the signaling network to favor their survival and proliferation.

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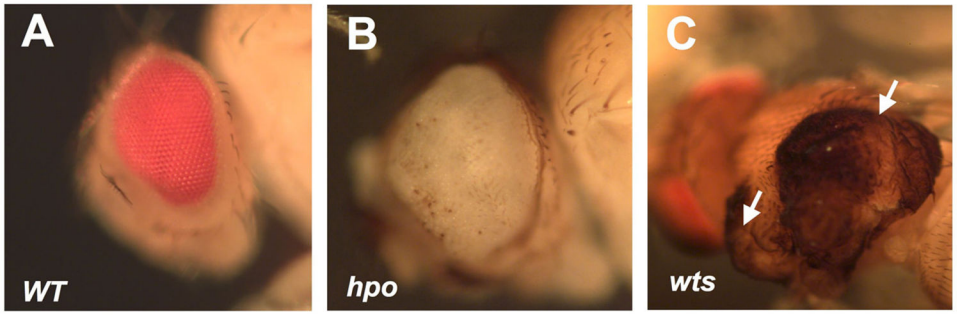

Figure 1. *hop* or *wts* mutant clones lead to tumor-like growth in mosaic flies

Wild type eye (A) or enlarged eye carrying *hpo* mutant clones (B*). wts* mutant clones (arrows) located on the notum resulted in tumor-like growth (C). Adapted from Jia et al.[10]

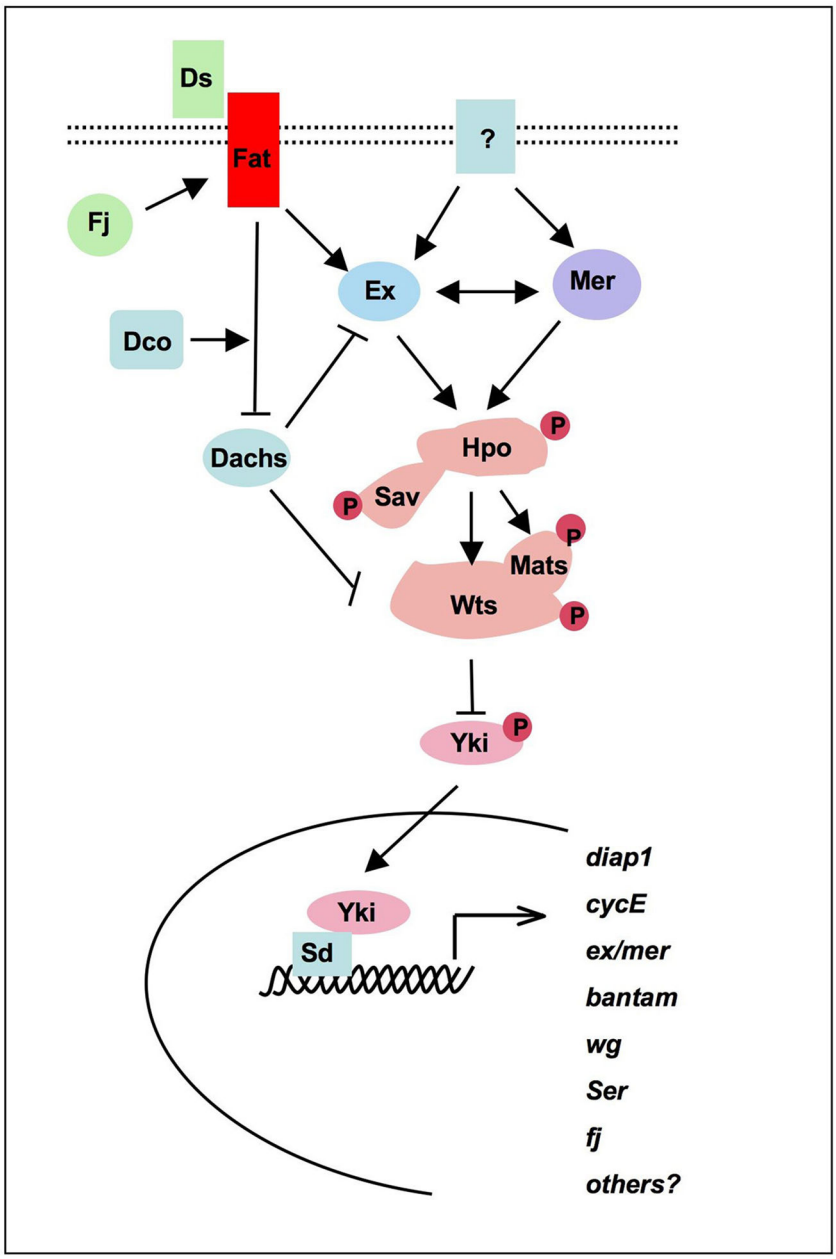

Figure 2. The *Drosophila* Hpo pathway

The Hpo kinase cascade consists of four core proteins: Hpo, Sav, Wts, and Mats. Sav binds and regulates Hpo. Hpo phosphorylates and activates Wts. Hpo also phosphorylates Mats to enhance its ability to activate Wts. Wts phosphorylates Yki and restricts its nuclear localization. Yki forms a complex with Sd to activate Hpo target genes. Ex and Mer act in a partially redundant manner to regulate the Hpo kinase cascade. Fat is a candidate receptor and may regulate the Hpo pathway through Dachs and Ex. Ds is a candidate ligand for Fat. Fj modulates Ds/Fat interaction through phosphorylating Ds and Fat in the Golgi.

Table 1

Conserved Hpo pathway components between *Drosophila* and mammals

Drosophila	**Mouse**	**Human**	**Protein type**
Dachsous (Ds)	Dchs1, Dchs2	DCHS1, DCHS2	Protocadherin
Fat	Fat1-Fat3, Fat4/Fat-j	Fat1-Fat3,Fat4/Fat-j	Protocadherin
Four-jointed (Fj)	Fjx1	Fjx1	Golgi associated kinase
Discs overgrown (Dco)	CK1ε/δ	CK1ε/δ	Ser/Thr kinase
Expanded (Ex)	Ex1/Frmd6,Ex2	Willin	FERM-domain
Merlin (Mer)	Merlin	NF2 (Merlin)	FERM-domain
Hippo(Hpo /dMst)	Mst1, Mst2	Mst1/STK4 Mst2/STK3	Ser/Thr kinase
Salvador (Sav)	WW45/Sav1	hWW45/SAV1	WW domain
Warts (Wts)	Lats1, Lats2	LATS1 and LATS2	Ser/Thr kinase
Mob as tumor suppressor (Mats)	Mob1, Mob2	MOBKL1A, MOBKL1B	NDR kinase family cofactor
Yorkie (Yki)	Yap TAZ	YAP TAZ/WWTR1	WW domain, transcriptional co-activator
Scalloped (Sd)	Tead/Tef1-Tef4	Tead1-Tead4	TEA DNA binding domain