REVIEW

Hippo signaling in organ size control

Duojia Pan[1]

Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

The control of organ (or organism) size is a fundamental aspect of life that has long captured human imagination. What makes an elephant grow a million times larger than a mouse? How do our two hands develop independently of each other yet reach very similar size? How does a liver precisely regenerate its original mass when two-thirds of it is removed? The recent discovery of a novel signaling network in *Drosophila*, known as the Hippo (Hpo) pathway, might provide an important entry point to these fascinating questions. The Hpo pathway consists of several negative growth regulators acting in a kinase cascade that ultimately phosphorylates and inactivates Yorkie (Yki), a transcriptional coactivator that positively regulates cell growth, survival, and proliferation. Components of the Hpo pathway are highly conserved throughout evolution, suggesting that this pathway may function as a global regulator of tissue homeostasis in all metazoan animals. Here, I provide a historical review of this potent growth-regulatory pathway and highlight outstanding questions that will likely be the focus of future investigation.

The development of a functional organ requires not only patterning mechanisms that confer proper identities for its constituent cells, but also growth-regulatory mechanisms that specify the final size of the organ. Developmental genetics in the past two decades have revealed at least seven core signaling pathways that mediate the majority of cell fate decisions in metazoans, namely, those mediated by Notch, Wnt, TGF-β, Hedgehog, receptor tyrosine kinase, nuclear receptor, and Jak/STAT (for review, see Barolo and Posakony 2002). In contrast, it is only in recent years that developmental biologists have started to dissect signaling pathways dedicated to size control. Most notable among these newly appreciated signaling cascades are the tuberous sclerosis tumor suppressor complex (TSC)–target of the rapamycin (TOR) pathway, which controls organ size by regulating cell growth (for review, see Pan et al. 2004), and the Hippo (Hpo) pathway, which controls organ size by coordinately regulating cell growth, proliferation, and apoptosis—the subject of this review.

In retrospect, the disparity in our understanding of growth and patterning might simply reflect the different methodologies used in dissecting these processes. A large body of our current understanding of pattern formation was built on classic genetic screens for homozygous mutant animals with embryonic patterning defects, using model systems like *Drosophila* (Nusslein-Volhard and Wieschaus 1980). Such screens are not suitable for studying size control since embryonic development in *Drosophila* is characterized by rapid cell division without cell growth or uptake of nutrients from the environment. Essentially, the size of a fruit fly embryo is already preset by the maternal contribution from its mother. In this respect, the imaginal discs of *Drosophila* provide a much more suitable system to study size control. These sac-like structures in the larval body are first allocated during late embryogenesis, proliferate exponentially during the larval stages, and increase their mass by ~1000-fold before differentiating into respective adult organs upon metamorphosis (for review, see Cohen 1993). Despite these attractive features, classic genetic screens based on homozygous mutant animals cannot be readily applied to imaginal discs, since mutations of many essential genes lead to earlier lethality and prevent the analysis of their role in imaginal disc development. Two important technical advances that revolutionized the studies of *Drosophila* imaginal discs were the introduction of the yeast FRT/FLP recombination system (Golic and Lindquist 1989) and the development of FRT/FLP-based techniques for generating mosaics, which allow one to analyze homozygous mutant clones in an otherwise heterozygous background (Xu and Rubin 1993). Such techniques not only enable sophisticated analysis at single-cell levels, but more importantly, allow systematic screens for genes that impact various aspects of imaginal disc development, including the control of imaginal disc size (Fig. 1).

Elucidation of a kinase cascade from Hpo to Wts

One of the very first genes isolated using the mosaic-based screens was the tumor suppressor *warts* (*wts*; also called *lats*), which encodes a kinase of the Nuclear Dbf-

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[1]**Correspondence.**
E-MAIL djpan@jhmi.edu; FAX (410) 502-3178.
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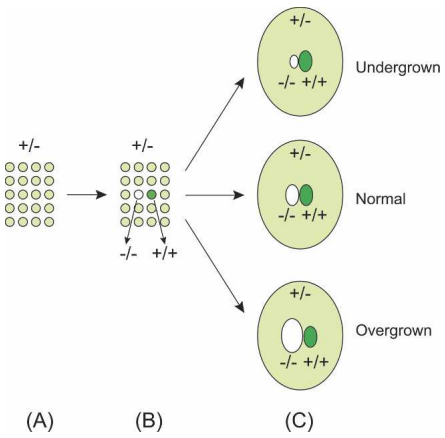

Figure 1. Genetic screens for growth-regulatory genes using mosaic flies. Starting from a heterozygous genetic background for any mutation (designated "+/−" in *A*), one can use the FRT/FLP technique to generate a single progenitor cell that is homozygous for the given mutation (designated "−/−" in *B*), as well a sibling cell that is homozygous for the control chromosome (designated "+/+" in *B*). Often the control chromosome carries a ubiquitous marker such that one can distinguish the −/−, +/+, and +/− cells based on the expression levels of the marker. (*C*) These progenitor cells proliferate to generate clones of cells, which can be scored in adult flies. Mutations of positive or negative growth regulators are expected to produce homozygous mutant clones that are smaller or larger than control clones, respectively. Most genetic screens so far have been focused on negative growth regulators since overgrowth is a much more specific phenotype.

2-related (NDR) family (Justice et al. 1995; Xu et al. 1995). Loss of *wts* leads to robust cell-autonomous overgrowth in a variety of epithelial structures such as the wings, the legs, and the eyes. Despite the dramatic effect on tissue growth, loss of *wts* does not appear to affect cell fate determination, thus distinguishing *wts* from genes that impact tissue growth indirectly via their effect on patterning. Interestingly, while *wts* mutant clones maintain a monolayer organization typical of imaginal discs, they do show a distinct cell shape abnormality wherein the apical surface of the *wts* mutant cells protrudes from the cell body, giving a dome-like appearance (Justice et al. 1995). The molecular basis for such "apical hypertrophy" remains elusive to date.

For several years since its discovery, Wts had largely remained an "orphan" tumor suppressor protein, without any upstream regulators, binding partners, or downstream effectors. This quiescent state was changed in 2002 by the identification of the *salvador* (*sav*; also called *shar-pei*) tumor suppressor gene. *sav* encodes a WW domain-containing protein, and its mutations result in a similar (albeit weaker) cell-autonomous overgrowth to that observed in *wts* mutant clones (Kango-Singh et al. 2002; Tapon et al. 2002). The study by Tapon et al. (2002) is particularly significant since it revealed that loss of *wts* or *sav* leads to increased cell proliferation as well as diminished apoptosis, thus providing the first evidence that these proteins coordinately regulate both cellular processes. They further observed that loss of *wts* or *sav* is associated with increased levels of the cell cycle regulator Cyclin E (CycE) and the cell death inhibitor Diap1 (Tapon et al. 2002). While this study implicated CycE and Diap1 as potential targets of Wts and Sav, the molecular nature of this regulation was unclear.

The next major advance in the genesis of the Hpo pathway was the identification of the Hpo tumor suppressor gene (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003), which encodes a Ste-20 family protein kinase with a similar loss-of-function overgrowth phenotype to that reported for *sav* or *wts* (Fig. 2). The study by Wu et al. (2003), in particular, represents the first elucidation of the Hpo kinase cascade (Fig. 3A). Using in vitro as well as cell culture-based assays, Wu et al. (2003) established a novel kinase cascade wherein Hpo phosphorylates and activates Wts, and that Sav potentiates this phosphorylation reaction. They went on to show that the Hpo kinase cascade controls Diap1 at the transcriptional level, a finding that contradicted that by Tapon et al. (2002), Harvey et al. (2003), and Pantalacci et al. (2003), who postulated a post-transcriptional regulation of Diap1 by phosphorylation. As discussed below, the large body of evidence so far favors the transcriptional control model proposed by Wu et al. (2003).

Besides Wts, the NDR family includes proteins that control cell cycle and cell morphogenesis in diverse species from yeast to man (for review, see Tamaskovic et al. 2003). Many NDR family kinases are known to function in a complex with a small regulatory protein of the Mob1 family (Mah et al. 2001; Tamaskovic et al. 2003), raising the possibility that the Wts tumor suppressor protein might also partner with a Mob1-like molecule. This possibility was nicely demonstrated by Lai et al. (2005), who identified a Mob1-related protein in *Drosophila* called Mats that not only binds to Wts but also potentiates its intrinsic kinase activity. Loss of *mats* leads to a similar overgrowth phenotype to that caused by loss of *hpo*, *sav*, or *wts*, further implicating Mats as a bona fide component of the Hpo pathway (Lai et al. 2005).

Taken together, the studies to this point illustrated a kinase cascade leading from the Ste20-like kinase Hpo to the NDR kinase Wts (Fig. 3A). Interestingly, each kinase is associated with, and stimulated by, a dedicated regulatory protein (Sav for Hpo and Mats for Wts), in much the same way that cyclins associate with and activate cyclin-dependent kinases. In addition, this kinase cascade appears to negatively regulate the transcription of targets such as *cycE* and *diap1*, a model that was largely borne out with the identification of Yorkie.

Identification of Yorkie as the nuclear effector of the Hpo signaling pathway

If the Hpo kinase cascade regulates the transcription of genes such as *diap1*, there should exist transcriptional regulator(s) that control *diap1* transcription and whose activity, in turn, is regulated by the kinase activity of the Wts protein. A search for this missing link between the

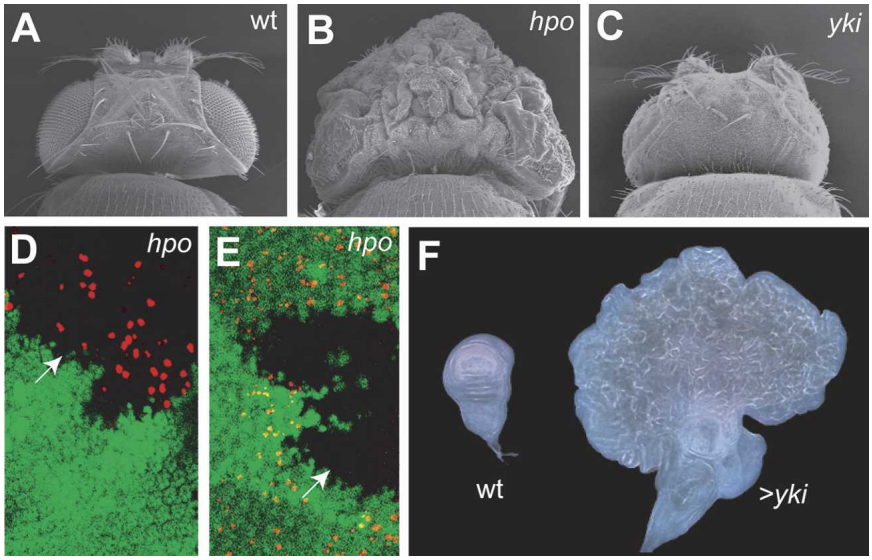

Figure 2. The Hpo signaling pathway controls organ size in *Drosophila*. Images reprinted from Wu et al. (2003), with permission from Elsevier, and Huang et al. (2005), with permission from Elsevier. *A–C* show images of wild type (*A*) and flies in which *hpo* (*B*) or *yki* (*C*) function is specifically inactivated in the head. While inactivation of *hpo* leads to massive overgrowth of the eye and head cuticles, inactivation of *yki* leads to the opposite phenotype. *D* and *E* show increased cell proliferation (*D*) and decreased cell death (*E*) in *hpo* mutant clones in the pupal eye. (*D*) While wild-type cells (green) had ceased cell proliferation (red), *hpo* mutant clones (black) continued to divide. (*E*) Conversely, normally occurring cell death can be detected in wild-type cells (green) but not in *hpo* mutant cells (black). *F* shows a wild-type wing imaginal disc (*left*) and a wing disc that overexpressed the *yki* gene (*right*). Yki overexpression leads to a dramatic increase in wing size (up to eight times the area of the wild-type wings).

Hpo kinase cascade and target gene transcription was accomplished by Huang et al. (2005), who identified the transcriptional coactivator Yorkie (Yki) as a critical substrate and downstream effector of Wts (Fig. 3A). Unlike the tumor suppressors of the Hpo pathway, which were isolated in phenotypic screens using genetic mosaics, Yki was identified in a yeast two-hybrid screen for Wts-binding proteins (Huang et al. 2005). Biochemical and genetic characterization of Yki demonstrates that Yki fulfills all the criteria expected of a Wts effector in growth regulation. Yki is phosphorylated and inactivated by Wts. Overexpression of Yki recapitulates loss-of-function *wts* phenotypes, such as increased *diap1* transcription and tissue overgrowth (Fig. 2). Conversely, loss of *yki* leads to tissue atrophy (Fig. 2) and diminished *diap1* transcription, and genetic epistasis analysis placed *yki* downstream from *hpo*, *sav*, or *wts*. Moreover, like the tumor suppressors of the Hpo pathway, loss or gain of function of *yki* does not affect cell fate determination despite its effect on imaginal disc growth. Huang et al. (2005) went on to show that YAP (yes-associated protein), the mammalian homolog of Yki, possesses similar activity to its *Drosophila* counterpart, suggesting that YAP likely functions as an oncogene instead of an apoptosis-promoting protein as previously reported (Basu et al. 2003). Taken together, these results identified Yki as a critical nuclear effector of the Hpo pathway (Fig. 3A; Huang et al. 2005).

The isolation of Yki is an important development in elucidating the Hpo signaling pathway. By linking the Hpo kinase cascade with a transcriptional regulator, it provides the most critical evidence supporting the original model by Wu et al. (2003), which posits that the Hpo kinase cascade regulates target genes through a transcriptional, instead of a post-transcriptional, mechanism. The fact that overexpression of Yki can recapitulate the *wts* loss-of-function overgrowth phenotype further demonstrates that Yki represents the most critical, if not the only, output of the Hpo kinase cascade in growth regulation. As a direct effector of the Hpo kinase cascade, Yki phosphorylation or Yki transcriptional activity provides a convenient assay to monitor Hpo signaling activity. Such assays are especially useful when testing novel upstream regulators of the Hpo pathway. Lastly, as the first physiological substrate identified for any NDR family kinase, molecular studies of Yki phosphorylation by Wts could provide general insights into substrate specificity for this family of protein kinases, which remains poorly understood at present.

Transcriptional targets of the Hpo signaling pathway

While the initial studies have identified *cycE* and *diap1* as transcriptional targets of the Hpo pathway, they are unlikely to be the only targets. Of note, the functional relevance of *diap1* and *cycE* expression that occurs upon loss of Hpo signaling has never been rigorously examined using genetic epistasis tests. Furthermore, *cycE* overexpression combined with inhibition of apoptosis does not result in the tissue overgrowth (Neufeld et al. 1998) that is characteristic of inactivation of *hpo* or activation of *yki*, suggesting that there should exist additional transcriptional targets of the Hpo pathway that contribute to its growth-regulatory function. A strong candidate is the microRNA molecule *bantam*, a positive regulator of imaginal disc growth that, like the known components of the Hpo pathway, possesses the dual activity of regulating cell proliferation as well as cell death (Brennecke et al. 2003). Two recent studies, by Thompson and Cohen (2006) and Nolo et al. (2006), have provided multiple lines of evidence implicating *bantam* as an additional target of the Hpo pathway. First, *bantam* expression is

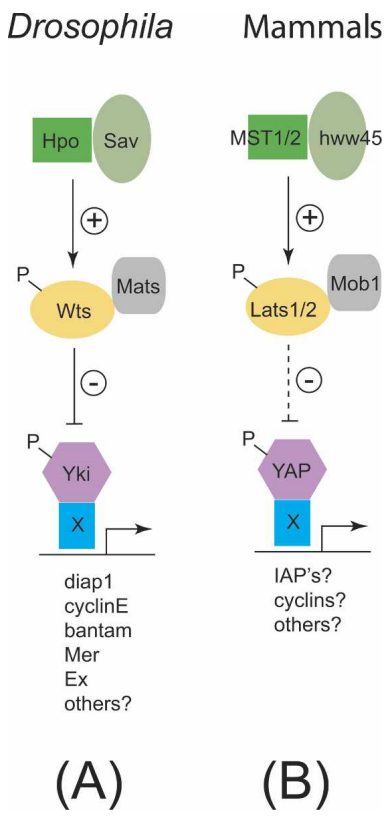

Figure 3. The Hpo kinase cascade in *Drosophila* (*A*) and mammals (*B*). Solid lines indicate validated and direct biochemical interaction, and the dashed line indicates suspected and unverified interaction. The corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. "X" denotes an unknown DNA-binding protein that partners with Yki or YAP to regulate target gene transcription. Modified from Huang et al. (2005), with permission from Elsevier. See text for details.

increased by Yki overexpression. Second, loss of *bantam* partially suppresses Yki-induced overproliferation, and *bantam* overexpression partially rescues the growth defect of *yki* mutant cells. The partial epistasis in both genetic settings is likely due to the contribution of other parallel targets such as *cycE* and *diap1*. Consistent with this hypothesis, Nolo et al. (2006) showed that the Hpo pathway regulates the transcription of *bantam* independently of *cycE* and *diap1*, and that simultaneous overexpression of all three target genes results in synergistic tissue overgrowth.

It is unlikely that *bantam*, *cycE*, and *diap1* will account for all the biological output of the Hpo pathway. For example, the cell shape defect (apical hypertrophy) that is characteristic of loss of *wts* or activation of *yki* cannot be explained by these known targets. The implication of the Hpo pathway in other biological processes (see below) also suggests the existence of additional, and perhaps tissue-specific, targets. Even with the known transcriptional targets, the available genetic data do not distinguish whether these genes are activated by Yki directly or indirectly through intermediary transcription regulators. Answers to this question will require molecular dissection of the regulatory regions controlling the transcription of *bantam*, *cycE*, or *diap1*.

Linking the Hpo kinase cascade with the extracellular world

A question that has long puzzled researchers in Hpo signaling is whether this pathway is normally regulated by extracellular cues, or alternatively, simply acts as a brake of tissue growth in a constitutive and unregulated manner. Several recent studies, all using a candidate gene approach, have provided several lines of evidence that potentially link the Hpo kinase cascade with the extracellular milieu.

The first breakthrough was provided by Hamaratoglu et al. (2006), who identified Merlin (Mer) and the related protein Expanded (Ex) as potential upstream regulators of Hpo. Both Mer and Ex are members of the "4.1, Ezrin, Radixin, Moesin" (FERM) domain-containing family of proteins, which generally function as adaptor proteins that link transmembrane proteins to the cytoskeleton or cytoskeleton-associated proteins (Chishti et al. 1998). The human ortholog of Mer is encoded by neurofibromatosis 2 (NF2), a tumor suppressor gene whose mutations lead to tumors in the central nervous system (for review, see McClatchey and Giovannini 2005). Previous studies in *Drosophila* have shown that Mer and Ex localize close to the adherens junction, and, in fact, these two proteins can heterodimerize with each other and appear to function redundantly as tumor suppressor genes that restrict the growth of imaginal discs (McCartney et al. 2000). However, the downstream effector of the Mer–Ex complex was unknown. Hamaratoglu et al. (2006) noted that cells doubly mutant for *mer* and *ex* are strikingly similar to those mutant for known tumor suppressor genes of the Hpo pathway, such as elevated *cycE* and *diap1* expression, and evasion of cell cycle arrest and programmed cell death. By examining *ex* overexpression in a *hpo* mutant background, and vice versa, they were able to place *mer* and *ex* upstream of *hpo*. Further corroborating their genetic analysis, Hamaratoglu et al. (2006) found that overexpression of Mer and Ex promotes the phosphorylation of Wts, although the mechanism by which this is accomplished remains to be determined: Neither Mer or Ex binds to Hpo directly; thus additional factors might be required to link Mer or Ex to Hpo (Fig. 4A; Hamaratoglu et al. 2006). An alternative model, which is compatible with the data presented by Hamaratoglu et al. (2006) although not considered by the authors, is that Mer and Ex might regulate Hpo indirectly by controlling the abundance of an upstream component of the Hpo pathway, such as its putative receptor. Indeed, this model was preferred by Maitra et al. (2006), who found that *mer*; *ex* double-mutant cells have increased steady-state levels of a variety of membrane proteins such as EGF receptor, Notch, and E-cadherin.

Using a similar candidate gene approach, three recent studies implicated the atypical cadherin Fat (Ft) as a potential receptor regulating the Hpo pathway (Fig. 4A; Bennett and Harvey 2006; Silva et al. 2006; Willecke et

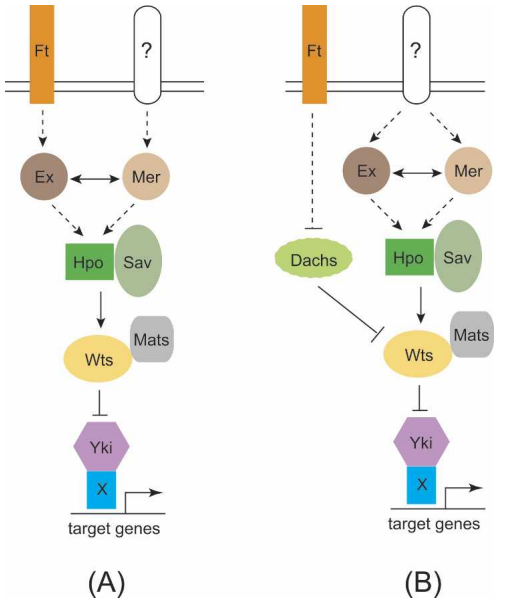

Figure 4. Two possible models linking the atypical cadherin Ft to the Hpo kinase cascade. (*A*) In the first model, Ft, Ex, and Hpo act in a linear pathway (Bennett and Harvey 2006; Silva et al. 2006; Willecke et al. 2006). (*B*) In the second model, Ft and Hpo mediate parallel inputs into Wts, with Ft and Hpo regulating the abundance and activity of Wts, respectively (Cho et al. 2006). Solid lines indicate validated and direct biochemical interaction, and dashed lines indicate genetic interaction.

al. 2006). *ft* is one of the first tumor suppressor genes isolated in *Drosophila* (Mahoney et al. 1991). However, the effector pathway regulated by Ft in growth control has largely remained elusive since its discovery. Collectively, the three recent studies provide several lines of evidence placing Ft, Ex, and Hpo in a linear pathway. First, *ft* mutant cells resemble *hpo* or *wts* mutant cells, such as up-regulation of *cycE* and *diap1*, increased cell proliferation, and decreased cell death. Second, overexpression of Ex or Hpo can suppress the loss-of-function phenotype of *ft*, thus genetically placing *fat* upstream of *ex* and *hpo*. Third, Ft is required for the apical membrane localization and stability of Ex. Lastly, Ft stimulates the phosphorylation of Hpo and Wts in cell culture assays. Interestingly, the Ft–Ex cascade appears to function in parallel to Mer, since Mer localization is not affected by Ft, and furthermore, *ft mer* double-mutant clones resemble *ex mer* double-mutant clones. These observations raise the possibility that Mer may transduce a signal from an unknown cell surface receptor to Hpo (Fig. 4A).

In contrast to the aforementioned studies, another recent report investigating the relationship between Ft and the Hpo pathway proposed a distinct model wherein Ft impinges on the Hpo pathway by controlling the abundance of the Wts tumor suppressor protein (Fig. 4B; Cho et al. 2006). This study was built on previous studies of Dachs, an unconventional myosin that has been shown to function downstream from, and antagonistically to, Ft (Mao et al. 2006). Using Dachs as an "anchor point," Cho et al. (2006) provide several lines of evidence linking the Ft–Dachs cascade to Wts, rather than Ex or Hpo. First, genetic epistasis places *dachs* downstream from *ft*, but upstream of *wts*. Second, *ft* mutant clones show decreased protein levels of Wts, but not of other Hpo pathway components such as Hpo, Sav, Mer, or Mats. Lastly, Dachs coprecipitates with Wts when overexpressed in *Drosophila* cell cultures. Based on these observations, Cho et al. (2006) proposed that Dachs might function as a scaffold to link Wts to proteins that promote Wts proteolysis. According to this model (Fig. 4B), the Ft–Dachs and Hpo–Sav cascades regulate Wts in a parallel manner, with the former affecting the abundance and latter controlling the activity of Wts. Additional studies will be required to determine whether this model, or the aforementioned linear model, represents the major mode by which Ft regulates the Hpo signaling pathway in vivo. For example, a prediction of the parallel model is that double mutants of *ft* and *hpo* should show a stronger overgrowth phenotype than either single mutant.

Unlike proteins such as Hpo, Sav, Wts, Mats, and Yki, which constitute the core of the Hpo kinase cascade, the connection of Ex, Mer, or Ft to the Hpo pathway is so far largely based on genetic evidence. Future studies directed at establishing a biochemical link between these proteins and the core kinase cascade are much needed to establish a bona fide biochemical pathway linking Ft–Ex–Mer to Hpo activation. This may not be a trivial endeavor since it is likely to involve unknown intermediary proteins.

Hpo signaling in mammalian tumorigenesis

Components of the Hpo signaling pathway are highly conserved throughout evolution (Fig. 3B). Accordingly, loss-of-function mutant flies for several components of the pathway can be rescued with their respective human counterpart (Tao et al. 1999; Wu et al. 2003; Lai et al. 2005). These observations raise the exciting possibility that the Hpo pathway might play an analogous role in mammals. While a direct role for Hpo signaling in developmental organ size control has yet to be demonstrated, there is increasing evidence implicating the Hpo pathway in mammalian tumorigenesis. For example, mice lacking a *wts* homolog develop soft-tissue sarcomas and ovarian tumors (St John et al. 1999), and the human orthologs of *sav* and *mats* are mutated in several cancer cell lines (Tapon et al. 2002; Lai et al. 2005). *NF2*, the human ortholog of *mer*, is a known tumor suppressor gene whose mutations lead to neurofibromatosis (for review, see McClatchey and Giovannini 2005).

Most recently, YAP, the mammalian homolog of Yki, has also been directly implicated in mammalian cancer. Using a mouse model of liver cancer initiated from progenitor cells harboring *p53* loss and *c-myc* overexpression, Zender et al. (2006) found a recurrent amplification at mouse chromosome 9qA1 that accelerates the rapid growth of tumors. Interestingly, the syntenic region of human chromosome 11q22 has been reported to be amplified in a variety of human cancers (Imoto et al. 2001; Dai et al. 2003; Bashyam et al. 2005; Snijders et al. 2005).

This amplicon (9qA1 in mice and 11q22 in humans) contains not only *Yap* but also *cIAP2*, a mammalian homolog of *diap1*; and functional analysis by Zender et al. (2006) revealed that both *Yap* and *cIAP2* contribute to tumorigenesis in this genetic context. Moreover, *Yap* was reported to transform immortalized mammary epithelial cells in vitro (Overholtzer et al. 2006). Thus, like its *Drosophila* counterpart (Huang et al. 2005), *Yap* functions as an oncogene in mammals. It is reasonable to speculate that besides gene amplification, other genetic perturbations, such as gain-of-function mutations in *Yap* or inactivation of upstream tumor suppressors, might also lead to *Yap* activation. Thus, the overall contribution of *Yap* misregulation to human cancer could be much higher than the frequency of 11q22 amplification detected in various tumors.

Hpo signaling in other developmental processes

A recurring theme in developmental biology is the use of a single signaling cascade in multiple, and sometimes seemingly unrelated, biological processes (e.g., see Bray 2006). The Hpo pathway is no exception. Besides its pivotal role in coordinating cell proliferation and apoptosis in proliferating cells, two recent reports have revealed unexpected roles for the Hpo pathway in post-mitotic cells in *Drosophila* (Fig. 5; Mikeladze-Dvali et al. 2005; Emoto et al. 2006).

In the first study, Mikeladze-Dvali et al. (2005) investigated the choice of rhodopsin expression in post-mitotic photoreceptor neurons. The bulk of the *Drosophila* compound eye contains two subtypes of randomly distributed ommatidia, each characterized by distinct rhodopsin expression in the inner photoreceptor cells R7 and R8. The pale (p) subtype expresses *rh3* in R7 and *rh5* in R8, while the yellow (y) subtype expresses *rh4* in R7 and *rh6* in R8. The decision of a given ommatidium to become y or p subtype is initially made stochastically by R7. Once an R7 commits to the p fate and expresses *rh3*, it sends an instructive signal to the underlying R8, rendering it to express the p-specific rhodopsin *rh5*. In the absence of the R7 signal (such as an *rh4*-expressing yR7), R8 expresses the y-specific rhodopsin *rh6*. The tight coupling between rhodopsin expression in R7 and the underlying R8 is required for robust color vision. Mikeladze-Dvali et al. (2005) found that Wts and a PH domain-containing protein Melted (Melt) play opposite roles to specify rhodopsin expression in R8, with Wts promoting the yR8 fate and Melt promoting the pR8 fate. Thus, in the absence of *wts*, or other Hpo pathway components such as *hpo* and *sav*, the yR8 subtype is misspecified into pR8. In contrast, in the absence of melt, the pR8 subtype is misspecified into yR8. Interestingly, *wts* and *melt* mutually repress the transcription of each other, forming a bistable regulatory loop that ensures a robust choice between the p (*melt*-expressing) and the y (*wts*-expressing) fates (Fig. 5A; Mikeladze-Dvali et al. 2005). The nature of the inductive signal from pR7 to R8 as well the mechanism by which *wts* and *melt* repress the transcription of each other are yet to be elucidated.

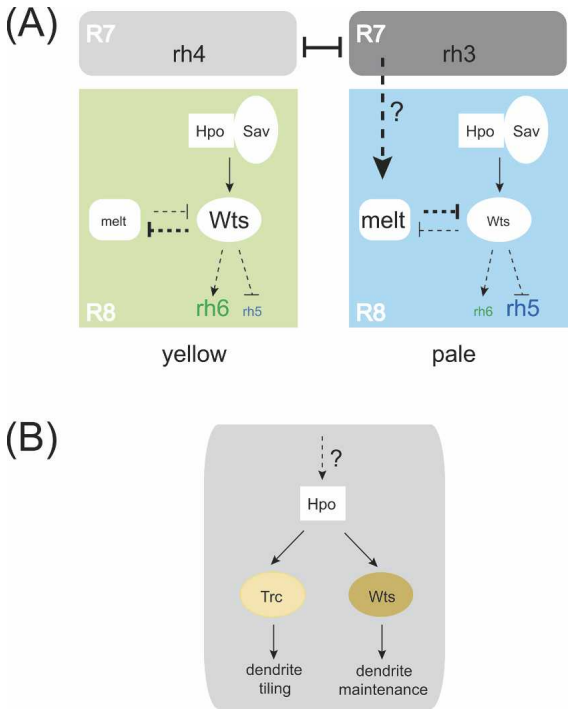

Figure 5. Examples of Hpo signaling in post-mitotic cells. (*A*) Regulation of the photoreceptor R8 subtype. Wts and Melt interact in a bistable loop to specify the yR8 and pR8. When an R7 expresses *rh4*, the underlying R8 does not receive any inductive signal, and by default, expresses Wts. Wts in turn represses *melt* and *rh5*, but activates *rh6* expression, specifying the yR8 fate. When an R7 expresses *rh3*, it sends an inductive signal to the underlying R8 and, by an unknown mechanism, relieves *melt* from the repression by Wts, thus swinging the system to the pR8 fate. Modified from Mikeladze-Dvali et al. (2005), with permission from Elsevier. (*B*) Dendrite morphogenesis. Hpo regulates Wts and Trc, respectively, for dendrite maintenance and tiling. Modified by permission from Macmillan Publishers Ltd: Nature (Emoto et al. 2006) © 2007. See text for details.

Emoto et al. (2006) provided another elegant example for the role of the Hpo pathway in post-mitotic neurons. While studying the morphology of class IV dendritic arboration sensory neurons in *Drosophila* larvae, Emoto et al. (2006) found that the tumor suppressor gene *wts* is required for the maintenance of dendrites. In *wts* mutants, dendrites initially form, but progressively lose branches at later stages. Interestingly, previous studies by Emoto et al. (2004) have shown that Tricornered (Trc), the other NDR family kinase in *Drosophila*, is required for the establishment of dendritic tiling, a process whereby dendrites of a similar type avoid crossing over each other, thus ensuring the complete and nonredundant coverage of the receptive field. Emoto et al. (2006) went on to show that Hpo activates both NDR kinases, and in this capacity, regulates complementary aspects of dendrite development: Trc for tiling and Wts for maintenance (Fig. 5B). Strikingly, Yki appears to be dispensable for dendrite maintenance, offering the only documented example in which Wts activity is not mediated by Yki (Emoto et al. 2006).

These recent examples of Hpo signaling in nondividing cells raise several important questions for future investigation. For example, what is the signal that regulates the Hpo pathway under these circumstances? Along this line, it will be important to determine whether the newly implicated upstream regulators of the Hpo pathway in proliferating tissues, such as *ft*, *ex*, and *mer*, are also required in these post-mitotic contexts. Another important direction is to define the relevant downstream effectors of the Hpo signaling pathway in the post-mitotic cells. In the case of R8 subtype specification, the Hpo pathway ultimately inhibits the transcription of *melt* and *rh5* and stimulates the transcription of *rh6*. It will be important to determine whether Yki directly mediates the transcriptional control of these target genes. In the case of dendrite maintenance, one needs to identify additional Wts substrate(s) that can fully account for the output of the Hpo pathway. Of particular interest is to determine whether the function of Wts in this process is mediated by a transcriptional mechanism as shown in proliferating tissues and R8 subtype specification, or a nontranscriptional mechanism.

Finally, it is worth noting that while this review has focused on the role of the Hpo kinase cascade in various settings, there are also examples in the literature that implicate single Hpo pathway components, rather than the canonical Hpo pathway, in certain biological processes. For example, a mammalian homolog of Hpo, MST1, has a widely documented proapoptotic function in cultured mammalian cells. This proapoptotic function appears to involve direct phosphorylation of substrates other than Wts, such as histone H2B (Cheung et al. 2003) or FOXO transcription factors (Lehtinen et al. 2006). In addition, the mammalian homologs of Wts have been implicated in the control of mitosis and cytokinesis, with their loss leading to multinucleated cells, centrosome amplification, and genomic instability (McPherson et al. 2004; Yang et al. 2004). Again, these reported functions of mammalian Wts proteins do not appear to involve an analogous Hpo–Wts–Yki cascade. Due to the scope of this review, such "noncanonical" Hpo (or Wts) activities will not be discussed further.

Outstanding questions

A striking feature of biological systems is that a quite small number of signaling pathways are used to regulate a myriad of biological processes. Thus, the discovery of an entirely new signaling cascade such as the Hpo pathway represents a major advance in understanding the signaling networks controlling metazoan development and physiology. Despite recent progress, our knowledge about this important growth regulatory pathway remains incomplete. In the following sections, I discuss several important questions that will likely be the subject of future investigation. I focus on the role of Hpo in proliferating tissues since this is the most widely studied aspect of Hpo signaling. This discussion is not intended to be comprehensive, but merely serves to highlight a few outstanding questions that are ready to be tackled with a combination of genetic, cell biology, and biochemical approaches.

Physiological regulation of the Hpo pathway

A major gap in our understanding of Hpo signaling concerns how this pathway is normally regulated in vivo. A prevailing hypothesis posits that the activity of the Hpo pathway is coupled with organ size or cell differentiation such that when an organ approaches its final size or when cells enter the differentiation phase of development, the Hpo pathway is activated to inhibit cell proliferation and to promote apoptosis. By impinging on both cell proliferation and apoptosis, the Hpo pathway offers a robust mechanism to sculpture final organ size. Unfortunately, so far there has been no direct evidence to prove or disprove this model, largely due to the lack of a reliable assay to monitor Hpo signaling activity in vivo.

Identification of Yki as the direct substrate of Wts suggests several possible venues to monitor Hpo pathway activity in vivo. One approach is to map the Wts phosphorylation site(s) on Yki, and to use phospho-specific antibodies against such site(s) to detect any spatial or temporal regulation of the Hpo pathway activity during development. Another approach is to dissect the regulatory region of known Yki targets with the goal of defining a minimal Hpo-responsive element (HRE). With such a DNA element, one should be able to construct a reporter construct that faithfully reflects Hpo signaling activity in vivo. Yet another possible venue is to take advantage of a fusion protein containing Yki and the DNA-binding domain of Gal4, which was initially developed to demonstrate the regulation of Yki transcriptional activity by upstream tumor suppressors in cell cultures (Huang et al. 2005). Hpo signaling activity can then be followed in vivo using flies carrying a ubiquitously expressed Yki-Gal4 fusion as well as a convenient UAS-driven reporter such as GFP. Besides these Yki-based approaches, reagents to monitor more proximal events in the Hpo signaling cascade, such as phosphorylation of Wts, can also be exploited to measure pathway activity under physiological conditions.

A related but largely unanswered question concerns the nature of the signal that ultimately regulates Hpo signaling in vivo. Conceptually, one can imagine several ways by which growth or survival of an individual cell may be coupled to the number of cells in the resident tissue. One possibility is that cells in a developing field may release as well as sense the concentration of a signaling molecule, in much the same way that bacteria use quorum sensing to coordinate their behavior according to cell number (for review, see Waters and Bassler 2005). A noted precedent for such paracrine signaling in organ size control can be found in myostatin, a TGF-β family protein that is secreted by muscle cells to inhibit their own growth (Lee 2004). Another possibility is that Hpo signaling might be regulated by the strength of cell–cell adhesion. Although cells adhere to each other irrespec-

tive of the size of the cell population, one can imagine that cells might become more crowded as organs grow, and such a crowding effect might lead to quantitative or qualitative changes in the adherens junctions between neighboring cells, which in turn might modulate Hpo signaling activity. Conceptually, such developmental changes of cell–cell adhesion might not be too dissimilar to contact inhibition, a well-documented phenomenon in cell culture whereby cells arrest proliferation when they come into contact with each other. Along this line, it is noteworthy that NF2, the human ortholog of Mer, has been implicated in contact inhibition in cultured mammalian cells (Morrison et al. 2001). Besides cell–cell adhesion, changes of any other biochemical or physical parameters that accompany organ growth, such as intracellular messengers or mechanical tension on cell membrane, might potentially regulate Hpo signaling during development.

The implication of Ft as a potential receptor of the Hpo kinase cascade provides a new conceptual framework to consider how this pathway may be regulated during development. Ft is a large (5147 amino acids) transmembrane protein containing 34 cadherin repeats, five EGF-like and two lamin A-G domains in its extracellular region, as well as an intracellular domain of 539 amino acids (Mahoney et al. 1991). Besides its role as a negative regulator of tissue growth, Ft is required for planar cell polarity (PCP) and proximal–distal patterning of appendages. Ft appears to function as a receptor for another atypical cadherin called Dachsous (Ds), and signals to these processes via its intracellular domain (Matakatsu and Blair 2006). Much of what we know about the regulation of Ft comes from studies of PCP, a process by which cells in the plane of an epithelium orient themselves along an axis orthogonal to the apical–basal axis (Yang et al. 2002; Casal et al. 2006). A prevailing model for PCP is that a morphogen gradient, such as wingless in the eye or hedgehog in the abdomen, leads to a graded expression of Ds, which, in turn, sets up an activity gradient of Ft. The polarity of a cell is then specified by differential Ft activity on opposite sides of the cell along the axis of PCP. A central tenet of this PCP model is graded Ft activity across the tissue. If this is the case, how can we reconcile the graded activity of Ft in PCP with the apparently uniform requirement for Ft in growth control? One possibility is that a low and basal level of Ft activity is required for growth control, while a higher Ft activity is required for PCP. Alternatively, it is possible that a ligand other than Ds is largely responsible for regulating Ft in growth control. Lastly, it remains possible that the distinct spatial requirement for Ft activity in growth control versus PCP simply reflects the different timing of the two events, in much the same way that Wts is required ubiquitously for growth control in proliferating cells but acts in a cell type-specific manner in post-mitotic R8 photoreceptors (Mikeladze-Dvali et al. 2005). A critical evaluation of these models relies on the development of tools that monitor Ft signaling activity in growth control, which are currently unavailable.

Biochemical and cellular mechanism of Hpo signal transduction

In many aspects, our current picture of the Hpo signaling pathway is incomplete. Not only are there missing components that remain to be discovered, even for the known components, but our understanding of their regulation is rudimentary. Here I highlight several obvious gaps concerning the known Hpo pathway components.

The first issue concerns the cellular mechanism of the Hpo kinase cascade. While the kinase cascade leading from Hpo to Wts and then to Yki is well established (Wu et al. 2003; Huang et al. 2005), we know very little about how this biochemical pathway is executed at the cellular level. In *Drosophila* imaginal discs, both Hpo and Wts appear to be enriched at the cell peripheral close to the plasma membrane (Cho et al. 2006; Silva et al. 2006). How do these cytoplasm/membrane-localized kinases inhibit Yki, a transcriptional coactivator that functions in the nucleus? At least two possibilities exist. First, phosphorylation of Wts by Hpo might promote the nuclear entry of Wts. Within the nucleus, Wts-mediated phosphorylation inhibits Yki function, either by directly inhibiting its transcriptional activity or promoting its degradation or its export to the cytosol. Along this line, it is worth noting that Lats1, a mammalian homolog of Wts, has been reported to be localized in the nucleus (Nishiyama et al. 1999). Alternatively, Yki may exist in both cytoplasm/membrane and nucleus at equilibrium. Phosphorylation of Yki at the cytoplasm/membrane leads to its degradation, shifting the equilibrium toward cytoplasmic localization, and thus reducing the effective concentration of Yki in the nucleus. A critical evaluation of these models requires reagents that monitor the localization and phosphorylation of Wts and Yki, as well as assays that monitor the transcriptional activity of Yki.

The second issue concerns the DNA-binding transcription factor(s) that partners with Yki to regulate target gene transcription. Like other transcriptional coactivators, Yki does not bind to DNA directly, but presumably functions by interacting with, and stimulating the activity of, DNA-binding transcription factor(s). The exact identity of this DNA-binding factor remains unknown at present. YAP, the mammalian homolog of Yki, has been reported to function as the coactivator for several transcription factors, such as the p53 family member p73, the Runt family member PEBP2α, and the four TEAD/TEF transcription factors (Yagi et al. 1999; Strano et al. 2001; Vassilev et al. 2001). Homologs of these proteins exist in *Drosophila* (Dmp53 for p73, Runt for PEPBP2α, and Scalloped for TEAD/TEF). It remains to be determined whether any of these proteins, or other unknown proteins, function together with Yki in the physiological context of imaginal disc development. Given the diverse biological outputs governed by the Hpo pathway, including cell proliferation, apoptosis, cell shape, R8 subtype specification, and dendrite morphogenesis, it is tempting to speculate that Yki might partner with multiple DNA-binding factors to regulate the complete spectrum of transcriptional targets. A particularly inter-

esting candidate is Orthodenticle (Otd), a homeodomain protein that activates *rh5* and inhibits *rh6* transcription by binding to conserved TAATCC sites in their promoters (Tahayato et al. 2003). Thus, at least genetically, Otd represents a prime candidate as a Yki partner in the context of R8 subtype specification.

As discussed earlier, it will also be important to establish the biochemical mechanisms by which the reported upstream regulators, such as Ft, Ex, and Mer, feed into the core Hpo kinase cascade. Unlike Hpo or Wts, the activities of these potential upstream regulators cannot be measured by straightforward enzymatic assays. Thus, tools must be developed that differentiate between the active and the inactive states of these molecules, which, unfortunately, are poorly understood at present.

Hpo signaling beyond Drosophila

As compared with *Drosophila*, much less is known about the composition and physiological function of the Hpo pathway in other species, especially as far as growth control is concerned. *Caenorhabditis elegans*, for example, has orthologs for all the Hpo pathway components, yet their requirement in tissue size control has not been documented.

Thus, an important direction in the future is to apply the insights learned from *Drosophila* and to investigate whether the Hpo pathway represents a universal mechanism to control organ size in all animals. Here I discuss several outstanding questions under this overall goal, using the mammals as an example.

First, it will be important to establish whether the biochemical and genetic interactions observed in *Drosophila* Hpo signaling can be recapitulated in mammalian systems. So far, the only biochemical interactions that have been reproduced in the mammalian cells are the phosphorylation of Lats1/2 (Wts homologs) by MST1/2 (Hpo homologs), the binding between MST1/2 and hWW45 (a Sav homolog), and the binding between Lats1 and Mob1 (a Mats homolog) (Fig. 3B; Chan et al. 2005; Callus et al. 2006; Hergovich et al. 2006). It remains to be determined whether YAP is phosphorylated and inactivated by Lats1/2 as in *Drosophila*, and if so, what the transcriptional targets of YAP are. Moving upstream, it will be important to investigate whether homologs of Ft (Fat4 in mammals), Ex (Willin in mammals), and Mer (NF2 in mammals) can be genetically or biochemically linked to the core components of the Hpo kinase cascade. These studies will not only extend the Hpo pathway in mammals, but also provide independent evidence strengthening the connection between these proteins and Hpo signaling in *Drosophila*.

A second important goal is to investigate the physiological function of Hpo signaling in mammals, especially its potential role in organ size control. Along this line, it is worth noting that the implication of Hpo pathway components in tumorigenesis alone does not necessarily prove a role for this pathway in organ size regulation, since perturbation of many cellular processes besides growth control can contribute to tumorigenesis (for review, see Hanahan and Weinberg 2000). Thus, a role for Hpo signaling in mammalian organ size control must be directly tested in a genetic setting analogous to what has been done in *Drosophila*. Knockout mice have been generated for several potential components of the mammalian Hpo pathway. Unfortunately, these mice are either viable and lack any overt overgrowth characteristic of the respective *Drosophila* mutants (*Lats1*) (St John et al. 1999) or embryonic lethal, thus preventing a critical assessment of their involvement in organ size regulation (*Lats2* and *Yap*) (McPherson et al. 2004; Morin-Kensicki et al. 2006). Therefore, knockout or transgenic models that manipulate these or other Hpo pathway components in a spatially and temporally restricted manner need to be developed. These mouse models should also reveal whether the Hpo pathway plays a role in other developmental processes, such as dendrite morphogenesis as documented in *Drosophila*.

The mammals should also provide a more suitable system to examine whether the Hpo signaling pathway plays any role in maintaining tissue homeostasis in adult life. Unlike fruit flies, whose adult tissues are largely quiescent, most mammalian tissues undergo constant renewal whereby old cells are replenished by newer ones descended from stem cells. Furthermore, the ability of certain mammalian tissues to regenerate themselves after insult (e.g., see Michalopoulos and DeFrances 1997) offers a unique opportunity to ask whether the Hpo pathway plays a role in stopping organ regeneration at the appropriate size.

Ultimately, one might be able to harness the power of Hpo signaling to engineer mammalian organs of predetermined size by modulating the activity of pathway components using transgenes or small molecules. In addition, chemicals that specifically target the Hpo pathway, such as those against the nuclear effector YAP, could be exploited for therapeutic intervention of human tumors caused by loss of Hpo signaling.

Concluding remarks

Organisms from *Drosophila* to mammals have evolved elaborate mechanisms to coordinate cell proliferation and cell death. In most circumstances, these two processes are coupled in a manner so that unscheduled cell proliferation, such as that caused by *myc* activation, is countered by increased cell death (for review, see Lowe et al. 2004). Such coupling between proliferation and apoptosis provides an important failsafe mechanism to prevent inappropriate proliferation of somatic cells. The Hpo signaling pathway appears to override this obligatory coupling between cell proliferation and apoptosis, since increased proliferation caused by inactivation of tumor suppressors (or activation of oncogenes) of this pathway is accompanied by an inhibition of cell death. In many aspects, these circumstances resemble certain cancer cells, which display both increased cell proliferation and suppressed cell death.

By simultaneously inhibiting cell proliferation while promoting apoptosis, the Hpo pathway might provide a

robust mechanism to quickly stop organ growth at the appropriate time in development, acting like an on/off switch. The robustness of this system comes with an inherent danger: any single-gene perturbation that compromises Hpo signaling activity could potentially lead to the detrimental outcome of uncontrolled growth. While this is less a concern for short-living organisms like *Drosophila* whose adult tissues are largely quiescent, it poses a more serious problem for long-living organisms with continuous cell replenishment. A possible solution to this dilemma is to evolve additional mechanisms that keep the system in check under conditions of aberrant Hpo activity. It will be interesting to identify such mechanisms, especially in higher organisms such as mammals.

Despite recent progress in elucidating the molecular underpinning of the Hpo signaling cascade, it is likely that we have only just begun to appreciate the composition and logic of this emerging pathway. From a cell signaling perspective, additional players of the Hpo pathway are yet to be identified that, together with the known components, constitute a complete signaling cascade linking the extracellular milieu to nuclear gene transcription. From a developmental biology perspective, we know little about how this potent regulatory pathway is coupled to tissue growth and differentiation programs during normal development. From an evolutionary perspective, it remains to be determined whether the Hpo pathway represents a universal size-control mechanism from *Drosophila* to man, and whether it offers a substrate upon which evolution acts to generate the diversity of organ or body size. It is an exciting time to tackle these questions: Biologists have at their disposal not only classical experimental tools such as genetics and biochemistry, but increasingly, modern technologies such genome-wide RNA interference screening, live imaging, and comparative genomics. With an integration of these approaches, studies of the Hpo signaling pathway are poised to provide critical insights into the long-standing puzzle of how organ size is determined during development and maintained in adult life.

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Duojia Pan

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