Chapter 14
Hippo Signaling and Organ Size Control

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Abstract Hippo signaling is a growth control pathway first described in Drosophila and more recently studied in mammals. At the core of the Drosophila Hippo signaling pathway is a cascade composed of the Hippo and warts serine threonine kinases whose function in the context of Hippo signaling is to restrict the activity of the transcriptional coactivator yorkie by phosphorylation and cytoplasmic retention. In mammals, a similar cascade is present with the mst1 and mst2 kinases serving the function of Hippo and the lats1 and lats2 kinases functioning as orthologs of warts. Mammals also have two yorkie-related genes, yap and taz. Emerging evidence suggests that a common theme of Hippo signaling in epithelial tissues is to regulate growth, either in a homeostatic or developmental framework, or in pathological situations such as cancer. Much initial and recent attention has focused on Hippo signaling in the context of organ size control. Indeed, how final organ size is achieved during animal development and how it is maintained in adults is a long standing and fundamental problem. In this chapter, basic concepts of organ size determination and the relationship between progenitor, stem cells, and regulation of organ size, both during development and in adult tissue homeostasis are reviewed in the context of Hippo signaling.

Keywords Hippo signaling • Organ size control • Stem cells • Development • Homeostasis

How organ sizes are set relative to each other and to overall body mass is a fundamental biological question that remains poorly understood. While there is a general trend that individual organ sizes vary as a function of overall body mass (Stahl 1965),

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there are significant deviations from this general rule. For example, while the brain of an elephant is large as would be anticipated by its overall body mass, the eye of the elephant is disproportionally small (Crile and Quiring 1940), accounting for poor visual acuity within these animals. Another example of organ size variation across species is brain-to-body mass ratio in vertebrates: small birds have one of the largest ratios at 1:12 (Sol et al. 2005) whereas the hippopotamus has one of the smallest ratios at 1:2,800 (Crile and Quiring 1940). In contrast, humans and mice have an intermediate and roughly equivalent brain:body mass ratio at 1:40 (Crile and Quiring 1940; Herculano-Houzel 2011). While there is considerable variation between species for individual organ:body weight ratio, including in the brain, within a given species there is a marked lack of variation of organ:body weight ratios between individuals. How different organ:body mass ratios are achieved with such precision has been the subject of considerable investigation, however, relatively little is understood mechanistically about what regulates these processes at the molecular and genetic level.

In principle, organ sizes could be set solely by defining a precise number of progenitor cells at a particular developmental stage, coupled with a robust program that ensures synchronous differentiation of a fixed number of progenitor cells at the end of embryogenesis (see Stanger 2008; Lui and Baron 2011 for excellent recent reviews on mechanisms that contribute to organ size determination in mammals and other organisms). Indeed, there is some evidence that certain animals and tissues follow this so-called deterministic mode of size regulation. In now classic experiments conducted by pioneering early experimental embryologists in the late 1800s and early 1900s, blastomeres of sea urchin (Driesch 1892) and frog embryos (Spemann 1938) were separated at the two cell stage and allowed to develop resulting in normally patterned larvae that were precisely half of the normal size. More recently, progenitor ablation studies in the mouse have revealed that reducing the number pancreatic progenitor cells during mid-to-late gestation results in a decreased pancreatic mass at birth (Stanger et al. 2007). These and other results (reviewed in Stanger 2008; Lui and Baron 2011) suggest that final organ mass or size might be directly related to defining the proper number of progenitor cells at an early stage.

In contrast, there is considerable evidence that final organ size can be independently of progenitor cell number and cell size. For example, tetraploid mouse (Henery et al. 1992) and haploid salamander embryos (Frankhauser 1938) are of comparable size to their diploid counterparts although they contain roughly half of the total number of cells in the case of tetraploids or twice the number of cells in the case of haploids. Similarly, in experiments where cell size and number were manipulated in Drosophila, normally sized imaginal discs were formed when discs contained either a larger number of smaller cells or a smaller number of larger cells (Weigmann et al. 1997; Neufeld et al. 1998). Furthermore, in the mouse, properly sized embryos are obtained upon aggregation of multiple morulae (Buehr and McLaren 1974) and properly sized mid-gestation embryos are formed following ablation of up to 70% of the inner cell mass cells at blastula stages (Tam 1988). Taken together, these results suggesting that pre- and early postimplantation mouse embryos have the ability to monitor cell number or total mass and adjust cell numbers to a

stage appropriate value. Finally, in genetic ablation experiments in the mouse liver, progenitor cell depletion has relatively little effect on overall liver mass at birth (Stanger et al. 2007). These examples of regulative growth suggest that mechanisms exist during development that sense organ size and adjust progenitor cell number accordingly. However, the molecular nature of this regulation remains mysterious in most cases.

Whatever mechanisms regulate organ growth during development, a number of lines of evidence suggest that they must be tightly regulated. Illustrating this fact are observations on the precision of organ size control. For example, in developing chicks the variance between the lengths of left and right wing skeletal elements in individual embryos is exceedingly small (Summerbell and Wolpert 1973). Also underscoring the role of precision in development, relatively small variations in progenitor cell number can in some instances lead to large alterations in body or organ size. In comparing embryos from animals with widely varying vertebral number, it was found that individual somite size and time of progenitor proliferation were key components that determined overall somite number and correlated with body length (Gomez et al. 2008). However, only several additional progenitor cell cycles are apparently necessary to go from a relatively small somite number in the chick (about 60) to a very large number in the corn snake (about 400). A simple mechanism that does not involve regulation and feedback control of progenitor cell proliferation and differentiation is unlikely to account for the robustness of these systems.

Regulated organ size determination is not limited to embryonic stages. In cases of diseased or damaged tissues compensatory growth or regeneration is often seen. When the heart is injured as a result of hypertension or infarction, cardiomyocytes undergo hypertrophy leading to increased mass by increasing cell size, not number (Hill and Olson 2008). In contrast, when the liver is subjected to partial hepatectomy where up to 2/3 of the liver is removed, a process of compensatory proliferation ensues leading to recovery of liver mass through increasing the number of cells in remaining liver lobes (Michalopoulos and DeFrances 1997). Kidney enlargement (via cellular hypertrophy) following unilateral nephrotectomy (Endele et al. 2007) and thyroid proliferation in cases of thyroid hormone insufficiency (Fierabracci 2012) are other instances where organs alter their sizes in response to injury or insufficient tissue function. Additional familiar examples of organ size regulation in adults include limb regeneration in starfish, crickets, and some urodele amphibians (Brockes and Kumar 2008). In these animals, limb amputation stimulates blastema formation followed by a process that in many ways resembles normal development, albeit on a larger scale. Remarkably, as in development, the regenerated limb stops growing when it has reached the proper size.

Despite distinct modes of organ size regulation in diverse tissues, several important molecules and pathway are known to contribute to overall organ size. One important group of molecules are termed chalones (derived from the Greek work khalon, meaning to slacken) that are circulating factors produced by a given tissue that negatively regulates that tissue's growth. Originally postulated as a feedback mechanism to regulate organ size (Bullough 1975), chalones have recently been brought to the fore by the identification of myostatin and leptin as being endogenous

growth regulators that function in muscle and fat respectively (reviewed in Gamer et al. 2003). Myostatin is a polypeptide produced by skeletal muscle cells that is secreted into the circulation and negatively regulates myoblast proliferation (McPherron et al. 1997). Homozygous inactivating mutations in myostatin lead to marked muscle mass enhancement as evidenced by a 40% increase in muscle mass in Belgian Blue cattle (Grobet et al. 1997; McPherron and Lee 1997), and other breeds that were selected for their impressive muscular build. Similarly, both heterozygous and homozygous mutations in myostatin have been found in a number of mammals (reviewed in Rodgers and Garikipati 2008), including humans, and these mutations lead to varying degrees of increased muscle mass suggesting a conserved mode of myostatin-mediated regulation. Like skeletal muscle, total adipose mass is modulated by another chalone-type signaling molecule leptin (Halaas et al. 1995; Pelleymounter et al. 1995). Leptin is produced by adipose tissue and enters the blood stream where it acts on cells in the hypothalamus to regulate the production of neuroendocrine hormones that control appetite and energy expenditure, thereby indirectly suppressing adipose tissue accumulation. Other documented examples of chalones include GDF11 for olfactory neurons (Wu et al. 2003) and BMPs for hair follicle cells (Plikus et al. 2008). However, for most organ systems, chalones have not been identified, calling into question whether this negative feedback mechanisms is a general modulator of organ size or is only employed in specific tissues.

A second mechanism that appears to be important for regulating compensatory growth in some tissues is metabolic regulation. In this case a metabolite (or in some cases metabolites) function as sensors that directly or indirectly control total organ mass as a function of concentration. Should the organisms demand for that metabolite increase, compensatory proliferation, and/or hypertrophy ensues, thereby increasing tissue mass and metabolite production until a proper homeostatic level is achieved. Evidence suggestive of this mode of regulation is apparent in the liver. Experiments involving transplantation of livers between dogs of different sizes clearly showed that the donor liver adjusts it size according to the host body mass (Kam et al. 1987). Parabiosis studies, where the circulatory system is fused between two animals, have shown that systemic factors play important roles in regulating liver size. In the rat, when one liver of a parabiotic pair is subjected to partial hepatectomy, both the operated and unoperated liver respond by increasing liver mass (Moolten and Bucher 1967). This effect is even more pronounced when one liver is completely removed. Although the endogenous factors that mediate compensatory growth and liver size have not been found, there is recent evidence that bile acid flux may be an endogenous regulator of liver mass. Increase in liver bile acids results in an increased liver size (Huang et al. 2006) and conversely, decreased liver bile acids leads to a delay in liver regeneration following partial hepatectomy (Ueda et al. 2002). Similarly, as previously mentioned, the thyroid, heart, and kidney can undergo metabolic size regulation as well as the adrenal cortex suggesting that multiple tissues employ this mode of size control, at least in the adult.

Other important growth regulators in metazoans include the IGF-AKT-mTOR pathway that functions in an evolutionary conserved role in diverse species from

insects to mammals (Bernstein 2010). However, IGF signaling by insulin-like growth factors largely affects overall body size (Sutter et al. 2007). Highlighting this role, a major IGF1 allele in dogs is largely responsible for the wide variation in sizes between different dog breeds. Manipulation of the IGF-AKT-mTOR axis in Drosophila likewise results in allometric or uniformly increased or decreased body size (Colombani et al. 2003). While this pathway can function cell autonomously to regulate growth in certain experimental situations (Weinkove et al. 1999), whether it normally does so during development or in the adult is less clear.

A new pathway that has been recently implicated in organ size determination in animals ranging from Drosophila to mammals is the Hippo signaling pathway reviewed in Halder and Johnson (2011). Components of the Hippo signaling pathway were identified initially in Drosophila by virtue of genetic screen for cell autonomous overgrowth defects in imaginal discs, larval precursors to adult tissues. Subsequently, additional components were identified by enhancer and suppressor screens and together were assembled into a signaling pathway via genetic and biochemical methods. Central to the Drosophila Hippo pathway is a kinase cascade where the serine-threonine Hippo kinase phosphorylates another serine-threonine kinase called warts. Warts in turn phosphorylates the transcriptional adaptor protein yorkie, thereby preventing its nuclear accumulation. Hence in the active state, yorkie is repressed and growth is suppressed. In contrast, when the core Hippo pathway kinases are inactive, yorkie accumulates in the nucleus and promotes growth. In addition to promoting growth, yorkie also activates multiple cell survival mechanisms and hence inhibits programmed cell death.

Extensive genetic analyses in Drosophila strongly suggest a fundamental role for Hippo pathway signaling in control of organ size (reviewed in Pan 2007). Imaginal disc cells that are mutant for warts or hippo kinases as well as cells that overexpress yorkie overgrow without respecting normal organ size control mechanisms. This effect is due in part to enhanced expression of positive regulators of the cell cycle such as cyclinE and coordinate upregulation of pro-survival factors such as dIAP. Not only are there increases in cell number during imaginal disc development, but cell numbers continue to increase following normal cessation of proliferation. For example, in Salvador mutant eye imaginal discs, excess interommatidial cells are not removed in during pupal stages, a process that involves apoptosis (Kango-Singh et al. 2002). Hence, Hippo signaling regulates imaginal disc size in Drosophila via a combination of pro-survival and proliferative cues.

Although not often explicitly stated, an underlying suggestion of these studies is that Hippo signaling is dynamically regulated by extracellular cues that sense organ size. According to this model, when progenitor cell proliferation is required, Hippo signaling activity falls below a growth inhibitory threshold. However, when final organ sizes are reached, or when progenitor proliferation is occurring at a rate higher than necessary, Hippo pathway signaling is upregulated, thereby slowing or stopping organ growth. While a model for dynamic regulation of organ size by modulation of Hippo signaling is attractive, it is only currently supported by fragmentary and incomplete evidence. Chief among the requirements for substantiating a Hippo-based mechanism for organ size control would be the identification of “organ size

checkpoints" (Leevers and McNeill 2005) that feed into the Hippo signaling pathway as well as demonstration of dynamic regulation of Hippo signaling in response to these inputs.

Much progress has been made into understanding upstream components that can positively or negatively impact Hippo pathway activity. Major regulators that have been reported include junctional complexes and the actin cytoskeleton. For example, a number of studies implicate E-cadherin, a component of the adherens junction in negatively regulating Hippo signaling (Nishioka et al. 2009; Kim et al. 2011). Likewise, apical-basal polarity complex components including scribble (Skouloudaki et al. 2009; Cordenonsi et al. 2011) and crumbs (Chen et al. 2010; Grzeschik et al. 2010; Ling et al. 2010; Parsons et al. 2010; Robinson et al. 2010; Varelas et al. 2010) have been demonstrated to play important roles in modulating Hippo signaling. Loss of cell polarity is often associated with deregulated growth and current evidence suggests that Hippo signaling may mediate this effect (reviewed in Martin-Belmonte and Perez-Moreno 2012). Finally, manipulation of F-actin levels has a pronounced effect on Hippo signaling (Sansores-Garcia et al. 2011; Wada et al. 2011) and provides an important link between the cytoskeletal architecture and growth control. Whether these diverse cytoskeletal and juxtamembrane complexes synergistically or independently regulate Hippo signaling remains unclear (Boggiano and Fehon 2012). However, taken together, these findings support a view that Hippo signaling responds to "cellular crowding" signals such as contact inhibition, mechanical stress, and/or apical-basal polarity to regulate organ size. Other extracellular modulators of Hippo signaling that have been reported include lysophosphatidic acid (Yu et al. 2012) and CD44 (Xu et al. 2010), although their roles in organ size control have not been explored.

Early on, it became apparent that the Hippo signaling pathway was evolutionarily conserved across diverse taxa, including mammals, at least at the level of sequence homology and biochemical interactions of core components. Evidence for a conserved role in growth control came from assessing the effects of manipulation of Hippo signaling, first in vitro in cultured cells, followed by overexpression and targeted deletion in vivo. The first in vivo reports employed mice engineered with transgenes that allowed for inducible expression of a mutant form of yap, one of two mammalian orthologs of yorkie (the other being taz), that is refractory to inhibitory phosphorylation by lats kinases, the mammalian orthologs of warts. In these studies (Camargo et al. 2007; Dong et al. 2007), a dramatic increase in liver size was observed clearly showing that enhanced yap activity can drive increased organ size. The yap-overexpressing livers comprised an increased number of cells, suggesting that cell proliferation was a key component of yap-induced increased liver mass. While these experiments showed that overexpression of yap can promote excessive liver growth, they did not demonstrate that Hippo signaling per se is required to modulate proper liver:body mass ratios. Subsequent mutational analysis of core components of the Hippo signaling pathway, including the adaptor protein Salvador (sav1) (Lee et al. 2010) and the Hippo kinase orthologs mst1/2 (Zhou et al. 2009; Lu et al. 2010; Song et al. 2010) showed that these upstream regulators of yap are indeed required to prevent excessive liver growth. Hence, Hippo signaling is active in the adult liver and functions

to negatively regulate liver size. As a whole, these yap overexpression and Hippo pathway component knockout studies demonstrate a conserved role for Hippo signaling in regulating the proper liver:body mass ratio. What they do not show is that Hippo signaling is dynamically regulated, either in embryonic or perinatal stages, and that this regulation is fundamental for setting proper liver:body mass ratios.

A major focus of these initial mammalian studies were on the liver, as this tissue exhibits dramatic responses to deregulated Hippo signaling. Whether all mammalian organs and tissues are subject to similar control by Hippo signaling were not systematically addressed and is an active area of investigation. Although this question has yet to be fully answered, several lines of evidence suggest that there are different responses to manipulating Hippo signaling in different tissues. For example, targeted deletion of sav1, mst1/2, or lats2 in cardiomyocytes (Heallen et al. 2011) all result in increased heart sizes during embryogenesis via yap regulation (von Gise et al. 2012), analogous to results obtained in the liver. However, targeted deletion of sav1 (Cai et al. 2010) or mst1/2 (Zhou et al. 2011) in intestinal epithelium using villin-cre, which is active in adult enterocytes and stem cells, does not result in increased size or mass of the intestine. Rather, there is a block in differentiation of intestinal epithelial cells and an expansion of progenitor cells in the case of mst1/2 and a defect in regenerative capacity in the case of sav1 mutant intestinal epithelium. Perhaps this result is not unexpected since intestinal size is not only a function of the intestinal epithelial component, where sav1 and mst1/2 deletion was targeted to, but requires inputs from both epithelial and stromal components. Another example where Hippo signaling activity is not directly correlated with growth control is in the preimplantation mouse embryo where Hippo signaling is required for the initial specification of the trophoblast and inner cell mass lineages (Nishioka et al. 2009). For the most part, this process is independent of growth or proliferation and is largely a cell fate decision. Hence, available evidence to date suggests that Hippo signaling is a critical growth regulator in multiple tissues and that Hippo signaling can restrain growth during embryonic, perinatal, or adult stages, depending on the tissue context. However, more work needs to be done to define specific tissue requirements for Hippo signaling in regulating organ sizes in mammals.

One theme that appears to be consistent across organisms and across tissues is a conserved role for Hippo signaling in regulating stem and progenitor cell proliferation. In the intestinal epithelium of both Drosophila (Karpowicz et al. 2010; Ren et al. 2010; Shaw et al. 2010; Staley and Irvine 2010) and mice (Camargo et al. 2007; Zhou et al. 2011), Hippo signaling is required for proper stem cell expansion, either during regeneration following injury or during normal homoeostasis. In the case of the mammalian liver (Lee et al. 2010; Lu et al. 2010), heart (Heallen et al. 2011), skin (Lee et al. 2008; Schlegelmilch et al. 2011), and chicken central nervous system (Cao et al. 2008), increases in progenitor cell proliferation and/or continued proliferation of fully differentiated cells are observed in deregulated Hippo signaling. In cultured mammalian cells, overexpression of yap, or in some cases taz, as well as knock down of upstream regulators such as lats1/2 and/or mst1/2 generally result in increased cell proliferation and capacity to grow to higher density. Moreover, in some cells, including breast cancer cells (Cordenonsi et al. 2011) and mouse

embryonic stem cells (Lian et al. 2010), Hippo signaling has also been shown to inhibit stemness and to promote differentiation. Given the relationship between organ size and progenitor cell number, at least in some tissues, these findings suggest that one fundamental role of Hippo signaling in organ size control may involve regulation of stem and progenitor cell number coupled with control of timing and extent of progenitor cell cycle exit and differentiation.

While Hippo signaling has been clearly implicated in organ size regulation in Drosophila and in mammals, there are many unresolved issues concerning the specific role(s) Hippo signaling plays in organ size determination. First, whether Hippo signaling is dynamically regulated in response to "organ size checkpoints" either during development or following regeneration remains to be determined. Second, how Hippo signaling impacts organ size is not clear in most circumstances where a direct role has been proven or suggested. However, control of Hippo pathway-regulated stem and progenitor cell proliferation and regulation of cell survival are likely to play important roles in a number of tissues. Finally, how Hippo signaling interfaces with other pathways that control stem and progenitor cell proliferation and organ size remains to be determined. Nevertheless, Hippo signaling has emerged as an important evolutionarily conserved pathway that functions to integrate multiple signaling that regulate growth in the context of developing and adult tissues. Future research will clarify the role of Hippo signaling as a key pathway in the determination of organ size as well as the precise mechanisms by which Hippo signaling maintains a delicate balance between proliferation and differentiation in many cells and organ systems.

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